MICROBIAL DIVERSITY 2013

MICROBIAL INTERACTIONS IN COMPLEX ECOSYSTEMS

MD **2013**

PROCEEDINGS OF THE SECOND INTERNATIONAL CONFERENCE ON MICROBIAL DIVERSITY 2013 - MICROBIAL INTERACTIONS IN COMPLEX ECOSYSTEMS, MD2013, 23-25 OCTOBER 2013, TORINO, ITALY

Microbial Diversity 2013
Microbial Interactions in Complex Ecosystems
MD2013

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Welcome

Dear Attendees at the Microbial Diversity 2013,

it is my pleasure to welcome you in Torino, first capital of Italy and city full of resources for visitors. From the Egyptian museum and the Risorgimento museum to the Royal Palace and the Palazzo Madama, Torino has fabulous museums. Don't miss the cinema museum in the Mole Antonelliana, the most distinctive building in Torino. You can also ride a breath-taking elevator to the base of the spire for a great view over the rooftops of the city.

After the success of the 1st International Conference on Microbial Diversity, 2011- "Environmental Stress and Adaptation", the Italian Society for Agricultural, Food and Environmental Microbiology (SIMTREA), with the patronage of the University of Torino and the Department of Agricultural, Forest and Food Sciences (AGRIFORFOOD), presents the second edition of the conference titled: "**Microbial Interactions in Complex Ecosystems**". The meeting gathers scientists in the fields of agricultural, food and environmental microbiology, coming from all over the world, in order to promote discussion and exchange knowledge and new findings on microbial interactions in complex ecosystems. Understanding of interactions of microbes in their own environment is the basis for the study of microbial ecology. Although the activity of microorganisms in nature manifests at macroscale, the interactions (physical, chemical and biological) take place at microscale. At this level, different microbial ecosystems can be recognized, affecting the final manifestation. It is well demonstrated that microbes interact through different mechanisms contributing to the determination of the ecology of microbial ecosystems both in space and time. Examples of such interactions are: quorum sensing, biofilms, symbiosis, competition and dominance through production of toxic or inhibitory compounds, such as organic acids and bacteriocins.

More than 200 participants, 2 keynote lectures, 6 invited speeches, 34 selected oral presentations and more than 150 posters are some of the numbers that characterise the MD2013, a forum that guarantees scientific update, discussion and exchange of information, which helps the advancement in science.

This book collects the abstracts submitted to MD2013. The topics presented are biofilms in complex microbial ecosystems; the potential of microorganisms in reclamation and remediation; competition, dominance and evenness: how microorganisms manifest their supremacy; communication among microorganisms; fight between microbes: antibiosis and other strategies; symbiosis of microbes with humans, animals and plants. Moreover, two chapters are dedicated to presentations of European and National Projects and of Young Researchers.

A special thanks to the sponsors of the conference, Biraghi Spa and Fontanafredda Srl, and to the scientific partners, the Federation of European Microbiological Societies (FEMS) and the International Committee on Food Safety and Hygiene (ICFMH), which contributed to the success of the conference.

I wish you all a very fruitful and successful conference in terms of scientific and personal interactions!

Luca Cocolin
Chair of the Organizing Committee of the MD2013

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MD2013 Scientific Programme

Wednesday, October 23, 2013

10:00 - 14:00 Registration

14:00 - 14:15 Welcome introduction

- President of the MD2013 Organizing Committee
- President of the SIMTREA
- Rector of the University of Torino
- Director of the Dep. of Agricultural, Forest and Food Sciences, Univ. of Torino
- Vice-Director of the Dep. of Agricultural, Forest and Food Sciences, Univ. of Torino

14:15 - 15:00 Keynote Lecture
Chairperson:
Luca Cocolin, University of Torino, Italy

LUC DE VUYST, Vrije Universiteit Brussels, (Belgium).
Metagenomics and meta-metabolomics analyses of spontaneous cocoa bean fermentation give further insight into its microbial interactions

15:00 - 15:30 Coffee break

15:30 - 17:00 - Session I:
Biofilms in complex microbial ecosystems
Chairpersons:
Daniele Daffonchio, Univeristy of Milano, Italy
Hans-Curt Flemming, University of Duisburg, Germany

15:30 - 16:00 **Plenary Lecture**
HANS-CURT FLEMMING, University of Duisburg (Germany)
Biofilms: how microorganisms organize their social life

16:00 - 16:20 **CANEK Novotny**, Academy of Sciences, Czech Republic
Effect of long-term bacterial and fungal stress on biofilms of ligninolytic fungi

16:20 - 16:40 **NIEROP GROOT Masja**, Wageningen UR, The Netherlands
Bacillus cereus *biofilm formation: role of sigma 54 in the interaction with a surface*

16:40 - 17:00 **CARDINALI Gianluigi**, University of Perugia, Italy
Cooperative biofilm formation of Enterococcus faecium *and* Trichosporon faecale *on stainless steel in static and dynamic conditions*

17:00 - 17:20 **BERNINI Valentina**, University of Parma, Italy
Gorgonzola cheese rind as complex biofilm matrix: strategies to reduce the contamination of Listeria monocytogenes *by using physical treatments*

17:30 - 18:30 - Poster & Special Sessions (parallel)

Special Session: **Young Researchers Presentations**
Chairpersons:
Marco Gobbetti, University of Bari Aldo Moro, Italy
Eugenio Parente, University of Basilicata, Italy

17:30 - 17:45 **BOTTA Cristian**, University of Torino, Italy
Characterization of potential probiotic bacteria from table olives fermentations: towards a new functional food

17:45 - 18:00 **CROTTI Elena**, University of Milano, Italy
In vitro, in vivo *and field probiotic effect of honeybee gut bacterial symbionts against* Paenibacillus larvae, *the causal agent of American Foulbrood disease*

18:00 - 18:15 **VIEL Alessia**, Univeristy of Padova, Italy
Population structure and geographical distribution of autochthonous S. cerevisiae *strains isolated in three different oenological areas in the North-East of Italy*

18:15 - 18:30 **RICHTER Andrea**, University College Dublin, Ireland
Integrating soil characteristics, land management and soil microbial communities

Thursday, October 24, 2013

08:30 - 10:20 - Session II:
The potential of microorganisms in reclamation and remediation
Chairpersons:
Sergio Casella, University of Padova, Italy
Gabriele Berg, University of Graz, Austria

08:30 - 09:00 **Plenary Lecture**
GABRIELE BERG, University of Graz, Austria
The plant microbiome: function and importance

09:00 - 09:20 **DE PHILIPPIS Roberto**, University of Firenze, Italy
Exopolysaccharides produced by cyanobacteria of induced biological sol crusts positively affects seed germination and seedlings fitness of desert shrubs, contributing to the restoration of desertified areas

09:20 - 09:40 **CULLEN William R.**, University of British Columbia, Canada
Quantitative real time PCR and arsenic mobility

09:40 - 10:00 **KUYUKINA Maria**, Russian Academy of Sciences Perm, Russia
Interactions between introduced hydrocarbon-oxidizing Rhodococcus *spp. in crude oil-contaminated soil monitored by direct PCR*

10:00 - 10:20 **BORIN Sara**, University of Milano, Italy
Unraveling the role of ecosystem development in the shaping of soil bacterial communities

10:20 - 10:40 Coffee break

10:40 - 11:25 **Keynote Lecture**
Chairperson:
Daffonchio Daniele, University of Milan, Italy

R. JOHN PARKES, Cardiff University, UK
Microbial life in the deep biosphere: a large but extreme habitat

11:25 - 13:35 - Session III:
Competition, dominance and evenness: how microorganisms manifest their supremacy
Chairpersons:
Erasmo Neviani, University of Parma, Italy
Nico Boon, University of Gent, Belgium

11:25 - 11:55 **Plenary Lecture**
NICO BOON, University of Gent, Belgium
Synthetic microbial communities: high throughput models to test new ecological hypothesis

11:55 - 12:15 **SØRENSEN Søren J.**, University of Copenhagen, Denmark
Social interactions in complex microbial communities - Survival of the fittest or the friendliest?

12:15 - 12:35 **CHAILLOU Stéphane**, MICALIS-INRA, France
Dynamic evolution during storage of meat and seafood products towards the selection of core and variable components of microbial spoilage

12:35 - 12:55 **CORSETTI Aldo**, University of Teramo, Italy
Transposon mutagenesis of Lactobacillus pentosus *C11 identified critical genes for growth in olive brine*

12:55 - 13:15 **SIMONCINI Nicoletta**, Stazione Sperimentale delle Conserve, Italy
Antagonist effect by native yeasts against a toxigenic strain of Penicillium nordicum *in dry cured meat model system*

13:15 - 14:30 Lunch

14:30 - 16:20 - Session IV:
Communication among microorganisms
Chairpersons:
Parente Eugenio, University of Basilicata, Italy
Arneborg Nils, University of Copenhagen, Denmark

14:30 - 15:00 **Plenary Lecture**
NILS ARNEBORG, University of Copenhagen, Denmark
Yeast-yeast interactions in wine

15:00 - 15:20 **ALBERGARIA Helena**, LNEG, Portugal
Dominance of Saccharomyces cerevisiae *in wine fermentations: secretion of antimicrobial peptides and microbial interactions*

15:20 - 15:40 **PERRONE Benedetta**, University of Torino, Italy
Transcriptomic approach for the identification of genes involved in the dominance phenomenon of Saccharomyces cerevisiae *strains*

15:40 - 16:00 **BELY Marina**, Bordeaux Segalen – ISVV, France
Torulaspora delbrueckii – Saccharomyces cerevisiae *aromatic interactions in Sauvignon Blanc wine*

16:00 - 16:20 **CALASSO Maria**, University of Bari Aldo Moro, Italy
Effects of the peptide pheromone Plantaricin A and cocultivation with Lactobacillus sanfranciscensis *DPPMA174 on the exoproteome and the adhesion capacity of* Lactobacillus plantarum *DC400*

17:30 - ... Departure for the visit of Fontanafredda Winery (Langhe Region) and Social Dinner (not included in the registration fee)

Friday, October 25, 2013

09:00 - 10:50 Session V:
Fight between microbes: antibiosis and other strategies
Chairpersons:
De Vuyst Luc, Vrije Universiteit Brussels, Belgium
Galvez Antonio, Universidad de Jaén, Spain

09:00 - 09:30 **Plenary Lecture**
ANTONIO GALVEZ, Universidad de Jaén, Spain
Ecological significance of bacteriocin production

09:30 - 09:50 **COTTER Paul D.**, Teagasc Food Research Centre, Ireland
Bacteriocin production and its impact on gut microbial diversity

09:50 - 10:10 **MORA Diego**, University of Milano, Italy
The overexpression of PmrB reduce the sensitivity of Streptococcus thermophilus *towards several antimicrobial molecules*

10:10 - 10:30 **SAVARD Tony**, AAFC/FRDC, Canada
Lactic starters may ensure safety in low sodium fermented vegetables: a hurdle technology combining amensalism and antibiosis

10:30 - 10:50 **LEROI Françoise**, IFREMER, France
Inhibition of Listeria monocytogenes *by the bioprotective strain* Lactococcus piscium *CNCM I-4031 : a cell-to-cell contact dependant system?*

10:50 - 11:10 Coffee break

11:10 - 11:30 Presentation of the FEMS grantees and of the awards-winning posters

11:30 - 13:00 Poster & Special Sessions (parallel)

Special Session: **Results of Selected EU and National projects**
Chairpersons:
Sergio Casella, University of Padova, Italy
Erasmo Neviani, University of Parma, Italy

11:30-11:45 **PIETRAMELLARA Giacomo**, University of Firenze, Italy
Soil aggregates: protective hot spots for microbes under extreme environmental

condition

11:45-12:00 **GOBBETTI Marco**, University of Bari Aldo Moro, Italy
Microorganisms in foods and in humans: study of the microbiota and the related metabolome as affected by omnivore, vegetarian or vegan diets

12:00-12:15 **VARESE Giovanna Cristina**, University of Torino, Italy
The MIRRI project: the role of collections of microorganisms in the development of Italian biotechnology

12:15-12:30 **ZOTTA Teresa**, Istituto di Scienze dell'Alimentazione (ISA) CNR, Italy
Genetic and physiological basis of aerobic metabolism in Lactobacillus rhamnosus *and* Lactobacillus paracasei*: basic and applied aspects*

12:30-12:45 **RADDADDI Noura**, University of Bologna, Italy
BIOCLEAN, New BIOtechnologiCaL approaches for biodegrading and promoting the environmEntal biotrAnsformation of syNthetic polymeric materials

12:45-13:00 **VANNINI Lucia**, University of Bologna, Italy
Effects of PBS-based packaging on the shelf-life and spoilage profiles of soft cheese

13:00 - 14:00 Lunch

14:00 - 15:50 Session VI:
Symbiosis of microbes with humans, animals and plants
Chairpersons:
Gobbetti Marco, University of Bari Aldo Moro, Italy
Van Sinderen Douwe, University College Cork, Ireland

14:00 - 14:30 **Plenary Lecture**
DOUWE VAN SINDEREN, University College Cork, Ireland
Bifidobacteria as part of the human gut microbiome: comparative and functional genomics of the early colonizers of our gut

14:30 - 14:50 **STEVENS Marc**, ETH Zurich, Switzerland
Using of genome data and RNAseq to unravel interactions between Bifidobacterium thermophilum *and* Salmonella *spec*

14:50 - 15:10 **CARDINALE Massimiliano**, University of Graz, Austria
Confocal microscopy as a tool to validate pyrosequencing results and to assess interactions within lettuce root microbiome

15:10 - 15:30 **SBRANA Cristiana**, Institute of Biology and Agricultural Biotechnology, Pisa

Mycorrhizal symbioses affect the production of health-promoting metabolites in host plants

15:30 - 15:50 **LAGO-LESTÓN M. Asunción**, Universidade do Algarve, Portugal

Tackling the specificity of the marine sponge microbiome: a biogeographical approach

16:00 Farewell

-President of the MD2013 Organizing Committee

-President of the SIMTREA

-Announcement of the 3rd International Conference on Microbial Diversity, 2015

-Closing remarks

-End of the Conference and Departure

MD2013 - Torino (Italy), 23-25 October 2013

KEYNOTE LECTURES

KL.1

METAGENOMICS AND META-METABOLOMICS ANALYSES OF SPONTANEOUS COCOA BEAN FERMENTATION GIVE FURTHER INSIGHT INTO ITS MICROBIAL INTERACTIONS

DE VUYST Luc*, ILLEGHEMS Koen, MOENS Frédéric, WECKX Stefan

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Introduction

Cocoa beans are the principal primary material for chocolate production (De Vuyst et al., 2010; Lima et al., 2011). To decrease the astringent, unpleasant flavour and taste of raw cocoa beans, they must be cured before they can be converted into attractive and full-flavoured chocolate. Curing involves fermentation of the cocoa beans, followed by drying and roasting. Therefore, the process of fermentation lays at the basis of the entire chocolate making process. It plays a significant role in determining the composition and flavour of chocolates and other cocoa-based products.

Primary material

Cocoa beans are the seeds of the fruits (cocoa pods) of the cocoa tree (*Theobroma cacao* L.). The main plant and cocoa bean varieties are Forastero, Criollo, and Trinitario, but nowadays mainly hybrids thereof are used in the cocoa-producing countries worldwide. Plant populations and genotypes determine harvest yields, resistance to diseases, and contribute to the composition and flavour of fermented dry cocoa beans (De Vuyst et al., 2010; Lima et al., 2011).

Inside the cocoa pods, the beans are embedded in a white, mucilaginous pulp. Pulp and beans within ripe healthy pods are microbiologically sterile. When the cocoa pulp-bean mass contents are removed from the opened pods, either manually or mechanically, the pulp becomes contaminated with a variety of microorganisms from the surrounding environment, many of which contribute to the subsequent spontaneous cocoa bean fermentation process (Camu et al., 2007; Nielsen et al., 2007).

Fermentation

The fermentation of the cocoa pulp-bean mass is still a spontaneous process that is carried out in heaps, boxes, baskets, or trays, on platforms, or otherwise, depending on the cocoa-producing region. It lasts for about four to six days, depending on local farm practices. Most of the world cocoa production is performed in boxes.

The heterogeneous natural cocoa bean fermentation process shows great variations in the course of both microbial counts and metabolite

concentrations of pulp and beans as well as in their nature. This is mainly due to the variable microbial environmental contamination of the initial cocoa pulp-bean mass and the applied agricultural and farm practices, which affect the acidity, composition, and flavour of cocoa and chocolate produced of the respective fermented dry cocoa beans (Ardhana and Fleet, 2003; Camu et al., 2007, 2008; Adeyeye et al., 2010; Caligiani et al., 2010; Garcia-Armisen et al., 2010; Papalexandratou et al., 2011a,b,c, 2013; Rodriguez-Campos et al., 2011). Therefore, chocolate manufacturers need to use fermented dry cocoa bean blends in their chocolate recipes to complement or to contrast the inconsistent compositional and flavour quality of spontaneously fermented dry cocoa beans (De Vuyst et al., 2010).

Fermentation course

The course of spontaneous cocoa bean fermentations has been studied for a long time, mostly through a classical microbiology approach, whether or not accompanied with the determination of certain metabolites (Ardhana and Fleet, 2003). Recently, systematic studies have been carried out on the cocoa bean fermentation process through a multiphasic approach, encompassing classical microbiology, metagenomics analyses (both 16S rRNA gene clone libraries and deep sequencing), and meta-metabolomics analysis. This allowed a thorough assessment of the natural cocoa bean fermentation process and gave deeper insights into its microbial interactions (Nielsen et al., 2005, 2007; Camu et al., 2007, 2008; Garcia-Armisen et al., 2010; Papalexandratou et al., 2011a,b,c, 2013; Illeghems et al., 2012). It turned out that successful cocoa bean fermentation requires a succession of particular microbial activities, namely yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB), which should not exceed four days (Figure 1; Papalexandratou et al., 2011a,b,c, 2013; Illeghems et al., 2012). Also, enterobacteria may play a role at the onset of the cocoa bean fermentation process (Garcia-Armisen et al., 2010; Papalexandratou et al., 2011a,b,c; Illeghems et al., 2012). When the fermentation lasts more than four days, bacilli and filamentous fungi may participate in the cocoa bean fermentation process too (Ardhana and Fleet, 2003; Papalexandratou et al., 2011a; Pereira et al., 2012, 2013). Whether these microorganisms have a desirable contribution to the fermentation process or should be considered as spoilers is still under debate.

At the onset of cocoa bean fermentation, yeasts are the dominating microorganisms. They are responsible for liquefying the pulp through depectinisation. This causes pulp drainage (sweatings), reduces pulp viscosity and hence allows air ingress. Also, yeasts produce ethanol from carbohydrates (mainly from glucose that originates from the hydrolysis of sucrose through pulp and/or yeast invertase activity). The yeast activities occur under anaerobic (due to tight packing of the beans in the heaps and boxes), acidic (presence of a high concentration of citric acid in the pulp),

and carbohydrate-rich (sucrose, glucose, and fructose in the pulp) conditions (Ardhana and Fleet, 2003; Nielsen et al., 2005, 2007; Camu et al., 2007, 2008; Papalexandratou et al., 2011a,b,c, 2013). Further, yeasts produce certain organic acids and volatile flavour compounds. The recent multiphasic studies of various cocoa bean fermentations have shown that *Hanseniaspora opuntiae/uvarum* is the predominant yeast during the initial phase of the fermentation (Jespersen et al., 2005; Nielsen et al., 2005, 2007; Daniel et al., 2009; Papalexandratou and De Vuyst, 2011; Illeghems et al., 2012; Papalexandratou et al., 2013). Also, *Hanseniaspora thailandica*, *Pichia kluyveri*, *Pichia kudriavzevii*, *Pichia manshurica*, *Pichia membranifaciens*, and *Saccharomyces cerevisiae* have been reported to play a role in the fermentation of cocoa pulp-bean mass, the *Pichia* species being substrate competitors and *S. cerevisiae* often dominating the main course of the fermentation process (Ardhana and Fleet, 2003; Daniel et al., 2009; Jespersen et al., 2005; Papalexandratou and De Vuyst, 2011; Papalexandratou et al., 2011c, 2013; Crafack et al., 2013; Pereira et al., 2012, 2013).

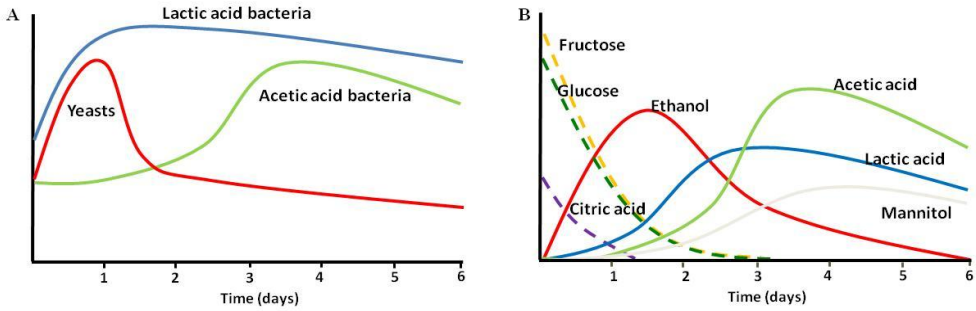

Figure 1. Microbial succession of a spontaneous cocoa bean fermentation process: (A) community dynamics; (B) substrate degradation and metabolite production kinetics.

As the pulp drains away, air penetrates into the fermenting cocoa pulp-bean mass, which creates ideal conditions for the growth of bacteria. Facultative anaerobic *Enterobacteriaceae* such as ddition to yeasts, and they may be responsible for the production of gluconic acid early into fermentation that can be further used by yeasts (Garcia-Armisen et al., 2010; Papalexandratou et al., 2011a,b,c). *Tatumella* species occur frequently and transiently (Garcia-Armisen et al., 2010; Papalexandratou et al., 2011a,b,c; Pereira et al., 2012, 2013). They may play a role in pectinolysis, in a Microaerophilic, acid-tolerant, ethanol-tolerant, and fructophilic LAB species, in particular *Leuconostoc pseudoficulneum*, *Leuconostoc pseudomesenteroides*, *Fructobacillus pseudoficulneus*, *Fructobacillus*

tropeaoli, and *Lactobacillus plantarum*, first dominate the fermentation, followed by *Lactobacillus fermentum* (Ardhana and Fleet, 2003; Camu et al., 2007; Nielsen et al., 2007; Papalexandratou et al., 2011a,b,c; Snauwaert et al., 2013; Crafack et al., 2013; Pereira et al., 2012, 2013). Glucose is fermented either homo- or heterofermentatively into lactic acid, acetic acid, and/or ethanol (Camu et al., 2007, 2008; Lefeber et al., 2010; Papalexandratou et al., 2011a,b,c, 2013; Adler et al., 2013). Fructose is either fermented to lactic acid, acetic acid, and/or ethanol (by fructose-loving LAB species) or used as alternative external electron acceptor (by strictly heterofermentative LAB species), in the latter case being reduced to mannitol. Citric acid is used as a co-substrate during heterofermentation and is converted into lactic acid, acetic acid, and/or flavour compounds such as diacetyl, acetoin, and 2,3-butanediol, enabling a slight increase of the pH of the pulp and hence favoring bacterial growth. Often, strictly heterofermentative *Lb. fermentum* strains are the prevailing LAB species during spontaneous cocoa bean fermentation, thanks to their strictly heterofermentative, citrate-converting, mannitol-producing and ethanol-, acid-, and heat-tolerant properties (Camu et al., 2007, 2008; Lefeber et al., 2010; Adler et al., 2013). During the aerobic phase, created by enhanced pulp drainage and air ingress upon fermentation, AAB species dominate, whereby *Acetobacter ghanensis* and *Acetobacter senegalensis* are replaced by *Acetobacter pasteurianus* (Camu et al., 2007, 2008; Papalexandratou et al., 2011a,b,c, 2013), the latter being the most prevalent AAB species involved in spontaneous cocoa bean fermentation, presumably because of its ethanol-oxidising capacity and acid and heat tolerance (Ardhana and Fleet, 2003; Camu et al., 2007, 2008; Nielsen et al., 2007; Lefeber et al., 2010; Papalexandratou et al., 2011a,b,c, 2013; Illeghems et al., 2013; Crafack et al., 2013). Also, *Acetobacter syzygii* and *Acetobacter tropicalis* occur frequently (Crafack et al., 2013; Pereira et al., 2012, 2013). AAB oxidise the ethanol produced by the yeasts into acetic acid and the lactic acid produced by the LAB into acetic acid and acetoin. Subsequently, the acetic acid is over-oxidised into carbon dioxide and water (Camu et al., 2007, 2008). Yeast, LAB, and AAB fermentations generate heat, in particular due to ethanol oxidation and acetic acid over-oxidation (De Vuyst et al., 2010).

Bioconversions

Ethanol, acetic acid and heat produced during fermentation penetrate into the cocoa beans and kill the embryo (De Vuyst et al., 2010; Lima et al., 2011). Also, increasing acetic acid concentrations and the concomitant lowering of the cocoa bean pH destruct internal membranes of the cocoa bean cotyledons upon further fermentation, so that substrates and enzymes from different cocoa bean compartments can migrate and mix. This process results in biochemical reactions catalysed by enzymes such as invertase, (dedicated) proteases, glycosidases, and polyphenol oxidase, the latter being active during

the aerobic phase, that lead to brown colour and flavour precursors. These are needed to further develop colour and flavour during drying of the fermented cocoa beans and they allow chemical reactions upon roasting of the fermented dry cocoa beans. At the same time that acetic acid penetrates into the cocoa beans, components such as alkaloids and polyphenols leak from the fermenting cocoa beans into the surrounding pulp, thereby reducing bitterness and astringency of the fermented cocoa beans. However, many more compounds are present in cocoa beans, whether or not modified upon fermentation, drying and further processing and whether or not leaking from the cocoa beans. The final chocolate composition and flavour result from the subsequent manufacturing operations and addition of other ingredients.

Conclusions

With the recent application of advanced scientific techniques in a multiphasic approach, detailed insights have been obtained in the temporal composition of the cocoa bean fermentation ecosystem and the ongoing metabolic processes. Such views will enable to estimate the importance of each of the members of the ecosystem and their relative contribution to the metabolic features. That way, functional starter cultures can be composed in a thought-out way, allowing controlled and even steered cocoa bean fermentation processes, thereby focusing on desired metabolic features that will result in high-quality chocolates and other cocoa-based products. Not only the chocolate industry will benefit from such an approach but also the farmers in the cocoa-producing countries will benefit from a high success rate of the fermentations performed.

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KL.2

MICROBIAL LIFE IN THE SUB-SEAFLOOR BIOSPHERE A LARGE BUT EXTREME HABITAT

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A general review of the sub-seafloor biosphere is presented. This includes an update and assessment of prokaryotic cell distributions within marine sediments (Fig. 1, (Parkes et al., 1994) and the impact of this on global sub-seafloor biomass estimates. These global estimates appear relatively robust to different calculation approaches and our updated estimate is 5.39×10^{29} cells in total, taking into consideration new data from very low organic matter South Pacific Gyre sediments (Expedition, 329 Scientists 2011, Kallmeyer et al., 2012). This is higher than other recent estimates, which is justified as several sediments, such as, gas hydrate deposits (Cragg et al., 1996) and oil reservoirs (Bennett et al., 2013) can have elevated cell concentrations.

The proposed relationship between elevated cell concentrations in sequential diatom rich layers at some sites to Milankovitch Cycles (Aiello & Bekins, 2010) demonstrates not only a dynamic deep biosphere but that it is an integral part of Earth System Processes over geological time scales. Cell depth distributions vary in different oceanographic provinces and this is also reflected in contrasting biodiversities. Despite this there are some clear common, sub-seafloor prokaryotes, for *Bacteria* these are the phyla *Chloroflexi*, *Gammaproteobacteria, Planctomycetes* and the candidate phylum JS1, and for *Archaea* uncultivated lineages within the phylum *Crenarchaeota* (Miscellaneous Crenarchaeotal Group and Marine Benthic Group B), *Euryarchaeota* (SAGMEG, Marine Benthic Group-D/*Thermoplasmatales* associated groups) and *Thaumarchaeota* (Marine Group I). In addition, spores, viruses and fungi have been detected but their importance is not yet clear. Consistent with the direct demonstration of active prokaryotic cells, prokaryotes have been enriched and isolated from deep sediments and these reflect a subset of the total diversity, including spore formers that are rarely detected in DNA analyses.

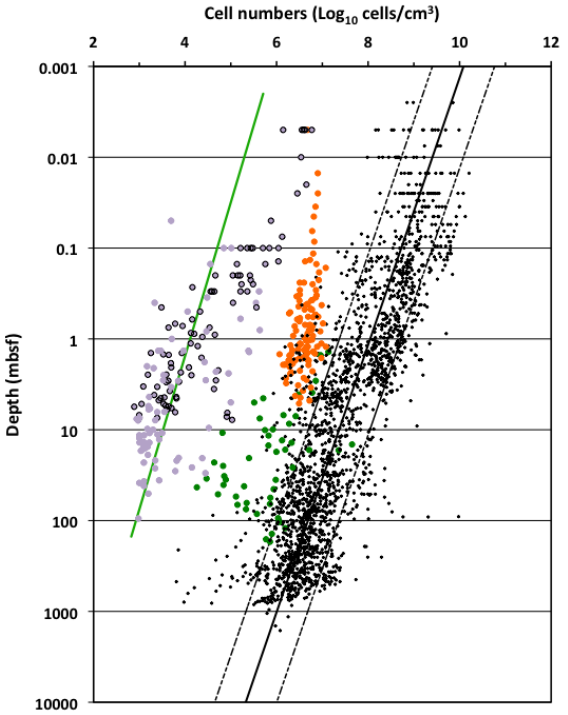

Figure 1. Depth (metres below sea floor) distribution of prokaryotic cells in sub-surface sediments at 106 locations, including 17 ODP/IODP Legs, black dots. Bold dashed black regression line is Log cells = 8.05 - 0.68Log depth. (R-sq = 0.70 & n = 2037) and light dashed lines are 95% lower and upper prediction limits. Orange circles are mud volcano breccia samples and green circles are hydrothermal samples. Mauve circles with a black outline are South Pacific Gyre samples, presented by Kallmeyer *et al*., 2012, plus additional data from an IODP Cruise (mauve circles) to the same sites (Expedition 329 Scientists, 2011), the green regression line is only through the later IODP cruise data. There is no significant difference (F = 0.79; d.f. = 1, 2105) in slope between this IODP cruise data regression (green line) and the regression (black line) through cell-depth distributions in "normal" marine sediments (black dots).

Activities are generally low in deep marine sediments (~ 10,000 times lower than in near-surface sediments), however, depth integration demonstrates that sub-surface sediments can be responsible for the majority of sediment activity (up to 90%), and hence, are biogeochemically important (Wellsbury et al., 2002). Unlike near-surface sediments competitive metabolisms can occur together (D'Hondt et al., 2004, Parkes et al., 2005) and metabolism per cell can be 1,000 times lower than in culture, and below the lowest known maintenance energies (Hoehler & Jorgensen, 2013). Consistent with this cell turnover times are approaching the

geological (100 – 1,000's of years). Prokaryotic necromass may be an important energy and carbon source, but this is largely produced in near-surface sediments as cell numbers rapidly decrease. However, this and deposited organic matter may be activated at depth as temperatures increase (Fig. 2). At thermogenic temperatures methane and other hydrocarbons, plus H_2 and CO_2 may be produced and diffuse upwards to feed the base of the biosphere (e.g. Nankai Trough [Horsfield et al., 2006] and Newfoundland Margin [Roussel et al., 2008]). Temperature activation of minerals may also result in oxidation of sulphides and the formation of electron acceptors (Bottrell et al., 2000), plus H_2 from low temperature (~ 55°C) serpentenisation (Mayhew et al., 2013). New mineral surface formation from fracturing, weathering and subduction etc can also split water via mechanochemistry producing both substrates (H_2) and oxidants (O_2, H_2O_2) for prokaryotes (Parkes et al., 2011). These and other biosphere: geosphere interactions may be important for sustaining a globally significant sub-seafloor biosphere.

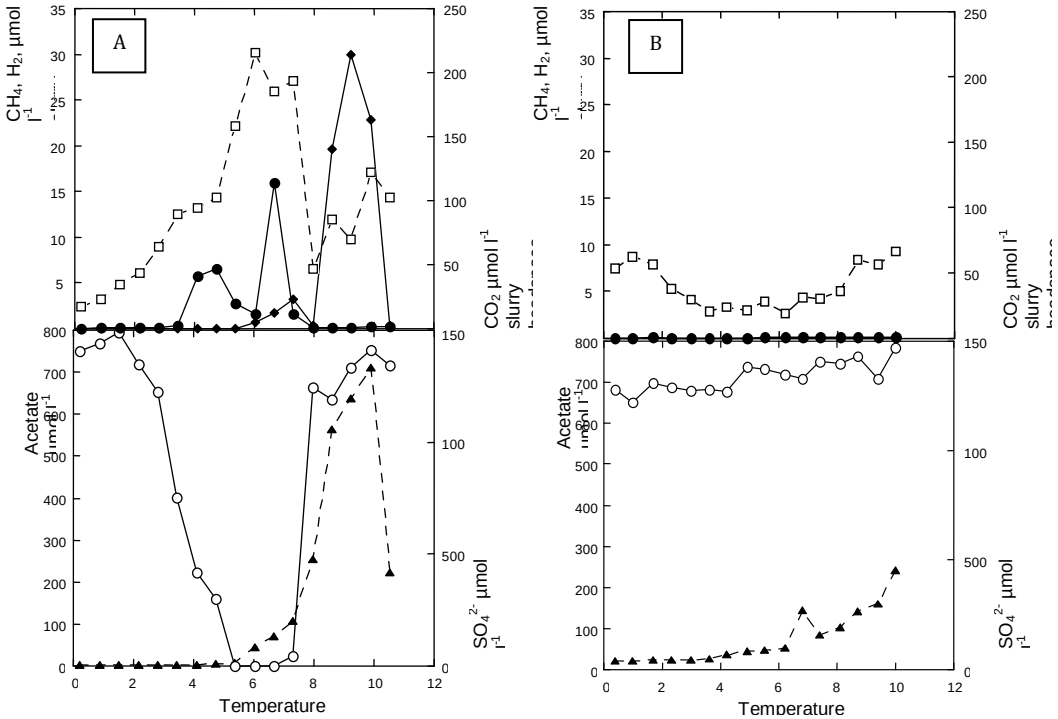

Figure 2. Headspace gas and sulphate and acetate changes in A. Basalt amended sediment slurries heated in a Thermal Gradient (83 days) compared to sterile controls (130 days) B. □ CO_2, ● CH_4, ▣ H_2, o sulphate, ⑩ acetate (Parkes, *et al.*, 2011). Geochemical changes reflecting activities are stimulated as temperatures increase in the presence of prokaryotes.

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PLENARY LECTURES

PL.1

BIOFILMS – HOW MICROORGANISMS ORGANIZE THEIR LIFE

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Overview

The biofilm mode of life is common to almost all microorganisms on Earth, be it continuously or as part of their life cycle. Although biofilm phenomena are very diverse, ranging from films on solid surfaces, aggregates at the water-atmosphere interface to flocs as "floating biofilms" and microbial mats and sludge as very thick biofilms, they all have common features. One of them is that they are embedded in a matrix of hydrated extracellular polymeric substances (EPS) which allows for developing emergent properties, not shared by planktonic cells. Biofilm cells can develop synergistic multispecies relationships, because they are immobilized in the matrix. The matrix itself traps exoenzymes, turning it into an external digestion system. In biofilms, intense intercellular communication takes place, regulating the expression of numerous genes. Furthermore, biofilm cells are significantly more resistant to stress such as radiation, toxic metals, disinfectants and antibiotics. On the other side, there is fierce competition among biofilm organisms, driving the dynamic, spatially and temporally heterogeneous structure of biofilms. The biofilm mode of life is the first recorded on Earth, it is extremely flexible and successful.

What are biofilms?

Microorganisms on Earth do not live as single cells in pure cultures - as they have been historically studied in microbiology - but rather in multispecies aggregates. Such aggregates display a huge variety of structural and functional properties. They may occur as films on interfaces ("biofilms" in the strict sense, e.g., on solid surfaces in water, soil, sediments, porous minerals and wet atmosphere-exposed surfaces), but also as flocs ("floating biofilms"), as aggregated particles in dust and clouds, and as sludge. But for all of them, the term "biofilm" has been accepted. Biofilms all have one important feature in common: the organisms live embedded in a matrix of extracellular polymeric substances (EPS) and establish complex interactions. Many types of naturally-occurring and engineered microbial aggregates have been subsumed under the heading of "biofilm", such as bacterial colonies, effluent treatment flocs, anaerobic digester granules, food-associated systems such as Kefir grains or the ginger beer plant, marine snow, mycelial balls, pellicles, and algal mat

communities. The list is long and includes even microbial aggregates in clouds – and all of them meet the above mentioned characteristics.

Biofilms play fundamental and often deleterious roles in medicine (Hall-Stoodley et al., 2004), as habitats for pathogens in the environment (Wingender, 2011) and in industrial biofouling (Flemming, 2011), where they are frequently involved in microbially influenced corrosion (MIC, Little and Lee, 2007) and biodeterioration of synthetic polymers (Flemming, 2010), wood, mineral materials and pieces of art. On the other hand, biofilms are often put to beneficial use because they are the carriers of biological purification of drinking water (Gimbel et al., 2006) as well as of waste water (Wuertz et al., 2003). It is clear that biofilms are ubiquitous and they have both very fundamental detrimental and beneficial aspects for our life at often largely underestimated dimensions. The prerequisites of their occurrence are minimal, requiring only sufficient humidity, nutrients (even in traces), and microorganisms. Aggregation and the association with surfaces of all kinds offer considerable ecological advantages for microorganisms. Practically all surfaces in non-sterile environments which provide sufficient amounts of water are colonized by biofilms, even at extreme pH-values, temperatures, salt concentrations, radiation intensities and pressure. Some examples are listed in Table 1.

Table 1. Examples for the range of environmental conditions in which biofilms exist (after Flemming and Wingender, 2003).

Conditions	**Examples**
Temperature	From – 12°C (cold saline water) to + 125 °C (hot vents)
pH value	From 0 (sulfur oxidizers) to > 13 (bacteria in natron lakes)
Hydrostatic pressure	From < 0.1 bar (vacuum systems) to 1,000 bar (barophilic bacteria at deep sea floor)
Redox potential	Entire range of redox stability of water; growth on electrodes
Salt concentration	From 0 (ultrapure water systems) to almost saturated salt solutions (hyperhalophilic bacteria in permafrost)
Nutrients	From < 10 µg L^{-1} to life directly on nutrients
Radiation	Biofilms on UV sources and nuclear power plants; *Deinococcus radiodurans* survives 10,000 Gy without being killed
Surface materials	Metals (including copper and silver), minerals, organic polymers, plant and animal tissue, bones etc.
Biocides	Growth in disinfectant pipes; "persister cells"

Biofilms can be considered the cradle of life (Schopf et al., 1983); it is a common concept that life has evolved at interfaces. Essentially, the first organisms were originally located at these interfaces and multiplied there. Interfaces remained "hot spots" in evolution and biofilms are considered the oldest and most successful form of life on Earth with fossils dating back

3.5 billion years and represent the first signs of life on Earth. It is very plausible to assume that the processes of endosymbiosis, which led to eukaryotic organisms, evolved in such biofilms, as well as photosynthesis, which provided the oxygen for Earth's atmosphere. Indeed, one possible origin of infectious diseases might have been the result of intense competition between biofilm microorganisms, including the emergence of predation of neighbouring cells.

Biofilms are also involved in the biogeochemical cycles of virtually all elements including most metals and they are carriers of the environmental "self-purification" processes. The basic underlying process is always the same: microorganisms on surfaces convert dissolved or particulate nutrients from the water phase and/or from their support into metabolites and new biomass. As mentioned earlier, this is the principle of biological filtration systems used in drinking water and wastewater purification as well as many other biotechnological applications.

Environmental roles of biofilms

Biofilms contribute strongly to primary production of biomass on earth, being especially represented in the rhizosphere and in microbial mats. They occur even within minerals ("endolithic biofilms") where they can survive very harsh conditions such as encountered in polar regions or the extremely dry Atacama desert. They also represent the "global cleaning company" for all biological material, which they often completely biodegrade (i.e., mineralize) to carbon dioxide and water. Most of the biodegradable biomass on Earth is not homogeneously dissolved but rather exists as heterogeneously distributed particulate matter. Particle degradation must be performed by attached microorganisms, i.e., biofilms, and their extracellular enzymes. Thus, biological degradation is chiefly the result of the activity of microorganisms in biofilms, in most cases performed by orchestrated interactions of different microbial species. Biofilms are driving the mobilization of minerals on a geological scale and, thus, providing metal ions for the biosphere.

Biofilms not only occur on solid surfaces but also at the interface between water and the atmosphere in global dimensions. The uppermost layer of surface waters is known as the neuston (Franklin et al., 2005). Here, hydrophobic substances accumulate and provide nutrients for hydrophobic organisms, forming biofilms. Due to microbial products such as amphiphilic molecules they influence surface tension, and, thus, the physico-chemical conditions of the mass transfer of gases between atmosphere and water. Therefore, the biofilms in the neuston layer are essentially influencing not only the global CO_2 exchange, but also the exchange, production and consumption of methane and dimethyl sulfoxide, which is believed to be involved in cloud formation (Ekström et al., 2010).

The Matrix: extracellular polymeric substances

Life in biofilms happens in a very complex microenvironment. The key

are the extracellular polymeric substances (EPS, Flemming and Wingender, 2010), well known as "slime". All manifestations of biofilms have in common that they are kept together by an EPS matrix. This matrix essentially consists of highly hydrated biopolymers such as polysaccharides, proteins, lipids, and extracellular DNA. The composition of the EPS matrix of a given biofilm is highly variable both in space and time. The success of the biofilm mode of life is possible through the matrix, made of EPS – in this matrix, the biofilm cells organize their life. Fig. 1 shows the matrix at different scales – it has to be taken into account, that the matrix is not homogeneous at all but clearly contains microdomains which are not depicted in this figure.

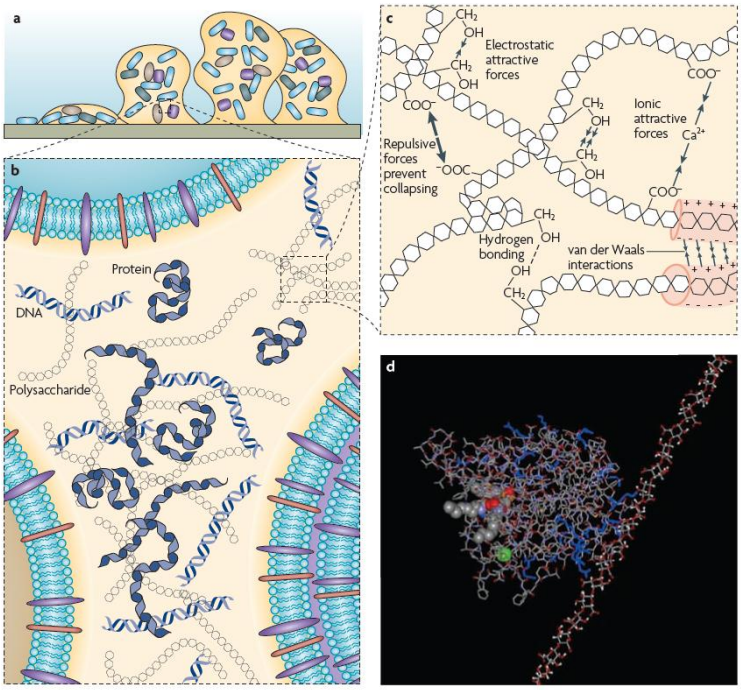

Figure 1. The extracellular polymeric substances matrix at different levels of resolution (Flemming and Wingender, 2010, with permission). **a** is a model of a bacterial biofilm attached to a solid surface. **b** shows ajor matrix components - polysaccharides, proteins and DNA - distributed between cells in non-homogeneously, setting up differences between regions of the matrix. **c** symbolizes classes of weak physicochemical interactions and the entanglement of biopolymers that dominate the stability of the EPS matrix. **d** is a molecular modeling simulation of interaction between the alginate (right) and lipase (left) of *Pseudomonas aeruginosa* in aqueous solution.

Emergent properties of the biofilm mode of life

When individual components in an environment come together and create

distinct, collective and interactive properties and functions, the results are called "emergent properties". As such, emergent properties are at the intrinsic core of the entire science of systems biology, as defined by the Encyclopedia of Science and Philosophy). This definition clearly applies for biofilms (Wimpenny, 2000; Sachs and Holowell, 2012). Among the emergent properties of biofilms which are not just achieved by simply adding single cell or by suspended populations are:

- Formation of the EPS matrix, functional architecture, mechanical stability of biofilms.
- Development of gradients of pH-value, oxygen concentration, redox potential, substrate and product concentrations.
- High variation of different habitats at small scale, supporting high biodiversity.
- Biofilm cells are kept in place and can form synergistic microconsortia.
- *Exoenzymes* are retained in the matrix and represent an external digestion system.
- Sorption of dissolved & particulate substances from water phase, providing nutrients.
- Facilitated gene transfer.
- Intense intercellular communication.
- Enhanced resistance to biocides, toxic metals, desiccation and other stress.

Jefferson (2004) states, that there are indeed similarities between biofilm bacteria and multicellular organisms. For instance, bacteria (including planktonic bacteria) can sense their surroundings, and this enables them to adjust their metabolic processes to maximize the use of available substrates and to protect themselves from detrimental conditions.

When bacteria are growing within a biofilm, changes in gene expression result in phenotypic heterogeneity within the biofilm, which can be interpreted as specialization or division of labor similar to cellular differentiation seen in multicellular organisms. In addition, bacteria secrete substances signals, which influence gene expression and may be a means by which cells communicate with one another. Thus, biofilms can be considered as interactive communities. There is even a growing body of evidence that bacteria exhibit altruistic behavior and can undergo a process similar to programmed cell death, again suggestive of multicellularity. However, there are fundamental distinctions between bacteria and multicellular organisms. For example, while bacterial cells can react and adapt to their environmental surroundings, they do not permanently differentiate (Jefferson, 2004).

Shapiro (1998) has lined out that coordinated multicellular behaviour can be observed in a variety of situations, including the development of *Escherichia coli* and *Bacillus subtilis* colonies, swarming by *Proteus* and *Serratia*, and spatially organized interspecific metabolic cooperation in anaerobic bioreactor granules. The organisms benefit from multicellular cooperation by using cellular division of labour, accessing resources that cannot effectively be utilized by single cells, collectively defending against antagonists, and optimising population

survival by differentiating into distinct cell types. The EPS matrix provides an organized space for these processes which allows the organisms to control their immediate environment. Inevitably, anthropomorphic terms have been used to describe the biofilm mode of life, e.g, "microbial landscapes", "city of microbes", and the EPS matrix as a "house of biofilm cells". All these analogies indicate how strongly the interactions in biofilms suggest complex and regulated processes. For microbial mats, the most appropriate metaphor may be biofilms as "microbial rainforests" due to their extremely complex biome of both structure and species diversity, with sunlight and water as the major limiting factors.

As a whole, biofilms are the success model of life in nature. They have spread not only over the entire crust planet but also in its depth where it has been associated with mineral oil production (Head et al., 2003) even in places where they were separated from any outside conditions for millennia. Literally, Earth is colonized by biofilms and microbial life is clearly not endangered. And if there is life on Mars, it is more than probable that it exists in the subsurface, e.g., in permafrost (Wagner et al., 2007) or subsurface mineral structures, and, thus, associated to surfaces in the form of biofilms.

Biofilms seem to be a universal mode of life.

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PL.2

THE PLANT MICROBIOME: FUNCTION AND IMPORTANCE

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Introduction

Plants have recently been recognized as meta-organisms due to a close symbiotic relationship with their microbiome. Comparable to humans and other eukaryotic hosts, plants also harbor a "second genome" that fulfills important host functions. These insights were driven by both 'omics'-technologies guided by next-generation sequencing and microscopic insights (Berg et al. 2013). However, the plant microbiome is well-structured and different in structure and function for each part (Berg et al., 2005b, Hirsch and Mauchline 2012).

The rhizosphere: microbial 'hot spot' of central importance in terrestrial ecosystems

Already in 1904, more than 100 years ago, Lorenz Hiltner defined the term 'rhizosphere' as root-surrounding soil influenced by root exudates (Hartmann et al., 2008). In addition, he discovered the importance of microbial root inhabitants for plant growth and health. Since that time, much has been learned about microorganism and plant host interactions, especially in recent years by means of new next generation sequencing (NGS) techniques, 'omics'-technologies, and microscopic methods (Sorenson et al. 2009; Jansson et al., 2011; Berendsen et al., 2012). The rhizosphere is of central importance not only for plant nutrition, health, and quality, but also for microorganism-driven carbon sequestration, ecosystem functioning, and nutrient cycling in terrestrial ecosystems. A multitude of biotic and a-biotic factors influence the structural and functional diversity of microbial communities in the rhizosphere (rev. in Berg and Smalla 2009). For example, microorganisms' response to root exudates (Bais et al., 2006) and root morphology was shown to shape rhizosphere microbial communities (Smalla et al., 2001; Berg et al., 2005c, 2006¸). In addition, plant defense signaling play a role in this process as well (Doornbos et al., 2012). Soil is the main reservoir for rhizosphere microorganisms, and many secrets and theories of microbial life in the rhizosphere were recently uncovered or confirmed due to the enormous progress in molecular and microscopic techniques. Haichar et al. (2008) used a stable isotope probing (SIP) approach to show that plant host

habitat and root exudates shape the soil bacterial community structure. In another example, Lundberg et al. (2012) as well as Bulgarelli et al. (2012) revealed that only a subset of the bacterial community in the soil is present around the plant roots of *Arabidopsis thaliana*. The main root inhabitants represent two bacterial phyla: Proteobacteria, which includes many growth-promoting members, and Actinobacteria, many of which are known for producing antimicrobial compounds. They hypothesized that *Arabidopsis* recruits a core group of microbes to benefit its basic functions, and an additional subset to help it thrive in specific environments. However, all these results reveal a new perspective on plants: they are meta-organisms comprised of the plants themselves and all microbial inhabitants.

The endosphere: an intimate relationship between plants and microbes

Not only is the densely colonized rhizosphere important, but each plant is also home to a complex and distinctive community of microbes consisting of billions of inhabitants (Vorholt 2012). Through a comprehensive look at the plant-associated community, hundreds of microbial species were identified as specific to crops, cultivars, and also for the micro-environment. Each plant can be divided into different micro-environments: the endorhiza (root), the phyllosphere (leaves), the spermosphere (seeds), the carposphere (fruit) etc., and we generally differentiate between the endosphere and ectosphere (Ryan et al., 2008). All these micro-environments provide specific biotic and a-biotic conditions for microbial life. However, an interesting question concerns the origin of the microbial inhabitants on plants as they seem to come from different sources. While soil is the main reservoir for rhizosphere microorganisms, a sub-set of rhizosphere inhabitants is also able to colonize the endorhiza (Berg et al., 2005b). Endophytes were long believed to originate only from the rhizosphere and soil, but molecular studies have shown that the above ground plant parts can be invaded by microbes as well (Berg et al., 2005b). New methods also revealed that seeds are colonized not only by (dormant) pathogenic bacteria, but they also harbor a beneficial seed microbiome. In addition, generative organs like pollen (Fürnkranz et al., 2012) and moss sporophytes (Bragina et al., 2012) harbor a core, beneficials-containing microbiome. Plants are in constant contact with diverse microbes blown by the wind or delivered via the water cycle, and some of them settle down and survive.

Conclusion

Collectively known as the plant microbiome, plant-associated microbes can help plants fend off disease, stimulate growth, occupy space that would otherwise be taken up by pathogens, promote stress resistance, and influence crop yield and quality. Therefore, the plant microbiome is a key determinant of plant health and productivity (Berg 2009, Mendes et al., 2012). In the future, due to climate change, the importance of the

microbiome especially the balance between plants and their microbiome will increase. Therefore, the stress-protecting function of plant-associated microbes can be exploited (Alavi et al., 2013).

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PL.3

SYNTHETIC MICROBIAL COMMUNITIES: HIGH THROUGHPUT MODELS TO TEST NEW ECOLOGICAL HYPOTHESIS

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Introduction

Microbial communities are primary drivers of element cycles on a planetary level and are essential for the functioning of all ecosystems on earth and in many industrial processes. A microbial community is a complex network, consisting of several populations, which can interact with each other by antagonistic or cooperative processes. Examples of natural processes in which complex communities contribute are: digestion in human and animal gut, C and N recycling, including the degradation of complex biopolymers or harmful pollutants, mitigation of plant diseases etc. Examples of industrial processes where complex microbial communities are exploited include food processing like making of wine and dairy products, production of energy (microbial fuel cells and production of bio-ethanol) and wastewater treatment for drinking water, reuse or discharge in receiving water bodies. In our current society, where sustainability and climate compatibility are at the fore, many of such microbial community-dominated processes are considered to be superior to processes based on chemical or physical reactions. Obviously, microbial communities and their managing are becoming increasingly interesting in both natural and industrial processes. However, in contrast to individual microorganisms for which we are getting to know a lot about gene expression, stress response, etc., the knowledge about the complex network that is formed by interacting microorganisms remains unsatisfactory. Most of the current research in microbial ecology is using modelling,

exploration or experimentation approaches to examine ecosystems (Figure 1). However since the visionary review paper of Prosser *et al.* six years ago (Prosser et al., 2007), it became clear that these three "schools of microbial ecology" should be united into conceptual and theoretical approaches.

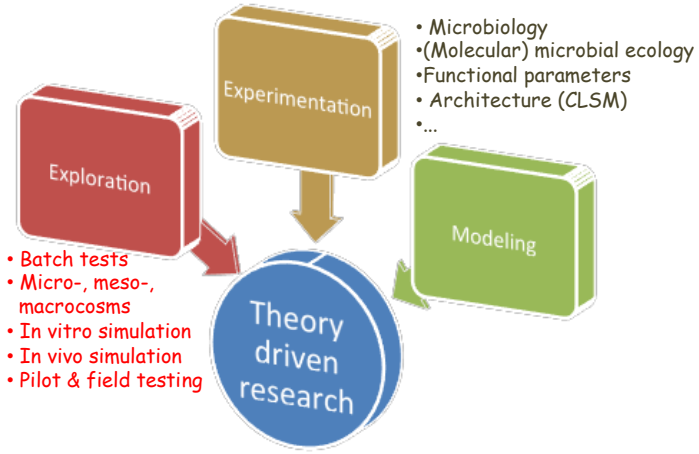

Figure 1. Exploration, experimentation and modelling approaches will be united to perform theory driven research

Understanding the interactions and their outcome within such a complex microbial community is essential for the development of a Microbial Resource Management (MRM) framework that would allow prediction and management of the behaviour of microbial communities (Read et al., 2011). The need for improved knowledge on interactions within microbial communities in different ecosystems has led to the development of synthetic ecosystems to generate theories and models for MRM, which will enable us to develop novel products and processes, and to improve our environment in the most sustainable way.

Results and discussion

Considering the current global biodiversity crisis, the biodiversity-stability relationship and the effect of biodiversity on ecosystem functioning have become major topics in ecology (Curtis and Sloan, 2004). Biodiversity is a complex term that includes taxonomic, functional, spatial and temporal aspects of organismic diversity, with species richness (the number of species) and evenness (the relative abundance of species) considered among the most important measures. With few exceptions, the majority of studies of biodiversity-functioning and biodiversity-stability theory have examined predominantly richness, showing that a minimal number of different species, i.e. more than 10, is needed to have a good ecosystem functionality. Using a total

of 1,260 microbial microcosms, our research group of UGent showed that initial community evenness is a key factor in preserving functional stability of an ecosystem (Wittebolle et al., 2009). It was found that the stability of the net ecosystem functioning in the face of selective stress, was strongly influenced by the initial evenness of the community (Figure 2). Therefore, when communities are highly uneven, or there is extreme dominance by one or a few species, their functioning is less resistant to environmental stress.

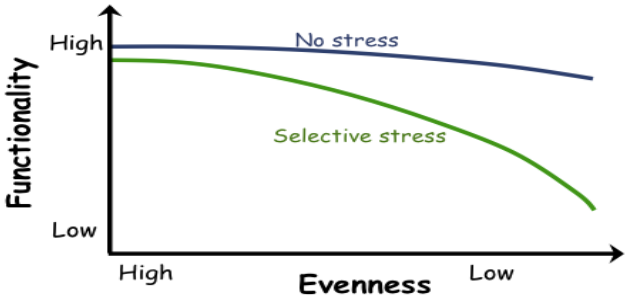

Figure 2. Microbial functionality in relation to the evenness. A selective stressor has a much more negative impact on the functionality of the unevenly distributed microbial community

However, as stated above, biodiversity also includes other aspects as the invasion potential. Biological invasion is widely studied, however conclusions on its outcome mainly originate from observations in systems that leave a large number of variables uncontrolled. This may lead to opposite conclusions on the role of a specific parameter, even in closely related ecosystems. Experiments conducted under controlled conditions give the opportunity to target some of these confounding factors, eventually explaining the inter-system variability. Also here a fully controlled system was used to evaluate the degree of invasion and the effect on the community functionality in relation to the initial community evenness under specific environmental stressors. More than 3000 assembled denitrifying bacterial communities with different levels of initial evenness but with the same richness were created and incubated with and without salinity stress (De Roy et al., 2013). The assembled community was challenged with a *gfp*-tagged, salt-resistant, non-denitrifying invader. After 20 hours of anaerobic incubation, the percentage of nitrite removal was used as a measure of functionality (i.e. denitrification). The invasion coefficient was determined by flow cytometry. In the absence of salt, invasion increased with an increasing Gini, a measure for the degree in evenness (with 0 an even and 1 an uneven community) (Fig 3a). The invader affected the performance of the community by lowering the overall functionality, independent of the Gini (Fig 3b) without influencing the growth of the community (Fig 3c). In the presence of salt (Fig 3d), the degree of evenness did not influence the

invasibility. However, the functionality was strongly influenced by the invader (Fig 3e). Under salinity stress and in the absence of invasion, nitrite was only partially reduced by the denitrifying communities with a high Gini. If the same communities were exposed to an invader, no negative correlation between functionality and the Gini coefficient was observed. The functionality was maintained at a high level over the complete range of evenness.

Conclusion

Under stress conditions an invasive species can preserve the indigenous functionality, while under non-stress conditions the functionality can be threatened. In the latter case evenness plays a crucial role in determining the community resistance to invasibility and in preserving ecosystem functionality.

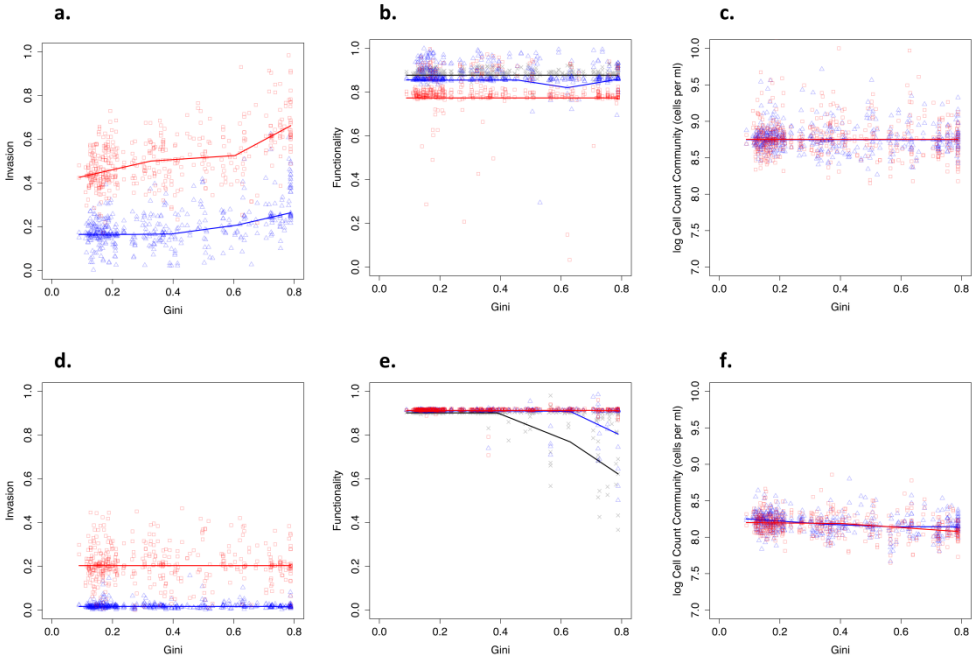

Figure 3. Invasion, community functionality and community growth in the absence and presence of salinity stress. The individual plots show invasion (a and d), its effect on functionality (b and e) and the growth of the community (c and f) in relation to the Gini coefficient in the absence (a, b and c) and presence (d, e and f) of salinity stress. Black, blue and red lines indicate the initial invader concentration of 0%, 0.1% and 1%, respectively.

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PL.4

YEAST-YEAST INTERACTIONS IN WINE

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Grape must and wine may be considered as a complex microbial ecosystem with a wide diversity of yeast species. In recent years, a growing number of studies have focused on the so-called non-*Saccharomyces* (NS) yeast species in winemaking for their suggested positive role in wine flavour development. It has therefore been suggested to use defined starter cultures of *Saccharomyces cerevisiae* (SC) and NS yeast species for mixed- or sequential culture wine fermentations in order to controllably achieve wines with enhanced and more complex flavours (Ciani et al., 2010). In fact, such defined wine starter cultures are now commercially available. To obtain, however, a better control of wine fermentations in which these starter cultures are used, it is crucial to gain more knowledge on the interactions taking place between SC and the NS yeast species. Typically, these interactions will result in the NS to arrest growth and die out during the early phases, and the SC to dominate the remaining part, of the fermentation. This paper will review and discuss some of the recent findings within this field of research, including the concept of quorum sensing. An overview of recent studies related to interactions of wine yeasts, focusing on growth and survival of NS, are shown in Table 1.

1. Toxic metabolites

More and more evidence are now at hand suggesting that ethanol produced by yeast during wine fermentations inhibit growth, and may even cause the death, of some NS yeast species in mixed cultures with SC, whereas it does not affect other NS yeast species and SC (Pina et al. 2005; Salvado et al., 2011). Thus, the difference in ethanol tolerance between SC and NS may explain the growth and survival interactions taking place between SC and some, but not all, of the NS. The role of other growth inhibitory yeast metabolites, such as e.g. short or medium chain fatty acids, acetaldehyde, and SO_2 on the succession of yeasts during wine fermentations is less described and merits much more investigation.

2. Fermentation conditions

It is well known that NS do not survive well under low available O_2 conditions, whereas SC does, and that this difference may explain, at least partly, the competitive advantage of SC over NS in wine fermentations (Hansen et al., 2001). Furthermore, in a recent study, mathematical models have been

used to assess the influence of temperature and ethanol on the fitness advantage of SC as compared to various NS yeasts isolated from wine fermentations (Salvadó et al., 2011). Based on these models, it is not ethanol that provides SC with an ecological advantage over NS (at least at ethanol concentrations below 9-10%), but rather the temperature increase produced by SC during fermentation. Thus, these authors suggest that low temperatures (below 15 ºC) should be used to favor growth and survival of NS during wine fermentations.

3. Nitrogen limitation

Recent data indicate that some NS have a higher nitrogen requirement than SC under anaerobic conditions (Blomqvist et al., 2012). The significance of this difference in nitrogen requirements for mixed-culture wine fermentations, however, still remains to be clarified.

4. Production of antimicrobial peptides by *S. cerevisiae*

SC may produce extracellular proteinaceous compounds having a toxic effect towards NS during wine fermentations (Perez-Nevado et al., 2006; Albergaria et al., 2010). These proteinaceous compounds have been characterized and identified as low molecular peptides having a size between 2-10 kDa, being non-heat labile and protease sensitive, and showing fungicidal activity against a range of NS (Albergaria et al., 2010).

5. Cell-cell contact

Cell-cell contact may also cause growth arrest and/or death of NS in mixed fermentations with SC (Nissen and Arneborg, 2003; Nissen et al., 2003; Renault et al., 2013). Very recently, this phenomenon has also been suggested to occur within different strains of SC (Perrone et al., 2013). The molecular mechanisms underlying the cell-cell contact phenomenon are, however, still to be elucidated.

6. Quorum sensing

As compared to bacteria, the phenomenon of quorum sensing has only been very scarcely studied in yeasts. Here, aromatic alcohols have been found to be quorum sensing molecules controlling morphogenesis (Hornby et al., 2001) and biofilm formation (Ramage et al., 2002). The dimorphic switch from unicellular to filamentous form regulated by quorum sensing in yeast was first observed in *Candida albicans* (Hornby et al., 2001) but has later been confirmed to occur also in SC (Chen and Fink, 2006). Recently, the aromatic alcohols regulating this phenomenon have also been shown to be produced by *Debaryomyces hansenii* (Gori et al., 2011) as well as by wine-related NS yeast species under model wine fermentation conditions (Zupan et al. 2013). Whether they play any role in yeast interactions during wine fermentations, however, remains to be investigated. Due to the fact that growth arrest and cell death of NS is only observed at high cell densities of SC in wine model, mixed-culture fermentations, a quorum sensing mechanism has been hypothesized to play a role in the succession of yeasts during wine fermentation (Nissen et

al., 2003; Perez-Nevado et al. 2006). This hypothesis, however, still remains to be experimentally verified, and the putative quorum sensing molecules regulating this phenomenon need, as yet, to be identified.

Table 1. An overview of recent studies on interactions of wine yeasts, focusing on growth and survival of non-*Saccharomyces* yeasts.

Non-*Saccharomyces*	Interaction	Explanation	References
T. delbrueckii *K. thermotolerans*	Early death of non-*Saccharomyces*	Low available oxygen conditions	(Hansen et al., 2001)
K. thermotolerans *T. delbrueckii*	Early growth arrest and death of non-*Saccharomyces*	Cell-cell contact Presence of viable *S. cerevisiae* at high cell concentrations	(Nissen and Arneborg, 2003; Nissen et al., 2003)
H. guilliermondii *H. uvarum* *K. thermotolerans* *K. marxianus* *T. delbrueckii*	Early death of non-*Saccharomyces*	Antimicrobial peptides produced by *S. cerevisiae*	(Perez-Nevado et al., 2006; Albergaria et al., 2010)
H. uvarum *T. delbrueckii* *P. fermentans* *K. marxianus* *C. zemplinina*	Growth competition between *S. cerevisiae* and non-*Saccharomyces*	Fitness advantage of non-*Saccharomyces* and *S. cerevisiae* by low and high temperature, respectively Inhibition of non-*Saccharomyces* growth by ethanol	(Salvadó et al., 2011)
*S. cerevisiae**	Growth competition within *S. cerevisiae* strains	Cell-cell contact	(Perrone et al., 2013)
T. delbrueckii	Early death of non-*Saccharomyces* and growth delay of *S. cerevisiae*	Cell-cell contact	(Renault et al., 2013)

*A study of different *S. cerevisiae* strains

As can be deduced from the above mentioned, the research field of yeast-yeast interactions in wine is still in its infancy, and most probably

it is not only one mechanism alone that regulates these interactions but rather several mechanisms that regulate them together. To unravel the full picture of this complicated issue, it will be necessary to e.g. conduct studies that combine the use of modern single cell techniques and functional genomics tools.

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PL.5

ECOLOGICAL SIGNIFICANCE AND APPLICATIONS OF BACTERIOCINS

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Introduction

Production of antimicrobial substances is important in competitive exclusion between microbial populations. Bacteria can release a variety of antimicrobial substances derived from cell metabolic activities (such as organic acids, free radicals, ammonia, reuterin or reutericyclin) or dedicated antimicrobial peptides which can be generated both by ribosomal and non-ribosomal synthesis. Bacteriocins can be defined as antibacterial proteinaceous substances of ribosomal synthesis, posttranslationally modified or not. Bacteriocin production seems to be widely disseminated among bacteria, especially among *Gammaproteobacteria* and *Firmicutes*. The genetic determinants for bacteriocin production and immunity are most frequently located on plasmids, some of which may be conjugative. Being strain-dependent, the genetic determinants for production of certain bacteriocins can be found in different strains of the same species or even in species of different bacterial genera (as in pediocin-like bacteriocins), suggesting a long history of evolution for this trait. Bacteriocin production and its associated immunity confer ecological advantages to producer strains in their microenvironments by displacing other strains of the same or even of different species. In stationary phase or other bottleneck situations, bacteriocin-killed cells may release essential nutrients and genetic material (Leisner & Haaber, 2012). For all these reasons, plasmids carrying bacteriocin genes are highly appreciated among bacteria, and each strain may be eager to get its own plasmid conferring bacteriocin production and immunity in order to better survive in microbial communities or even to co-exist with other producer strains of the same species.

Biofilm formation is another trait of relevance for bacterial survival, with practical implications. For example, in probiotic bacteria, adhesion to mucoses is one of the mechanisms by which they can outcompete other bacteria, including pathogenic bacteria. Production of antimicrobial substances in biofilms and by cells adhered on mucosal surfaces is considered to be relevant for displacement of bacterial pathogens, and bacteriocin production is often considered to be a probiotic

trait in this context. It is well known, from studies carried out in culture media and in foods, that bacteriocins act synergistically or have an additive effect in their antimicrobial activities when combined with other antimicrobials. Even the inhibitory spectrum of bacteriocins can be modified in combination with selected antimicrobials such as chelators of divalent cations. Interestingly, lactic acid bacteria may simultaneously secrete organic acids and bacteriocins. The precise role of this combined antimicrobial action on other bacterial populations in biofilms is not known, but it is well recognized that many bacteriocins have stronger antimicrobial activity under acidic pH conditions.

Applications derived from microbial antagonism: bacteriocins

Bacteriocins and their produced strains have been extensively studied for food preservation, as exemplified by nisin or enterocin AS-48, among others (Thomas et al., 2000; Gálvez et al., 2011). The antimicrobial activity of bacteriocins (and/or their producer strains) against biofilms (including the inhibition of biofilm formation and/or killing of cells embedded in biofilms) is a novel field of research. Interest in this research has been fueled by the fact that human pathogenic bacteria in biofilms are far more resistant to clinically used antibiotics. Biofilms can form on surfaces of any indwelling device, including heart valves, prosthesis implants, catheters, and others, becoming permanent reservoirs of bacteria as well as bacterial toxins. Biofilm formation is also considered problematic for dental health. In the food industry, biofilm formation is still an unresolved problem. Biofilms on processing equipment can increase corrosion rate, reduce heat transfer, and increase fluid frictional resistance. Most important, biofilms act as reservoirs in which human pathogenic and food spoilage bacteria can persist against cleaning and disinfection processes. Bacterial colonization of different kinds of materials (such as the surfaces of stainless steel, tubings, cutting boards..) that may come in contact with foods is thoroughly documented. The increased resistance to disinfection processes may be aggravated when biofilms are formed on surfaces that are more difficult for mechanical cleaning such as cracks, holes, or tube connections. The planktonic cells released from these colonization microenvironments may enter the food chain and, if proper conditions for growth occur, they can proliferate and compromise the food safety and stability. Recent studies suggest that bacteriocins can potentially be used to fight bacterial biofilms in the food industry, and interest in the use of combinations of antimicrobial compounds to improve biofilm removal has intensified.

Enterococci are well known commensals of the gastrointestinal tract of warm-blooded animals, including humans, and also found in foods where they play a role in ripening (as is the case of many traditional fermented cheeses). Enterococci have an outstanding capacity to produce bacteriocins, but only a few strains

have been described so far capable of producing enterocin AS-48. This bacteriocin is a cyclic peptide produced by *Enterococcus faecalis* and *Enterococcus faecium*. Most strains producing AS-48 have been derived from cheeses or ewe´s milk, but also as the predominant enterococci in the uropygial glands of hoopoe (*Upupa epops*) birds, where they may play a role in protecting the uropygial gland ecosystem and also in preventing proteolytic bacilli from colonization and degradation of bird feathers (Ruiz-Rodríguez et al., 2013). Enterocin AS-48 has a broad spectrum of antimicrobial activity against Gram-positive bacteria (*Listeria monocytogenes, Staphylococcus aureus* and species of genera *Bacillus, Geobacillus, Paenibacillus* and *Alicyclobacillus*) and is also active on Gram-negative bacteria (such as *Escherichia coli* or *Salmonella enterica*) when added in combination with outer-membrane permeabilizing agents. AS-48 has bactericidal activity, and in many cases it also has a secondary, bacteriolytic effect. The genetic determinants and structure of the mature peptide are well-established. Processing of the bacteriocin pre-peptide results in a cycled molecule with a head-to tail peptide bond, being the first circular bacteriocin characterized. Enterocin AS-48 can be purified easily by cation exchange chromatography and can be produced as a dry-powder preparation. It has been tested for preservation in different food systems, including meats, dairy products, seafoods, and vegetable foods and beverages (Abriouel et al., 2010). The lethal effect of AS-48 can be enhanced in combination with other antimicrobials or treatments such as heat, pulsed electric fields or high hydrostatic pressure. In the combined treatments, added bacteriocin also provides post-treatment protection during the product shelf life.

Recent works suggest that enterocin AS-48 is an interesting candidate as an antibiofilm substance for use in the food industry. A comparative study was carried out on a cocktail of *L. monocytogenes* cells in planktonic and in sessile state on polystyrene microtiter plates. Enterocin AS-48 was applied singly or in combination with different types of biocides, including representatives of quaternary ammonium compounds (QACs), biguanides, polyguanines, bisphenols, and oxidizing agents (Caballero Gómez et al., 2013a). Synergistic effects between bacteriocin and biocides were detected on planktonic cells, reducing the minimum inhibitory concentration of the bacteriocin on the cocktail of strains from 1.0 to 0.1 or 0.2 mg/l. Sessile cells were far more resistant to AS-48 as well as biocides (by 10 to 100-fold). Bacteriocin concentrations of 25 or 50 mg/l reduced the viable populations of *Listeria* only by 0.5 to 1.5 log units after 60 min contact. Inactivation of sessile listeriae increased remarkably when bacteriocin and biocides were combined, allowing the use of lower concentrations of antimicrobials. For QACs, best results (consisting in complete inactivation of biofilm populations) were obtained for combinations of enterocin AS-48 with cetrimide, hexadecylpyridinium

chloride, or didecyldimethylammonium bromide. Complete inactivation of the sessile listeria were also obtained for combinations of bacteriocin with hexachlorophene, chlorhexidine, or poly-(hexamethylen guanidinium) hydrochloride. Sygergistic effects were also detected in combinations with benzalkonium chloride and triclosan, but not with oxidizing agents. These synergistic effects were explained taking into consideration that enterocin AS-48 permeabilizes the bacterial cytoplasmic membrane, leading to depletion of the membrane electrochemical gradient. Most biocides have chaotropic effects on bacterial membranes, modifying lipid-lipid and lipid-protein interactions and leading to loss of the bilayer structure. Others (like triclosan) interfere with bacterial lipid biosynthesis, which has an indirect effect on membrane functionality. Accumulation of cell damage caused by biocides and AS-48 on bacterial membranes and depletion of energy supply required for cell damage repair could explain the observed increases in antimicrobial activities in the bacteriocin-biocide combinations.

In experiments carried out with a cocktail of *Bacillus cereus* strains, addition of enterocin AS-48 (25 mg/l) increased the inactivation of planktonic cells by QACs, bisphenols, biguanines and polyguanine. Increased inactivation of the bacilli was also observed for the combination of enterocin AS-48 with P3 oxonia, but not by P3 topax 66 commercial disinfectants (Caballero Gómez et al., 2013b). In the sessile state, the bacilli were more resistant to biocides and also to the bacteriocin-biocide combinations. Hexadecylpyridium chloride was the most active biocide on biofilms in the single treatments. In the combined treatments with 50 mg/l bacteriocin, hexadecylpyridinium, polyhexamethylen guanidinium chloride and P3 oxonia achieved complete inactivation of bacilli populations. Bacterial endospores from *B. cereus* are resistant to AS-48, but they become gradually sensitive in during the course of germination. When a cocktail of endospores was challenged with biocides and enterocin AS-48 for 60 min at temperatures of 22ºC, 40ºC, and 60ºC, enterocin AS-48 did not significantly reduce viable spore counts or increase the lethal effect of biocides. However, treatments at 60ºC with benzalkonium chloride, hexadecypyridinium or P3 oxonia achieved complete inactivation of bacterial endospores, both singly and in combination with bacteriocin. Significant reductions of viable counts (1 to 2 log cycles) were also obtained for some treatments with cetrimide, triclosan or polyhexamethylen guanidinium chloride.

S. aureus is a food poisoning bacterium but it is also known as a human pathogen capable of forming biofilms on catheters and other indwelling devices. Methicillin-resistant strains are of major clinical significance and, most worrying, are becoming associated to food environments. Control of staphylococci during cleaning and disinfection is equally important to the food industry. Enterocin AS-48 was tested singly or in combination with biocides against a cocktail of six *S.*

aureus strains (including three methicillin-resistant strains) in planktonic state as well as in biofilms formed on polystyrene microtiter plates (Caballero Gómez et al., 2013c). Inactivation of planktonic cells increased significantly when enterocin AS-48 (25 mg/l) was tested in combination with benzalkonium chloride, cetrimide and hexadecylpyridinium chloride and non-significantly in combination with didecyldimethylammonium bromide, triclosan, hexachlorophene, polyhexamethylen guanidinium chloride, chlorhexidine or P3-oxonia. In the sessile state (24 h biofilms), staphylococci were the most resistant bacteria to enterocin AS-48, and bacteriocin treatments at 50 mg/ml did not reduce biofilm populations. Inactivation of sessile cells also required higher biocide concentrations in most cases, except for P3-oxonia. Inactivation of sessile staphylococci increased remarkably when biocides were applied in combination with enterocin AS-48, especially when the bacteriocin was added at 50 mg/l (Figure 1). During storage, the concentrations of sessile as well as planktonic cells in the treated samples decreased remarkably for benzalkonium chloride, triclosan and polyhexamethylen guanidinium chloride, but P3-oxonia failed to inhibit proliferation of the treated biofilms as well as growth of planktonic cells. The observed inhibitory effects during storage were potentiated when the biocides were combined with 50 mg/l enterocin AS-48.

A different approach to deal with the problem of biofilm formation was the use of adsorbed bacteriocin. Polystyrene microtiter plates conditioned with enterocin solutions (0.5 to 25 mg/l) decreased the adherence and biofilm formation of the *L. monocytogenes* cell cocktail, avoiding biofilm formation for at least 24 h at a bacteriocin concentration of 25 mg/l. When tested on *B. cereus*, polystyrene surfaces dosed with enterocin AS-48 (25 or 50 mg/l) remained free of detectable bacilli from 2 to 24 h after being inoculated with a cocktail of endospores. However, stainless steel surfaces dosed with 50 mg/l bacteriocin did not prevent bacterial growth from endospores. These results suggest different adsorption/desorption coefficients for enterocin AS-48 depending on the substrate material. For *S. aureus*, polystyrene-adsorbed bacteriocin delayed biofilm formation, but did not prevent bacterial growth after 24 h of incubation. These results were explained by the higher resistance of sessile staphylococci to the bacteriocin.

Conclusions

Results from these studies illustrate the influence of ecological conditions (planktonic or sessile bacterial cell state) on the efficacy of enterocin AS-48. This bacteriocin could be applied as enhancer of biocide activity against planktonic and sessile cells of *L. monocytogenes*, *B. cereus* and *S. aureus*. The combinations of enterocin AS-48 and biocides could open new possibilities for disinfection, reducing the concentration of biocide (generally

obtained by chemical synthesis) while adding a natural product (the bacteriocin). The synergistic effect observed on methicillin-resistant staphylococci suggest new possible strategies to combat dissemination of antibiotic-resistant bacteria through the food chain based on combinations of bacteriocins and biocides.

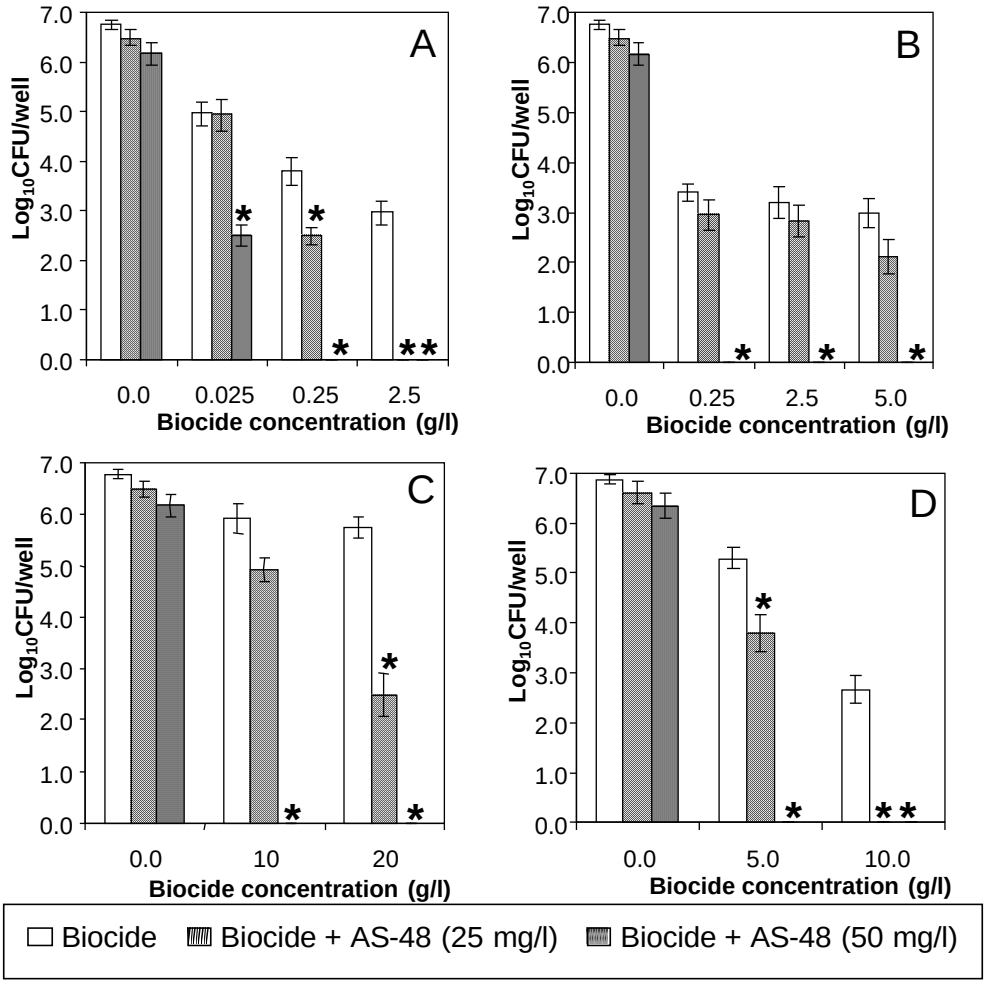

Figure 1. Effect of biocides applied singly or in combination with enterocin AS-48 (25 or 50 mg/l) on the viability of 24-h biofilms formed by a cocktail of six *S. aureus* strains on polystyrene microtiter plates. The biocides tested were benzalkonium chloride (A), cetrimide (B), chlorhexidine (C) or hexachlorophene (D). Asterisks denote statistically significant reductions ($p < 0.05$) of viable cell concentrations in the biofilms.

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PL.6

MICROBES AND THE HUMAN GUT MICROBIOME: A BIFIDOBACTERIAL PERSPECTIVE

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Abstract
The human gut is colonized by several hundreds of trillions bacteria which are believed to be essential for health and well-being of the host. Though high throughput sequencing technologies have allowed an unprecedented view regarding the composition and diversity of this gut microbiota, the precise mechanisms by which they interact with their host and convey these beneficial effects are only partially understood. Bifidobacteria are typical inhabitants of the gastro intestinal tract (GIT) of humans and other mammals, and are particularly dominant in healthy, breast-fed infants. In recent years a wealth of information has become available on the prevalence and diversity of bifidobacteria in the infant GIT, while various mechanisms have been elucidated by which they colonize and interact with their host.

Bifidobacteria: how many and how diverse?

In 1899 Henri Tissier isolated for the first time bifidobacteria from feces of breast-fed infants; these microorganisms were initially named *Bacillus bifidus communis* (Tissier, 1900), because of their particular y-shape morphology. In fact, the term *bifidus* in Latin means 'forked in two parts'. The possible formation of a connection between lactic acid bacteria (LAB) and the propionic acid bacteria led Orla-Jensen in 1924 to propose a new classification that considered this *Bacillus bifidus* as a new separated genus with the name *Bifidobacterium* (Ventura et al., 2006).

At the time of writing, the genus *Bifidobacterium* encompasses 47 species, including four taxa (*Bifidobacterium longum*, *Bifidobacterium pseudolongum*, *Bifidobacterium animalis*, *Bifidobacterium thermacidophilum*) that are further divided into subspecies, all of which share more than 93 % identity in their 16S rRNA sequences (Miyake et al., 1998). Among the 47 currently recognized bifidobacterial species are four species that were isolated from the digestive tract of a bumble bee, i.e., *Bifidobacterium actinocoloniiforme*, *Bifidobacterium bohemicum*, *Bifidobacterium coagulans* and *Bifidobacterium bombi* (Killer et al., 2010; Killer et al., 2011; Killer et al., 2009), as well as five

bifidobacterial taxa identified from feces of common marmoset (*Bifidobacterium reuteri*, *Bifidobacterium callitrichos*, *Bifidobacterium saguini*, and *Bifidobacterium stellenboschense*) and one species that was isolated from feces of a red-handed tamarin (*Bifidobacterium biavatii*) (Endo et al., 2012).

With the recent advent of the genomic era, the availability of gene sequences of many possible alternative molecular markers aside from the 16S rRNA gene, allowed bacterial taxonomists to build so-called phylogenetic supertrees, which provide a more robust image of the evolutionary development of a specific bacterial taxonomic unit. When this approach was applied to the *Bifidobacterium* genus, six distinct phylogenetic groups were identified, which include *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Bifidobacterium pullorum*, *Bifidobacterium pseudolongum*, *Bifidobacterium boum* and *Bifidobacterium asteroides* (Ventura et al., 2006). Notably, the achieved supertree allowed the identification of the putative ancestor of the genus *Bifidobacterium*, which was determined to be most closely related to representatives of the *B. asteroides* phylogenetic group (*Bifidobacterium asteroides*, *Bifidobacterium bombi*, *Bifidobacterium indicum*, *Bifidobacterium coryneforme*, *Bifidobacterium actinocoloniiforme*, *Bifidobacterium bohemicum*). It therefore seems that bifidobacteria took up residence in the insect gut and subsequently specialized to occupy the gastrintestinal tract of mammals, reptiles and birds.

Bifidobacterial genomes reveal that they utilize diet- and host-derived carbohydrates

The first bacterial genome sequence of a bacterial pathogen was published in 1995, and within 15 years, more than 1000 microorganisms were fully sequenced and deposited in the NCBI database. This large amount of genomic data has shed light on the genetic basis of bacterial pathogenesis and has lead to the formulation of a new genomics-based discipline called pathogenomics (Pallen and Wren, 2007). Only relatively recently, genomic efforts have focused on the decoding of genome sequences from human gut commensals, including health-promoting bacteria, such as bifidobacteria. In recent years, 27 bifidobacterial genomes have been completely sequenced of the 47 bifidobacterial taxa so far recognized (Kim et al., 2009) (Lee et al., 2008; Schell et al., 2002; Sela et al., 2008; Bottacini et al., 2012; Turroni et al., 2010; Ventura et al., 2009; O'Connell Motherway et al., 2011), with another 25 strains whose genome sequences are still unfinished (NCBI source). This huge amount of genomic data provides intriguing information about specific characteristics of bifidobacteria (e.g., metabolic capabilities, genetics and phylogeny) and provides basic scientific information regarding the molecular mechanisms responsible for their adaptation to a specific ecological niche. Bifidobacterial genome sequences can notably contribute to the

understanding of genetic adaptation to specific niches such as the (breast-fed) infant gut in the case of *B. longum* subsp. *infantis* ATCC15697 (Sela et al., 2008), the oral cavity as was shown for *B. dentium* Bd1 (Ventura et al., 2009), or the human gut in the case of *B. bifidum* PRL2010 (Turroni et al., 2010). Also, *in silico* analysis of the recently sequenced genome of *B. asteroides* PRL2011 reveals the genetic adaptation of this bifidobacterial strain to the insect intestine (Bottacini et al., 2012). *In silico* analyses of the genome sequence of *B. longum* subsp. *infantis* ATCC 15697, which revealed the presence of a large genomic locus whose encoded protein products are involved in the metabolism of Human Milk Oligosaccharides (HMO) (Sela et al., 2008). This cluster consists of a 43 kb DNA region, encoding specific intracellular glycoside hydrolases, such as fucosidases, sialidases, β-hexosaminidase and β-galactosidase, extracellular solute binding proteins and permeases, predicted to hydrolyze and internalize human milk-derived oligosaccharides thereby generating monosaccharides that enter the central metabolic pathway involving the typical fructose-6-phosphate phosphoketolase enzyme (Sela et al., 2008).

Another dominant infant gut bifidobacterial species is *B. bifidum* (Turroni et al., 2009). Various physiological investigations revealed that members of the *B. bifidum* species are able to metabolize host-derived glycans, such as mucin (Ruas-Madiedo et al., 2008). The complete genome sequence of *B. bifidum* PRL2010 was published in 2010 (Turroni et al., 2010) and revealed interesting metabolic strategies followed by *B. bifidum* to metabolize mucin-derived carbohydrates. *In silico* analyses coupled with functional genomic analysesa highlighted the existence of specific *B. bifidum* enzymatic pathways involved in the utilization of host-derived glycans (Turroni et al., 2010).

The availability of the complete genome sequences of the oral opportunistic pathogen *B. dentium* Bd1 represents another key example of the importance of genomics in understanding the biology of bifidobacteria (Ventura et al., 2009). Recent studies of oral bifidobacteria related to dental caries in adults and children revealed that *B. dentium* represents about eight percent of the culturable bacteria isolated from carious lesions (Ventura et al., 2009). Genome analysis of *B. dentium* Bd1 displayed that this strain possesses the necessary metabolic capabilities to utilize a large variety of carbohydrates, including both simple sugars as well as complex carbohydrates (Ventura et al., 2009). This may reflect the ecological origin of this microorganism; in fact during every meal the human oral cavity would be expected to contain a large amount of simple carbohydrates that are preferentially utilized by the microbiota encountered in this GIT compartment. At the same time *B. dentium* Bd1 is able to survive in the human fecal material, where the only carbon source is represented by complex carbohydrates.

Bifidobacteria and host interactions: pili and surface polysaccharides.

Recently, the genome sequence of *B. breve* UCC2003 has been fully decoded (O'Connell Motherway et al., 2011), revealing the presence of a gene cluster predicted to be involved in the production of two different cell surface-associated EPSs (Fanning et al., 2012). Surface EPS produced by UCC2003 influenced *in vivo* persistence of bifidobacterial cells, while EPS-producing *B. breve* UCC2003 cells stimulate only a weak adaptive immune response compared with EPS-deficient mutants lacking this cell envelope-associated structure. Specifically, EPS production was shown to be linked to the evasion of the B-cell mediated adaptive immune response. In addition, the presence of surface EPS-expressing UCC2003 cells in a murine model reduced colonization levels of the murine pathogen *Citrobacter rodentium*. These findings indicate a crucial and beneficial role for bifidobacterial surface EPS in modulating various aspects of host-microbe interaction, including host-mediated immune tolerance of the commensal, while providing protection against a pathogen in an as yet unknown manner (Fanning et al., 2012).

Another key structure produced by gut bacteria, which are implicated in host-microbe interactions is represented by pili. Pili are proteinaceous appendages present on the surface of both Gram negative and Gram positive bacteria. However, their presence was only very recently established in bifidobacteria (Foroni et al., 2011; O'Connell Motherway et al., 2011; Turroni et al., 2013). In this context, it has been shown that bifidobacterial genomes belonging to *B. bifidum*, *B. longum* subsp. *longum*, *B. adolescentis*, *B. dentium*, *B. animalis* subsp. *lactis* and *B. breve* encompass one to seven predicted sortase-dependent pilus gene clusters, each of which are predicted to encode one major pilin subunit plus one or two minor pilin subunits, as well as a so-called sortase, a protein specifically dedicated to covalently assemble these pilin subunits (Foroni et al., 2011; Turroni et al., 2013). Other pili-like structures, recently identified in bifidobacteria, are represented by a member of the so-called type IVb or tight-adherence (Tad) pilus family, which was recently shown to be specifically expressed by *B. breve* UCC2003 under *in vivo* conditions in a murine (O'Connell Motherway et al., 2011). Mutational analysis of the corresponding *tad* locus of *B. breve* UCC2003 shown that this cluster is essential for efficient *in vivo* murine gut colonization. Interestingly, the *tad* locus is conserved among all the so far sequenced bifidobacterial genomes, which supports the notion of a ubiquitous pilus-mediated host-colonization and persistence mechanism for intestinal bifidobacteria (O'Connell Motherway et al., 2011).

Questions to be answered and a look at the future

For millennia mammals have existed with their commensal partners, and adaptive co-evolution has formed very complex links between the gut microbiota and their host. Imbalances

in the gut microbiota may contribute to certain human intestinal diseases. Furthermore, it is generally accepted that gut bacteria such as bifidobacteria clearly affect the regulatory network of the immune system, which compounds the intricate connection between gut microbiota composition and host health. Here, we have provided a number of telling examples of molecules, such as pili and capsular or surface polysaccharides, that mediate bifidobacterial host microbe interaction. The first decade of genomic exploration the biology of gut commensals, such as bifidobacteria, has afforded unprecedented insights into the genetic adaptation of these microorganisms to the human gut through the decoding of their genome sequences (probiogenomics). The next decade holds the promise of being even more rewarding as new discoveries on the molecular mechanisms underpinning host-microbe interactions are generated by means of functional genomics efforts.

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SELECTED ORAL LECTURES

O1.1

EFFECT OF LONG-TERM BACTERIAL AND FUNGAL STRESS ON BIOFILMS OF LIGNINOLYTIC FUNGI

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Introduction

Biodegradation power of ligninolytic fungi (LF) for removal of organopollutants in water and soil have been well established and efficient strains selected. When applied to bioremediation of contaminated water LF are often used as biofilms colonizing a solid support. These biofilms have to be applicable over long periods under non-sterile conditions exposed to other microorganisms, namely bacteria. The knowledge of interactions between fungi and bacteria in such biofilms has been rather poor and the effect on their remediation performance mostly unknown (e.g. Mikesková et al., 2012). The presence of bacteria in submerged cultures of *Trametes versicolor* suppressed fungal degradation probably due to a competition for the C source and resulted in vacuolization of the hyphae and bacterial growth on the mycelial surface (Borchert and Libra, 2001). In contrast, fixed-film cultures of *Phanerochaete chrysosporium* exhibited high and stable degradation efficiency(Gao et al.,2008). Interactions with bacteria and fungi can result in increased levels of laccase in LF, sometimes leading to higher biodegradation rates (Baldrian, 2004; Hiscox et al.,2010). In studies carried out in soil, however, the presence of bacteria often lead to a lower laccase production and decreased PAH degradation by LF(Andersson et al.,2000).

Our purpose was to study interactions between *Irpex lacteus* and *Dichomitus squalens*, preselected as efficient textile-dye LF degraders, and model pro- and eukaryotic microorganisms, *Escherichia coli* and *Saccharomyces cerevisiae*. Long-term effects of bacterial/yeast stress on fungal biofilms that were either pre-formed on an inert carrier or were in the course of formation in the presence of the bacterium or the yeast were observed. The effects on growth inhibition, production of fungal extracellular enzyme activities involved in degradation, and on degradation capacity of LF biofilms measured as decolorization of the anthraquinone dye Remazol Brilliant Blue R (RBBR) were monitored.

Material and Methods

Microorganisms: *I. lacteus* CCB 931, *D. squalens* CC B750 (Culture Collection of Basidiomycetes ASCR, Prague); *E. coli* CCM 3988, *S. cerevisiae* CCM8191 (Czech Collection of Microorganisms, Brno). Growth inhibition between microorganisms was measured on agar plates inoculated with a mycelium-covered agar disk and drops of a bacterial culture (10^{10} CFU.ml^{-1}). The plates were grown at 28ºC and inhibition evaluated as a zone width between the fungal and bacterial colonies. Standard enzyme assays: LiP (verathryl alcohol assay), MnP (2,6-dimethoxyphenol assay) and laccase (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid assay), see Novotný et al. (2012).

In order to prepare pre-formed fungal biofilm cultures the LF was let to colonize polyamide mesh for 1 week before a 1-d bacterial or yeast culture grown in nutrient broth- (NB, Difco) or malt-extract/glucose (MEG) medium (Novotný et al., 2012), respectively, was added at 10^6 CFU/ml and static growth started at 28 ºC. Nascent fungal biofilms were prepared in a similar way, only the bacterial inoculum was added simultaneously with the fungal inoculum that was prepared by homogenization of a 7-d static liquid culture growing on a mineral medium (Novotný et al., 2012). This low-nitrogen mineral medium was also used in all experiments with fungal biofilms. Three replicate cultures were used in the biofilm experiments together with corresponding biotic and abiotic controls. One week after the formation of fungal biofilms was started, RBBR (150 mg.l^{-1}) was added to the biofilm cultures and the decolorization measured spectrophotometrically. Subsequent decolorization cycles were investigated by applying additional portions of the dye during the experiment. The bacterial and yeast cell counts were determined by plating on NB or MEG plates, respectively.

Scanning electron microscopy analysis of biofilms: samples were fixed in OsO4 vapor or with 3% glutaraldehyde, dehydrated through alcohol series, critical-point-dried, putter-coated with gold and analyzed with an Aquasem scanning electron microscope.

Results and discussion

Pre-formed 7-d-old biofilms of *I. lacteus* and *D. squalens* growing in the low-nitrogen mineral medium pH 6 were exposed to *E. coli* or *S. cerevisiae* (final concentration 10^6 cells.ml^{-1}) and their efficiency to decolorize RBBR was measured in subsequent decolorization cycles. Fig. 1 shows that no inhibitory effect of the bacterium was observed in three sequential decolorization cycles by *D. squalens* whereas in *I. lacteus* a small inhibition up to 20% was observed in 2nd and 3rd cycles (not shown). These results were in agreement with those of Gao et al. (2008) obtained with immobilized *P. chrysosporium* whose degradation efficiency was high and stable irrespective of the presence of bacteria. In control fungal cultures without the bacterium or yeast, the decolorization efficiency was 90-100% within 12 days. When the yeast was used as the competing organism, no inhibition of the decolorization by the two LF was

observed. No decolorization by the bacterium or yeast control cultures was detected.

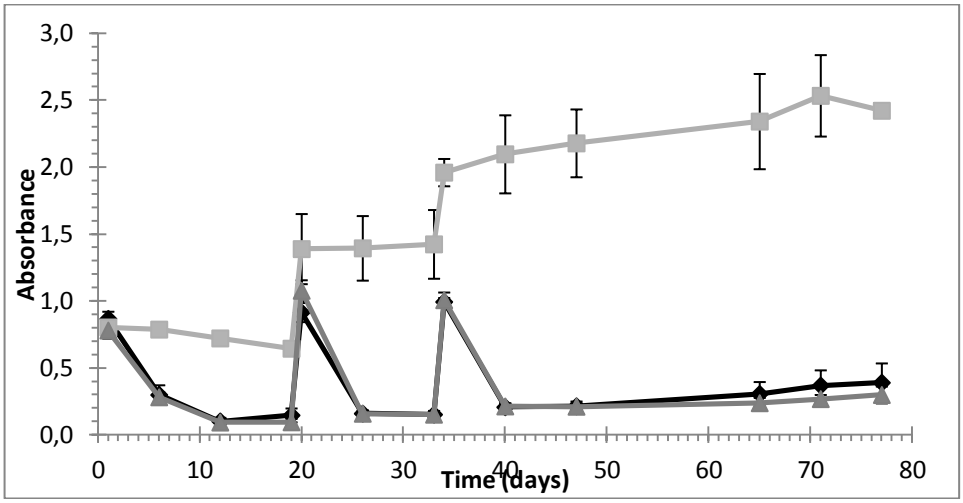

Figure 1. Decolorization of RBBR dye in three subsequent cycles by pre-formed biofilm cultures of *D. squalens* exposed to *E. coli* in low-nitrogen mineral medium. The dye was added at Days 0, 30 and 35. *D. squalens* + *E.coli* + RBBR (▲-▲), *D. squalens* + RBBR (♦-♦), *E. coli* + RBBR (■-■).

The enzyme activities of extracellular peroxidases and laccase involved in dye degradation were monitored in the course of the decolorization experiments. The bacterium did not influence laccase- or MnP levels of *D. squalens* (Table 1). No increase of laccase was observed in the presence of the competing microorganisms in either *D. squalens* or *I. lacteus*, comparable to the findings of Baldrian (2004) and Hiscox et al. (2010). The presence of the yeast did not affect laccase- and peroxidases levels in the two LF either.

The amounts of the bacterium and the yeast were monitored during the decolorization experiments. *E. coli* present alone in the low-nitrogen mineral medium pH 6 did not survive for more than 3 weeks. In contrast, in the presence of *I. lacteus*, the initial *E. coli* viable cell counts increased 2-3 times. In co-cultures with *D. squalens*, no bacterial cells were detectable in the culture liquid after 2 weeks. However, live bacterial cells were always found adsorbed to fungal hyphae in the biofilms as demonstrated by fluorescence microscopy. The yeast survived when cultivated alone in the mineral medium but the live cell amounts did not increase. A significant sorption of yeast cells to fungal hyphae was also observed.

Scanning electron microscopy demonstrated no harmful effect of long-term bacterial presence on the structure of fungal biofilms.

Table 1. Maximal enzyme activities in pre-formed biofilm cultures of *D. squalens* exposed to *E. coli* in low-nitrogen mineral medium measured in the presence and absence of RBBR dye.

Culture type	Maximal enzyme activities (units per litre)		
	Laccase	MnP	LiP
D. squalens	197.4±55.6	23.8±14.0	32.1±19.5
D. squalens+RBBR	262.0±20.4	12.3±6.8	31.0±7.1
D. squalens+*E. coli*	205.6±33.8	25.5±26.1	26.7±2.2
D. squalens+*E. coli*+RBBR	258.6±29.4	9.3±2.2	56.2±23.3
E. coli	0	0	0

Note: MnP, manganese-dependent peroxidase; LiP, lignin peroxidase.

Conclusions

The study demonstrated resistance of LF biofilms to bacterial and yeast stress and showed that their degradation capacity was maintained during a long-time exposure to bacterial or fungal infection. Degradation efficiencies of pre-formed biofilms and those formed in the presence of *E.coli* were similar and did not decrease up to 3 months in the presence of the bacterium. Bacterial and yeast cells had a strong tendency to adsorb to the surface of fungal biofilms. Behavior of fungal biofilms in the presence of bacterial and yeast stress was favorable with respect to their application in bioremediation and to a possibility of tailoring mixed biofilms for biodegradation purposes.

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O1.2

BACILLUS CEREUS BIOFILM FORMATION: ROLE OF SIGMA 54 IN THE INTERACTION WITH A SURFACE

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Introduction

The biofilm lifestyle is common for many microorganisms, including spore forming pathogenic Bacillus cereus. Biofilms can be difficult to eradicate from surfaces including stainless steel used in the food industry and microbes embedded in biofilms often display increased resistance to antimicrobial agents because the self-produced matrix of extracellular polymeric substances functions as a protective barrier. The objective of this project is to provide insight in factors contributing to biofilm formation and the molecular mechanisms that are involved in biofilm development. Insight may provide new leads to prevent establishment of biofilms and develop more effective strategies to eradicate them.

Approach

Previous studies mostly with gram-negative bacteria suggested the alternative sigma factor Sigma 54 (also referred to as RpoN or SigL) to play a central role in the physical interaction of bacteria with their environment including biofilm formation. The gene encoding Sigma 54 is also present in all members of the Bacillus cereus group. This triggered us to construct a targeted mutant in rpoN encoding Sigma 54 in Bacillus cereus ATCC14579. Next to transcriptome analysis, the mutant and its complemented counterpart were phenotypically characterized for biofilm formation and a range of other relevant functionalities.

Results and Conclusion

Biofilm formation of Bacillus cereus ATCC14579 and its mutant derivative was evaluated on stainless steel coupons in BHI medium. The wild type strain forms biofilm preferentially on the liquid-air interface. The mutant derivative was dramatically affected in biofilm forming capacity including loss of air-liquid biofilm formation and tendency to form submerged biofilms at reduced levels. Other cellular characteristics were affected in the mutant including growth and sporulation characteristics, cell and colony morphotype, swimming and swarming behaviour. Combining

genome sequence information, Sigma 54 regulon prediction, comparative transcriptome analysis and phenotyping of wt, mutant and complemented strain, has provided novel insights in the role of this alternative sigma factor in B. cereus performance.

Keywords: Bacillus cereus; biofilms; sigma 54

O1.3

COOPERATIVE BIOFILM FORMATION OF *ENTEROCOCCUS FAECIUM* AND *TRICHOSPORON FAECALE* ON STAINLESS STEEL IN STATIC AND DYNAMIC CONDITIONS

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Abstract
In the present study we evaluated the ability to form biofilm on stainless steel of the opportunistic pathogenic microbes *Enterococcus faecium* and *Trichosporon faecale*, alone and in combination. The two strains employed in the study have been previously isolated from the same biofilm debris found onto a plastic mold in a dairy industry.

Introduction
Some microorganisms have a natural tendency to adhere to surfaces as a survival mechanism, producing biofilms (Abee et al., 2011). Biofilm cells are more resistant to cleaning and disinfection processes (Kostaki et al., 2012) and represents a big concern for hygiene in food industrial environment and a risk for public health (Srey et al., 2013; Carpentier et al., 1993). While much has been written about the bacterial biofilm (Van Houdt et al., 2010), less is known in the case of fungi and very little about cooperative formation of biofilms by members of these two kingdoms**.**

Material and methods
Adhesion tests were performed using 1 cm^2 stainless steel washers, in order to have a standardized surface usable for both static and dynamic tests, thanks to the possibility of medium flow through the washers in dynamic conditions. In the static experiments, the washers were inoculated in a 10^8 cells/mL cell suspension followed by a thorough wash to eliminate all cells not firmly attached to the surface. These washers were maintained immersed in diluted medium mimic of realistic situations in food industry. The biofilm evolution was determined over the time by SEM observation of the washer surface and by counting the viable cells detached from the surface with glass bead washing. Dynamic experiments were carried out by flowing appropriate media through the washers after an inoculation phase. In this case, biofilm evolution was observed by counting the

cells adhering (sessile) and those freely suspended in the medium (planktonic).

Results and discussion

Biofilm formation on stainless steel in static conditions was observed after 72 h for both *E. faecium* ($3.77*10^5$ CFU/cm^2) and *T. faecale* ($1.95*10^3$ CFU/cm^2). In the subsequent days, the two species showed a generalized decrease of the cell density. *E. faecium* decreased by three orders of magnitude in six days, indicating that this bacterium has the capability to adhere to stainless steel, but most of the cells either dye or leave the biofilm, in a way that we are trying to elucidate with further analyses. On the contrary, the fungus remained substantially stable over the period between the third and ninth day (Figure 1a and 1b). The co-culture of the two strains showed an increasing trend over the time for both species in an almost parallel way. Namely, the bacterium increased the density by one order of magnitude between the 3rd and 9th day, whereas the fungus increased by almost four orders of magnitude in 9 days (Figure 1c). At the end of the experiment the bacterium and the fungus showed cell densities of $1.0*10^6$ and $7.9*10^3$ CFU/cm^2 respectively. These data together show that the fungus grows well on the washers and form a sort of "priming layer" onto which the bacterium can grow well. The SEM observations (data not shown) confirmed these observations.

Biofilm formed by single cultures, in dynamic conditions during five days experiments, showed a two-fold increase of sessile *E. faecium* cells (from $6.7*10^5$ to $2.1*10^6$ CFU/cm^2), while the planctonic cells decreased during the first two days from $7.4*10^8$ to $9.3*10^7$ CFU/ml, then stabilized in the next three days (Figure 1d).

The sessile *T. faecale* increased by almost one order of magnitude during the experiment (from $1.2*10^3$ to $9.7*10^3$ CFU/cm^2) while the planctonic cells decreased during the first three days from $1.3*10^6$ to $6.3*10^4$ CFU/ml, then in the next three days returned to the density of the third day (Figure 1e).

The co-culture (Figure 1 f) of the two strains showed that the planktonic cells decreased in the first two days and then reached almost the original densities, with the bacterial cells three to four orders of magnitude more dense than the fungi. The sessile cells of the bacterium decreased by almost one order of magnitude from $7.7*10^5$ to $9.4*10^4$ CFU/cm^2. The fungal sessile cells reached a maximum after 2 days, whereas at the experiment beginning and at the end of the experiment the density were $2*10^3$ and $1.33*10^3$, respectively.

These data suggest that the biofilm formation if favored by the co-culture, as showed by static experiments. The mixed biofilm structure is probably more fragile as suggested by the decreasing trends of the dynamic experiments. Moreover, the fact that bacteria, more than fungi, decreased during the dynamic experiments could be explained by supposing that the latter formed a structure adherent to the support, which included the *E. faecium*, that in turn adhered to the fungal cells and not directly to the stainless steel.

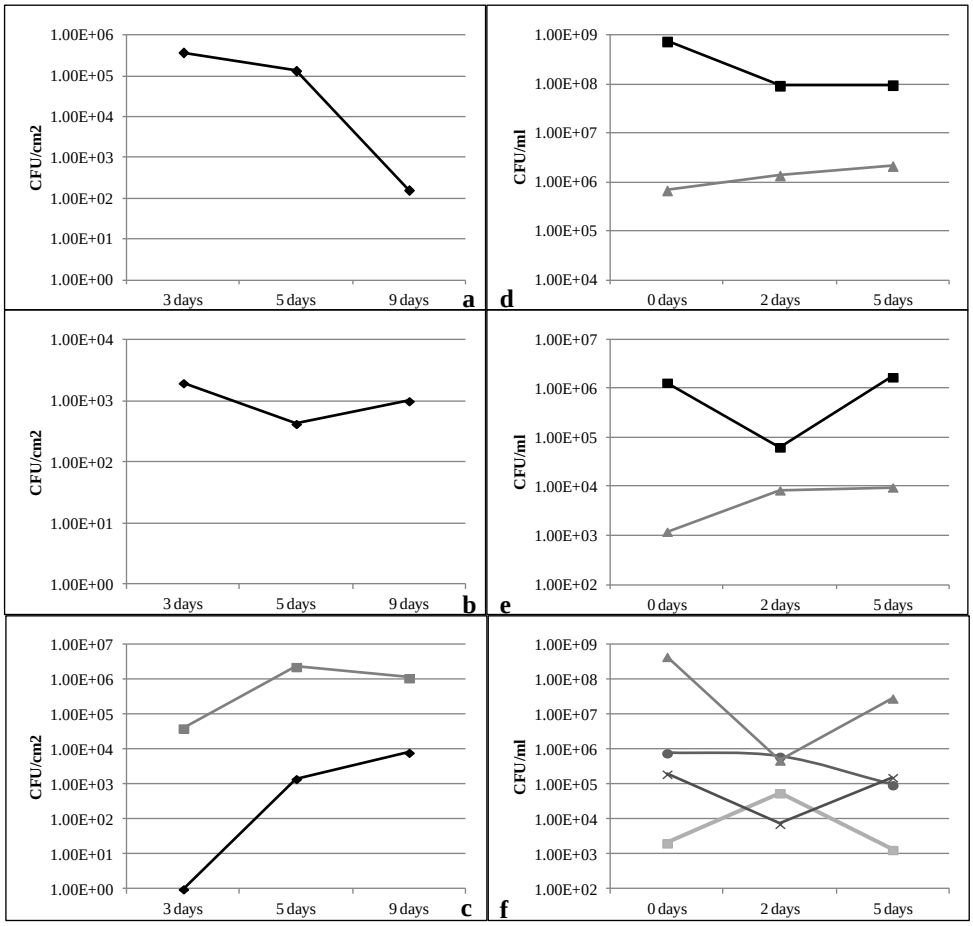

Figure 1. Biofilm formed in static and dynamic conditions by single cultures and by co-culture of *Enterococcus faecium* and *Thricosporon faecale* strains.
Legend. Panels a, b and c: biofilm formed in static condition by *E. faecium*, *T. faecale* and by the co-culture of the two strains, respectively. **Squares** represent *T. faecale* while **triangles** the *E. faecium* cells, respectively. **Panels d and e:** biofilm formed in dynamic condition by *E. faecium* and *T. faecale*, respectively. **Squares** represent the planktonic while **triangles** the sessile cells, respectively. **Panel f:** biofilm formed in dynamic condition by the co-culture of the two strains. **Triangles:** planktonic cells of *E. faecium*; **Crosses:** planctonic cells of *T. faecale*; **Circles:** sessile cells of *E. faecium*; **Squares:** sessile cells of *T. faecale.*
Data refer to the biofilm removed from the washers (sessile cells in static and dynamic conditions) and to the planktonic cells in dynamic experiments.

Conclusion

Microbes are often present in nature and in industrial environments as biofilm and very rarely as pure cultures, making the mixed biofilm one of the most likely form of microbial life and

presence in various environments. In spite of their importance, still little is known on biofilm formed by more than one species. Modelling in laboratory binary cultures is rather different because of various technical problems and limitations. In the case of multiple cultures in biofilm, any modelling should at least consider the possibility that each of the cultures adheres to the support or to another culture acting as a sort of priming in the biofilm formation mechanisms. The role of each culture is therefore depending on its affinity to the substrate, to the priming culture or to both. The experiments presented in this paper showed the importance of studying the effects of the interaction between microbial species, separately and in conjunction, in order to study the affinity of each culture and the reciprocal interaction. The data presented indicate that the bacterium *E. faecium* is present in higher numbers than the fungus *T. faecale*, although the mass ratio between fungal and bacterial cells (ca 500 – 1000 times) suggests that even with three orders of magnitude, the cell masses present in the biofilm could be almost equal.

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O1.4

GORGONZOLA CHEESE RIND AS COMPLEX BIOFILM MATRIX: STRATEGIES TO REDUCE THE CONTAMINATION OF *LISTERIA MONOCYTOGENES* BY USING PHYSICAL TREATMENTS

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Introduction

Gorgonzola is one of the most important "Protected Denomination of Origin" (PDO) Italian cheeses. It is a blue-veined, mould-ripened cheese made with pasteurized cow's milk inoculated with *Lactobacillus* and *Streptococcus* starter cultures, together with *Penicillium roqueforti* that develops as an internal blue-green mould. During ripening of blue-cheeses, *P. roqueforti* proteolytic activity lead up to pH raising, influencing the heterogeneity of microflora and favouring a possible contamination by ubiquitous dairy microorganisms and food-borne pathogens (Mucchetti and Neviani, 2006). Among these, *Listeria monocytogenes*, due to its characteristics and to the product ones, can survive and multiplicate. Anyway, in Gorgonzola and in other blue-veined cheeses the contamination, if present, seems to be limited to the rind (GOLIS Project, 2004; Bernini et al., 2013). For this reason, to avoid the risk of ingestion, the Consortium for the Protection of Gorgonzola Cheese declares the rind not edible. Different strategies for decontamination of food surfaces have been investigated: activity of bacteriophages (Schellekens at al., 2007), use of antimicrobial producing strains as surface starter cultures (Izquierdo et al., 2009; Siafaras et al., 2008), high-pressure processing (Carminati et al., 2004; Cheftel, 2005), washing with high-pressure water spray (Mucchetti et al., 2008), atmospheric pressure plasma (Song et al., 2009).
The aim of this research was to evaluate, by microbiological challenge testing, the efficacy of a physical treatment (patented by Biraghi S.p.A, Milk Industry) based on specific heat sources in reducing cheese rind microflora and, in particular, *L. monocytogenes*.

Material and methods

The efficacy of the patented treatment (Number TO2007A000822) was evaluated on 64 cheeses: 32 creamy and

32 piquant variety. The treatment consists in stages of exposure of the product to different temperatures: 1) cold step, 2) rapid increase of temperature on the surface by specific heat sources, 3) low temperature step. A mixed inoculum of five *L. monocytogenes* strains (Lm2, Lm3c, Lm8, Lm60, Lm90) previously isolated from Gorgonzola cheese rinds and identified in The Fodder and Dairy Productions Research Centre (CRA-FLC) of Lodi (Italy) was used. Appropriate dilutions of the mixture were spreaded on the upper cheese rinds in order to obtain contamination levels ranging between 4 log cfu g^{-1} and 9 log cfu g-1. Contaminated samples were let to dry to allow the attachment of the cells to the rind. Before and after the treatment, a quarter part of the rind was gently scraped with a knife and analyzed for the following enumerations: Total Mesophilic Bacteria (TMB), Yeasts and Moulds (Y&M), Mesophilic Lactic Acid Bacteria (MLAB), *L. monocytogenes* (Lm). A non destructive sampling method based on the use of sponge bags (A) was compared with the traditional but destructive one based on rind scraping (B). 10 samples were checked to test the absence of naturally occurring *L. monocytogenes*. After the treatment, samples were also checked by selective enrichment for eventual undetectable quantitative levels of *L. monocytogenes*. Efficacy (E) was determined: $E = 100- (n_t/n_o)*100$, where n_0 = cfu g^{-1} before the treatment and n_t = cfu g^{-1} after the treatment.

Results and discussion

Method B allowed higher bacterial counts for TMB, MLAB and Y&M before the treatment but highlighted a lower reduction after the treatment in comparison to method A. In samples A microflora mostly decreased from 2 up to 4 log cfu g^{-1} and, in some samples, the reduction was higher than 4 log cfu g^{-1}. No samples B reached this high decontamination level but almost all showed a reduction lower than 2 log cfu g^{-1} (Figure 1).

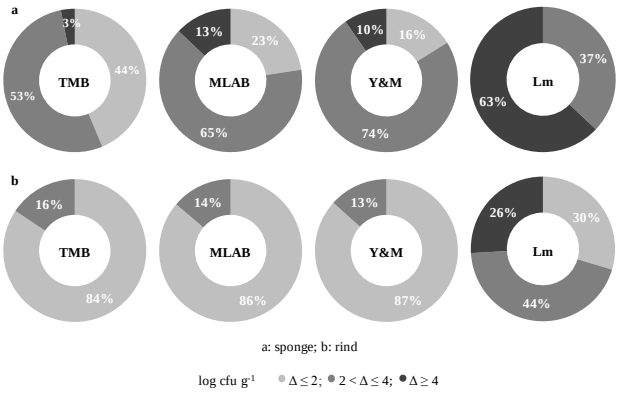

Figure 1. Percentage of samples with different reduction intervals of TMB, MLAB, Y&M and Lm. Reduction interval values (Δ) expressed as log cfu g^{-1}.

Overall, the effect of decontamination, evaluated as percent reduction, of non pathogenic microflora ranged from 95,08% in samples B up to 98,48 % in samples A (Figure 2). This difference could be due to the microbial colonization of the lay located just few millimetres below the surface, that can be sampled only by scraping the rind and that seems to be less influenced by the treatment applied. Compared to the non pathogenic microflora, *L. monocytogenes* was more affected by the physical treatment and, independently by the sampling method, was always reduced of more than 3 log cfu g^{-1}. Anyway, as already emerged, the best decontaminating effect of the treatment was obtained for the more superficially located cells with an average value of reduction of 99,94% (Fig. 2). In particular, the 63% of samples showed more than 4 decimal reductions (Fig. 1).

Mucchetti et al. (2008) obtained a variable decontaminating effect, ranging from 72% up to 99%, of *Listeria innocua* using different water pressures and Carminati et al. (2004) too have previously noticed a remarkable resistance of *L. monocytogenes* to pressures up to 500 MPa. A reduction higher than 99,999% was achieve pressurizing cheese rinds at 700 MPa for 15 min, involving however long time and high cost of equipment. A very variable reduction from 1,70 up to 8 log cfu g^{-1} was achieved for *L. monocytogenes* inoculated on cheese slices using atmospheric plasma, with many limitations due to volume, size and shape irregularities of the product (Song et al., 2009).

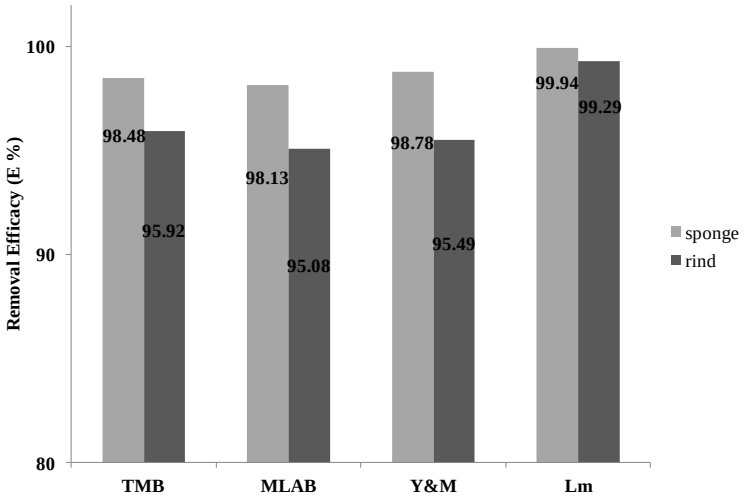

Figure 2. Removal efficacy (E %) of TMB, MLAB, Y&M and Lm.

The treatment seems to be principally effective on microorganisms located at the surface of the food: the irregular and not completely smooth surface of

Gorgonzola rind could presents niches for microorganisms recovery and protection to adverse conditions. Considering that on Gorgonzola surface *L. monocytogenes* is usually less than 3 log cfu g^{-1} (GOLIS Project, 2004), this treatment demonstrated to be useful to control pathogen food risk associated and improve cheese safety, since the pathogen reduction obtained was always higher than 3 cfu g^{-1}.

Conclusions

The treatment evaluated could represent an important physical hurdle to control pathogen food risk associated and improve cheese safety. The technology applied offers the advantages of short time exposures, good applicability of the equipment in the plant, no shape or volume limitations for the treated product, no additives or antimicrobials added, limited industrial costs. So, it is reasonable to consider a possible application for superficial decontamination of other food products.

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O2.1

EXOPOLYSACCHARIDES PRODUCED BY CYANOBACTERIA OF INDUCED BIOLOGICAL SOIL CRUSTS POSITIVELY AFFECT SEED GERMINATION AND SEEDLINGS FITNESS OF DESERT SHRUBS, CONTRIBUTING TO THE RESTORATION OF DESERTIFIED AREAS

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Introduction

Biological Soil Crusts (BSCs) are complex, cyanobacteria-dominated microbial communities, commonly found in arid and semiarid areas of the world. The resilience of crustal organisms under constrained conditions such as drought and high solar irradiation is ensured by microbial-produced extracellular polysaccharides (EPSs), which also accomplish for a wide array of key ecological roles. As an essential component of dry land ecosystems, BSCs impinge on the onset of vegetation, establishing two-way complex interactions with plants. The removal (or the immission) of BSCs in a given ecosystem can effectively shift the state of the ecosystem itself (Bowker, 2007), so that their induction on degraded soils is considered a feasible approach to maintain, and possibly amend, land fertility.

In Hobq desert, Inner Mongolia, China, various experimental sites constituted by moving sandy dunes were inoculated with cyanobacterial cultures in different years (Chen et al., 2006). As a result, these former hyper-arid sites currently provide a unique and valid study setting, characterized by the presence of induced BSCs (IBSCs) at different developmental stages, associated with grass and shrubs.

Within this frame, this study was aimed at assessing the effects of cyanobacterial inoculation in terms of phototrophic abundance and C pool increase. Moreover, under laboratory conditions, the effect of cyanobacterial EPSs on seed germination, seedling-ion uptake, photosynthetic activity and oxidative system of *Caragana korshinskii*, a desert sub-shrub widely diffused in the area under study, was tested.

Results showed that IBSC induction resulted beneficial in enhancing SOC (Soil Organic Carbon) and in increasing the abundance of phototrophic organisms and vegetation cover. Notably, cyanobacterial EPS resulted gainful to seedling growing and metabolism of *C. korshinskii*, also contributing to give them some protection against the damaging effects

of reactive oxygen species (ROS), generated under UV-irradiation, salt stress and desiccation, typical constraints of desert environments.

Material and methods

Samples of IBSCs and of the corresponding underlying soil (20 cm below the soil surface) were collected in July 2011 in the region of Shapotou, Zhongwei County, China (37° 27' N, 104° 57' E). Collected samples were 4, 6 and 8 years old IBSCs. Total carbohydrate content in the samples was quantified after treating the samples with 10 ml 2M sulfuric acid at 100°C. After centrifuging at 6000 x *g* for 15min, the total carbohydrate content in the supernatant was quantified by using the phenol-sulfuric acid assay (Dubois et al., 1956). Chlorophyll *a* was extracted from soil with 95% ethanol and quantified according to the method of Chen *et al.* (2006). Controls (Ck) are represented by samples collected in non-inoculated areas close to IBSCs.

Polysaccharide from the cyanobacterium *Phormidium tenue*, which is dominant in IBSCs in the considered study setting, was used in the present study for adding to plant seeds. The wild seeds of *C. korshinskii* Kom. were also harvested from the same sites. The polysaccharide produced by *P. tenue* was prepared following the method described by Huang et al. (1998). After being washed and disinfected with 0.5% potassium permanganate, seeds of *C. korshinskii* were put on filter papers infiltrated with solutions at different concentrations of polysaccharide (2, 10, 30, 60, 120, 240 mg/L). Non treated seeds were used as controls. Chlorophyll *a* and *b* in the leaves of *C. korshinskii* were extracted with 80% acetone and determined according to Lichtenthaler (1987). Seed germination tests were conducted according to Chon et al. (2002), while germination rate, germination energy, germination index and vigor index were measured according to Chen et al. (2005). Superoxide dismutase (SOD) activity was determined according to Chen et al. (2009), Malondialdehyde (MDA) content and reactive oxygen species (ROS) generation were determined according to Tang (1999).

PSII physiological status was assessed determining a number of functional and structural parameters derived from the fluorescence transient, tested through JIP test (Xia et al., 2004).

Results and discussion

Chlorophyll *a* was used as an index of the abundance of phototrophic microorganisms in the soil. For all the IBSCs samples, Chlorophyll *a* was found to be mostly concentrated within the crust thickness, with the content positively correlated with the age of the crusts (R^2=0.95, P>0.05), while in the samples collected 20 cm below the crust surface, Chlorophyll *a* contents resulted minimal (site 1, 3) or null (site 2) (Figure 1).

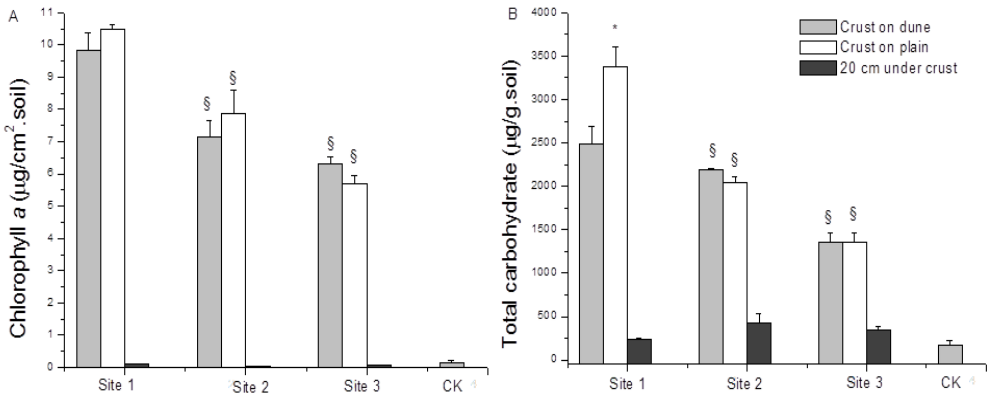

Figure 1. Chlorophyll *a* and total carbohydrate content in 8 (site 1), 6 (site 2) and 4 (site 3) years old induced BSCs. Controls (CK) are represented by samples collected in non-inoculated points. Samples were always processed in triplicates and error bars are reported in the figure.

Total carbohydrate content at the topsoil increased significantly with the age of the crusts (R^2=0.96, P<0.05) with amounts significantly (P<0.05) higher than the contents of the soil samples collected 20 cm below IBSCs (Figure 1). These results clearly point out the key role played by the crustal community in providing carbohydrate immission in the crusts.

The effect of cyanobacterial exocellular carbohydrates was tested by treating *C. korshinskii* seeds with polysaccharide produced by *P. tenue*. Germination, germination energy and germination index increased significantly (P<0.05) with the addition of 10-60 mg/L polysaccharide solution, while vigor index increased in the presence of 2-10 mg/L (Table 1). In all the cases seed germination parameters resulted correlated with the polysaccharide concentration, assuming a Gaussian distribution (P<0.01). According to JIP test, polysaccharide addiction resulted also beneficial to the photosynthetic efficiency. Indeed, polysaccharide concentration in the range 60 to 120 mg/L led to an improvement of the fluorescence yield at phase J, I and P of *C. korshinskii*. F*v*/F*m*, ET_0/ABS, ET_0/TR_0 and RC/CS_0 resulted correlated with the amount of polysaccharide in solution, assuming a Gaussian distribution (P <0.01). The usual production of ROS by photosynthetic organisms living in arid environment, which is harmful to the normal metabolism due to oxidative damages to photosynthetic system, resulted strongly inhibited by the treatment with polysaccharides, which is most likely due to the capacity of EPS to scavenge cellular $OH^{\cdot}$. Indeed, ROS production decreased from 2.75 µmol/g fresh weight (samples without

polysaccharide addition) to 0.59 µmol/g fresh weight (presence of 120 mg/L polysaccharide). The decrease in ROS production was coupled with an increase in SOD activity, which ranged from 42 U/g fresh weight (0 mg/L polysaccharide) to 105 U/g fresh weight (120 mg/L polysaccharide), and a decrease in MDA content, ranging from 0.020 µmol/g fresh weight (0 mg/L polysaccharide) to 0.015 µmol/g fresh weight (120 mg/L polysaccharide). The increase in SOD activity most likely acted scavenging free O_2^- radicals.

Table 1. Variation of germination rate, germination index, germination energy, vigor index and root length of *C. korshinskii* seeds after 7 days of treatment with different amounts of polysaccharide produced by *P. tenue*
P<0.05; **P<0.01. Values significantly different compared with the control, represented by untreated seeds.

Polysaccharide (mg/L)	*Germination rate (%)*	*Germination energy (%)*	*Germination index*	*Vigor index*	*Root length (cm)*
0	80.67± 0.48	71.44± 0.62	2.51± 0.10	3.76± 0.28	1.50± 0.11
2	83.72± 1.22*	74.78± 0.91*	2.66± 0.22	4.78± 0.29*	1.80± 0.05*
10	88.33 ± 1.06*	81.86± 0.55**	3.03± 0.23*	6.35± 0.55**	2.11± 0.16**
30	89.89± 0.94**	82.78± 0.65**	3.05± 0.17*	5.78± 0.29**	1.91± 0.05*
60	92.56± 1.02**	83.67± 0.61**	3.16± 0.13**	5.68± 0.42**	1.80± 0.09*
120	85.11± 1.95*	76.00± 1.78*	2.81± 0.10*	4.94± 0.36*	1.76± 0.07*
240	76.67± 0.79	68.89± 0.75	2.68± 0.06	4.37± 0.30	1.63± 0.07

In general these results pointed out the reliability of using cyanobacterial inoculation for soil rehabilitation. In first place, inoculation led to an increase in the C soil pool and photosynthetic organism amount, compared to non-inoculated spots. The presence of green microalgae and cyanobacteria, beside increasing C content through EPS excretion, also contributes to supplement soil with N, vitamins and growth factors. The EPS actually represents a trophic resource for crustal heterotrophs. Moreover, it was also found that cyanobacterial EPSs have positive effects on seed germination and establishment of *C. korshinskii*, proving to be capable of influencing plant establishment in disturbed landscape.

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O2.2

QUANTITATIVE REAL TIME PCR AND ARSENIC MOBILITY

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Introduction

Fifty years of gold mining in Yellowknife, North West Territories, Canada, has resulted in a huge unresolved arsenic contamination problem in the area. Microorganisms, surviving in this extremely polluted environment, could play a significant role in redox transformations of arsenic compounds leading to their mobilization from sediments and underground workings. Quantitative real-time PCR has the possibility of becoming a molecular tool for indicating the potential for arsenic to become mobilized in environmental samples.

The focus of our study was quantification by real-time PCR of three target genes that are responsible for arsenate As(V) reduction and arsenite As(III) oxidation: the *arrA* respiratory arsenate reductase gene, the *arsC* arsenate reductase gene, and the *aroA* arsenite oxidase gene. It is generally believed that As(III) is more mobile in the environment than As(V).

We report results for the quantification of these genes from samples collected in the Yellowknife area: soil samples collected underground from the Miramar Con Mine (now flooded) and surface sediment samples obtained from Baker Creek, which received water from the Giant Mine tailings ponds. The residues and water from the production circuit were stored in ponds and the overlying water was periodically released into Baker Creek after treatment with iron oxy(hydroxides) to remove arsenic from solution. Other arsenic input to Baker Creek occurred in accidental discharges of sludge containing arsenic, cyanide, copper, lead, nickel, zinc and other chemicals. Over the years these inputs have resulted in a high concentration of arsenic in the sediments of Baker Creek. Release of arsenic from this source continues to contribute to the exposure of the Yellowknife community and the burden in the Great Slave Lake. To enhance sensitivity of the qPCR for genes present in low copy numbers, nested real time PCR protocols were used for the *arrA* and *arsC* amplification.

Materials and Methods

Underground soil and water samples were collected in Con Mine tunnels at depths of 1.6 km, 1.5 km, 1.4 km and 1.07 km in sterile 50-ml Falcon tubes and plastic bags. Baker Creek samples were collected from several locations in the creek bed upstream and downstream of the tailing ponds in 2008 and 2011. "Dry" samples were obtained from exposed surfaces and "wet" from nearby submerged sediments. Duplicates were taken in all cases and shipped on ice to UBC as soon as possible where they were stored at -80ºC. To minimize the risk of sample contamination, all further handling of samples and PCR preparation procedures were conducted aseptically in a biological safety cabinet. The amplifiable DNA for analysis of the genes was purified directly from samples by a process developed to prevent heavy metal, cyanide and chloride inhibition of enzymes in qPCR. Soil samples (5g) were washed on 0.10 µm Stericup filters (Millipore) with sterile deionized water and the removal of salts was monitored with 0.1M AgNO3 solution until a white precipitate was absent. DNA was extracted using a PowerSoil DNA Isolation kit (MoBio). For the *arsC* quantification several groups of primers have been designed based on the *arsC* sequences available at the time and validated against positive and negative controls as described previously (Sun et al., 2004). Real-time PCR was performed in a 20 µl reaction mixture that consisted of 1 ng of DNA, 1x BioRad SsoFast EvaGreen Supermix, 250 nM primer set for *arsC* (600 nM for *aroA*). Reactions were carried out in the iCycler (Bio-Rad). Real-time PCR conditions were 95°C for 3 mins, 40 cycles of 95°C for 15 sec (denaturation), 60°C for 15sec (annealing), and 72°C for 15 sec (elongation). The primer sets and PCR conditions to amplify the *aroA* and the *arrA* genes were used as described by Inskeep et al. (2007) and Perez-Jimenez et al. (2005).

Results and Discussion

We have previously reported on the use of quantitative PCR for the identification and quantification of the *arsC* gene in environmental samples (Sun et al 2004). This work was based on the hypothesis that the *arsC* gene codes for arsenate reduction, a process that in its simplest interpretation could lead to an increase in arsenic mobility in the environment being examined because arsenate binds more strongly to metal oxide(hydroxides) than arsenite (Cullen and Reimer, 1989).

The DNA extracted from all the Yellowknife samples by using known protocols proved to be unsuitable for the application of PCR. This necessitated the development of a reliable extraction method that included both genomic and plasmid DNA. (The samples contained not only high level of total arsenic, but also had high amounts of heavy metals and some cyanide, contaminants that inhibit enzymes involved in amplification processes). Control and spiked experiments verified that plasmid DNA was not missed during the extraction.

The number of *arsC* and *arrA* genes in Baker Creek samples was either very

low or undetectable with all available primers. Nested rtPCR protocols for the *arsC* and the *arrA* were also performed, but the gene count was low for the *arsC* and not detected for the *arrA*. However, in Con mine sediments one of the *arsC* primers showed the presence of 212000 copy numbers per 1 ng of total DNA at the 1.07 km level, 5380 copy numbers at 1.4 km, and 104000 at 1.6km. In related work we have detected the *arrA* in other environments such as arsenic containing hot springs. Others have reported (Zobrist et al., 2000) that some strains that are able to synthesize the arrA protein are able to respire both soluble As(V) and also As(V) sorbed onto hydrous ferric oxide.

Our study of the bacterial redox capacity for arsenic in the environment was extended to include the isolation and quantification of the *aroA* gene that codes for arsenite oxidation. For this work we used two sets of primers (designated *aroA*-1 and *aroA*-2) developed by Inskeep et al. (2007) for conserved regions in seven arsenite oxidase genes. The results from Baker Creek (wet and dry sediments) using primer *aroA*-2 are shown in Table 1. Surprisingly, different primers for the aroA gene designed for the same group of bacteria, provided different count and in Table 2 we present the result of quantification of the *aroA* gene in Con Mine samples with both primers.

Table 1. Copy numbers of the aroA gene per 1 ng of total DNA in Baker Creek samples collected in 2008 and 2011.

Samples	*wet-2008*	*dry-2008*	*wet-2011*	*dry2011*
Upstream,station1	77700	60100	30800	28700
Upstream, st.2	96700	111000	48	11400
Discharge, st.1	51300	104000	5640	10300
Discharge, st.2	51000	97900	13300	ND
Creek mouth	177	97300	22500	25600
Downstream,st.1	148000	101000	1530	ND
Downstream, st.2	113000	169000	10200	16900
Downstream, st.3	158000	143000	549	16100

Table 2. Copy numbers of the *aroA* gene per 1 ng of total DNA in Con Mine samples with two different primer sets ("*aroA*-1" and "*aroA*-2").

Samples	*aroA*-1	*aroA* -2	As conc., ppb
3500-1	759	696	56
3500-5	2500	1370	57
4500-3	1510	162	881
4500-6	76	9	454
4900-8	18300	583	1719
5300-34	4850	1090	3159

Conclusions

The high numbers of *aroA* genes involved in arsenic oxidation as determined in our analysis reveal intensive arsenic oxidation activity in deep mine high arsenic brines and tailing ponds, indicating that the mobility of arsenic in this environment is low. The presence of the *arsC*, *arrA* and *aroA* genes and their ratio could serve as functional indicators of the propensity for arsenic reduction or oxidation in environmental samples; however, such gene quantification results need cautious interpretation and many factors such as sample size, suitability of the primers for particular samples, and quality of the DNA need to be considered.

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O2.3

INTERACTIONS BETWEEN INTRODUCED HYDROCARBON-OXIDIZING *RHODOCOCCUS* SPP. IN CRUDE OIL-CONTAMINATED SOIL MONITORED BY DIRECT PCR

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Biological remediation of terrestrial ecosystems contaminated with crude oil receives increasing attention with enhanced awareness of the potential harmful effects of petroleum hydrocarbons on human health and the environment. The genus *Rhodococcus* is a promising group of actinobacteria suitable for biodegradation of recalcitrant oil constituents, e.g. PAH and heterocyclic compounds. Upon revealing new catabolic abilities of *Rhodococcus* species and isolation of environmental strains degrading a wide range of contaminants, these bacteria have been increasingly explored for bioremediation of contaminated soils, waters and air (Kuyukina and Ivshina, 2010). Prospective molecular genetics approaches to be applied to bioremediation involve oligonucleotide primers and DNA probes constructed for target biodegradation organisms and genes to estimate their *in situ* functional activities (Whyte et al. 2002). Moreover, correct prognosis of bacterial survival and biodegradation activity in contaminated environments requires studies of interactions between introduced cultures and indigenous microorganisms using physiological and molecular approaches (Watanabe and Hamamura 2003). We previously developed 16S rDNA-targeted PCR primers for ecologically important *Rhodococcus* (*R. globerulus, R. erythropolis, R. opacus* and *R. ruber*) species, which enabled their direct detection in mixed cultures and environmental samples (Bell et al., 1999). Species-specific and functional *alk* gene primers were used for the monitoring of introduced mixed *Rhodococcus* cultures in crude oil-contaminated soil during field bioremediation trial. Additionally, physiological tests, e.g. viability measurements and respirometry analysis were performed to evaluate the survival and catalytic activities of introduced strains.

The results obtained demonstrate that introduced *Rhodococcus* cultures survived successively in oil-contaminated soil, leading to increased counts of hydrocarbon-degrading

bacteria and an increased rate of hydrocarbon degradation. Indeed, it was found that *R. erythropolis* was initially presented in oil-contaminated soil (Fig. 1), and its number increased by almost 10 times in the hydrocarbon-oxidizing bacterial population. This finding supports the idea that *R. erythropolis* is a ubiquitous soil bacterium, which can be successfully enriched by the incubation of soil with crude oil. Also the results obtained suggested a good adaptation of *R. erythropolis* to survive within the abundant indigenous soil population, unlike *R. ruber* predominating in less populated soil. Particularly, the number of *R. ruber* in the hydrocarbon-oxidizing bacterial population increased only by 2.5 times after 2 months, and by 3 times after 4 months. Relative abundance of 1.5×10^7 cells/g was registered for the introduced *R. ruber* strain after 6 months of incubation in initially low populated oil-contaminated soil. Relative prevalence of several *Rhodococcus* species in oil-degrading microbial consortia may be also explained by their ecological behavior, particularly the *r-K* scheme, which suggests that evolution favors either adaptation to high rates of reproduction (*r* strategists) or optimal utilization of environmental resources (*K* strategists) (Margesin et al. 2003). Bacteria, such as pseudomonads, which rapidly grow in nutrient-rich media, are *r* strategists. Others, such as rhodococci, tend to be more successful in resource-limited, crowded environments are *K* strategists. Apparently, populations of *K* strategists would be more stable and permanent members of the communities of chronically polluted biotopes, like oil-contaminated soils. Taking into consideration high *R. ruber* resistance to harsh environmental conditions (Kuyukina and Ivshina, 2010) this *Rhodococcus* species could be assumed as a good candidate for augmentation of oil-contaminated soils deficient in microorganisms due to extreme climate (e.g. polar and desert soils) or high of toxicant levels (e.g. oily waste dumps). Comparative respiration dynamics for the soil samples received different experimental amendments is shown in Fig. 2. Respirometry results confirm an essential role of rhodococcii in oil-contaminated soil bacteriocenosis. In particular, after two months, cumulative oxygen consumption and carbon dioxide production in *Rhodococcus*-amended soil samples was higher than in control soil. Moreover, maximum respiration was registered in oil-contaminated soil inoculated with poly(vinyl alcohol) cryogel-immobilized bacteria; total amounts of O_2 consumed and CO_2 released by this soil were 2-3 times higher than in soils received liquid bacterial culture (see Fig. 2).

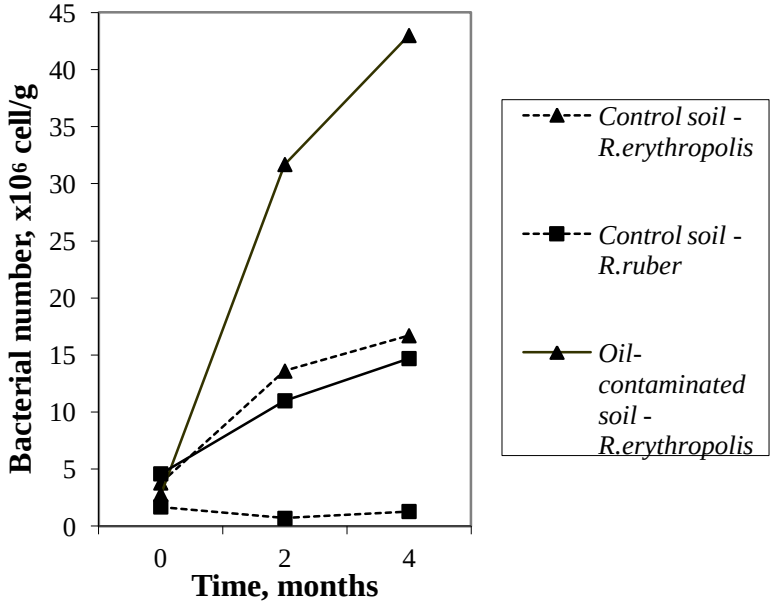

Figure 1. Dynamics of *R. ruber* and *R. erythropolis* in hydrocarbon-oxidizing bacterial population of control and oil-contaminated soils with different amendments.

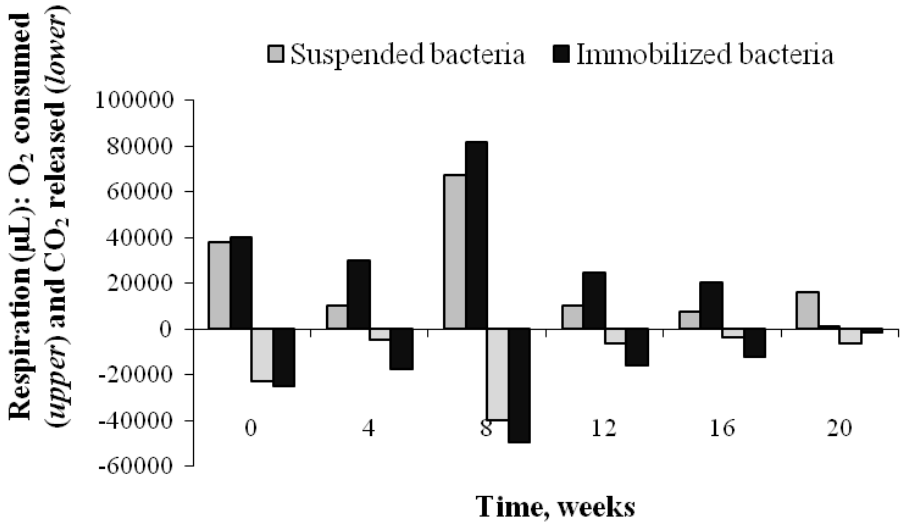

Figure 2. Respirometry dynamics of oil-contaminated soils with different amendments.

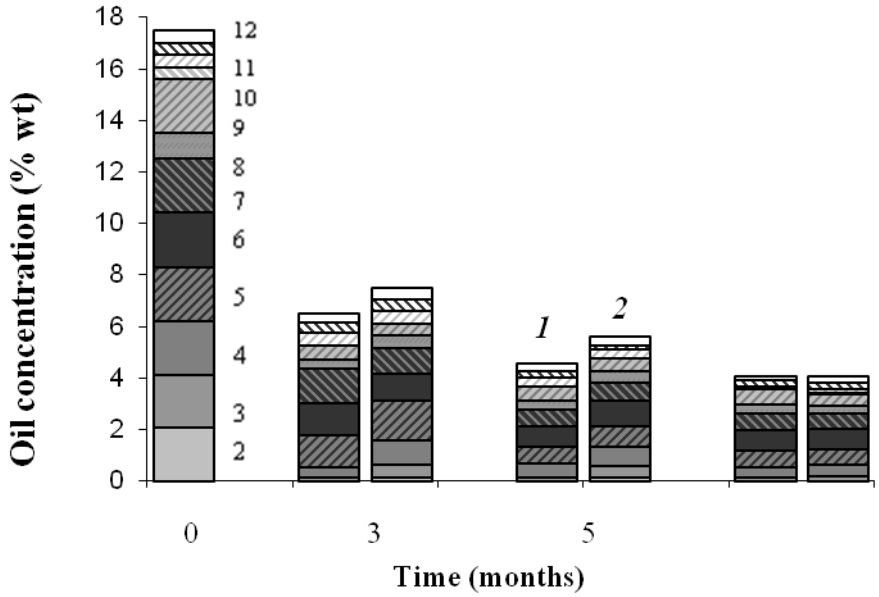

Figure 3. Dynamics of oil biodegradation in soils with different amendments: *1* – immobilized bacteria; *2* – liquid culture. Main oil components detected were as follows: n-decane (1), n-undecane (2), n-dodecane (3), n-tetradecane (4), n-hexadecane (5), n-heptadecane (6), n-nonadecane (7), pristane (8), naphthalene (9), acenaphthene (10), phenanthrene (11), anthracene (12).

This finding suggests that application of gel-immobilized bacteria in bioaugmentation protocols has considerable advantage over liquid bacterial preparations, especially for long-term soil bioremediation. Revealed increase in physiological activity (measured by respirometry) of mixed *Rhodococcus* consortium is explained by synergistic metabolic interactions resulted in enhanced degradation of crude oil without toxic intermediate accumulation.

After 5 months of experiment, residual crude oil concentrations (measured as chloroform-extractable TPH) in *Rhodococcus*-amended soils were 50-60% lower than in control soil. As it can be seen from the Fig. 3, oil hydrocarbons were degraded to the greater extend in soils with cryogel-immobilized bacteria compared to liquid preparations. These results confirmed the impact of *Rhodococcus* augmentation in oil degradation.

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O2.4

UNRAVELING THE ROLE OF ECOSYSTEM DEVELOPMENT IN THE SHAPING OF SOIL BACTERIAL COMMUNITIES

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Abstract
Microbial primary succession and plant biocoenosis establishment are key events in land reclamation, nevertheless the underlying mechanisms driving the succession are still poorly understood. Aim of the work was to determine the role of environmental factors and ecosystem development in shaping soil microbiota. The forefronts of receding glaciers are natural laboratories where to study microbial diversity in relation to geochemical factors and ecosystem development stage. Chronosequences can be identified on glacier moraines, where the increasing distance from the glacier edge corresponds to the increase of time of permanence out of ice, soil structuring and ecosystem development. We studied a first site on the moraines of the Lobuche glacier (Mount Everest area, Nepal), identifying several niches characterized by a different level of biotic colonization, from a bare mineral substrate to complex biological soil crust. Bacterial phylogenetic and functional diversity was studied by DNA-fingerprinting methods and a high variability was observed, demonstrating that the presence of spatially isolated microhabitats in soil matrices can strongly influence data interpretation. We then focused on a second site on the moraines of the Midtre Lovéenbren glacier (Svalbard Islands, Norway) where a chronosequence was previously characterised. It was composed by 7 sites subjected to a primary succession process, exposed to the terrestrial habitat since 5 to 2000 years. The sites were not isolated by distance but by the age of ecosystem evolution, distinguished by increasing levels of nutrient availability, soil fertility and plant colonization. Together with environmental factors, plant cover and diversity have been demonstrated to strongly influence the structure of soil microbiota. Aiming to consider separately the role of plants and the role of soil development in the primary bacterial succession, we identified a

vascular plant, namely *Saxifraga oppositifolia*, present along the whole Midtre-Loveénbreen chronosequence and analyzed the bacterial communities associated to its rhizosphere and the corresponding bulk soil. We demonstrated that soil development was the discriminating factor in the earlier successional stage, while the rhizosphere effect was significant only after 20 years out of ice covering. Rhizosphere communities showed higher similarity between 20 and 100 years of soil development, differentiating in the subsequent successional stages. The results showed the presence of biogeographic patterns in rhizosphere microbiota, differently influenced by the interaction with the plant and by environmental factors related to different stages of ecosystem evolution.

Introduction

Drylands on Earth cover approximately 40% of the land surface (Reynolds et al., 2007) and include both hot and cold deserts, which extension raise at different latitude and altitude is exacerbated by global warming. Deserts are model biomes where to study the mechanisms of ecosystem development, gaining information with potential application in the frame of soil reclamation and reverse desertification. Climate change outcomes are particularly drastic on Arctic and altitude environments where the mean temperature increase during the last century is consistent with glacier retreat. Ice melting determines the exposure of a barren mineral substrate subjected to the processes of primary colonization and pedogenesis with physiochemical and biological transformations initially carried out by pioneer microbial key players (Hodkinson et al., 2003). The forefront of receding glaciers is therefore a natural laboratory where to study the process of soil formation. Chronosequences can be identified, where the distance from the forefront is a proxy for i) increase of time of permanence out of ice, ii) soil structuring and ecosystem development. Aim of the work was to study bacterial diversity in soil ecosystems characterized by different development status, evaluating the role of environmental parameters in shaping soil microbiota. We choose different moraine environments: a high altitude and low latitude glacier, *i.e.* Lobuche (Mount Everest area, Nepal), and a low altitude and high latitude glacier, *i.e.* Midtre Lovéenbren (Svalbard Islands, Norway).

Material and methods

Study sites and soil sampling: Lobuche glacier (25°N, 86°E) moraines were explored in September 2009 and station NP09LOG1 was identified, presenting in a total area of approximately 50 m^2, 7 niches containing protosoil with different surface morphology. Air temperature and humidity and total and photosyntethically active radiation (PAR) were measured by Par Photon Flux Sensor (Decagon Device, Pullman, USA) provided with datalogger. A previously characterized chronosequence (Hodkinson et al., 2003) was identified on the Midtre Lovéenbren glacier (78°N, 12°E)

moraines, composed by 7 sites along a 1.8 km transect, released from ice covering since 4 to 2000 years ago. In September 2006 superficial biological soil crusts and 1-5 cm deep soil were aseptically sampled and shipped freeze to the lab.

Molecular and chemical analyses: total nucleic acids were extracted from 0.5 g of each sample with the commercial PowerSoil® DNA Isolation Kit (MoBio Inc., CA, USA) following the instructions of the manufacturer. Amplified Ribosomal Intergenic Spacer Analyses (ARISA) and 16S rRNA clone libraries were applied on the extracted DNA as previously reported (Borin et al., 2010). Standard soil chemistry analyses were performed as previously reported (Borin et al., 2010).

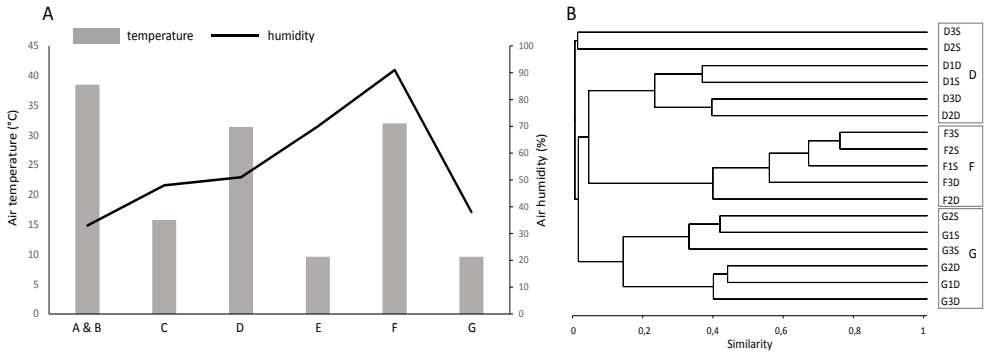

Figure 1. Biological and physiochemical characterization of different sites within the moraines of the Lobuche glacier. A) maximal air temperature and humidity of the 7 sampled sites (A-G). B) Cluster analyses of ARISA fingerprinting on BSC (S) and deep layer (D) in sites D, F, G.

Results and discussion

On the moraines of the Lobuche glacier in the NP09LOG1station, 7 sites were identified, containing a mineral proto-soil covered by a typical black-grey biological soil crust (BSC), evidence of the ongoing process of soil ecosystem development. The sites were differentiated by slope and irradiation, air and crust relative humidity, minimal and maximal air temperature (Figure 1A), BSC extent and morphology. The BSC upper layer and the below mineral substrate were separately collected to analyse the microbiota involved in primary colonization. The overall bacterial diversity was analysed by ARISA fingerprinting, demonstrating that each site hosted a peculiar bacterial community and that within each site the surface BSC hosted a community different from the deeper layer (Figure 1B). The observed differences in phylogenetic diversity were linked to environmental factors, as demonstrated by differences within the sites in cation exchange capacity (CEC) and nutrient soil content, indicators of soil quality and hence to its development stage.

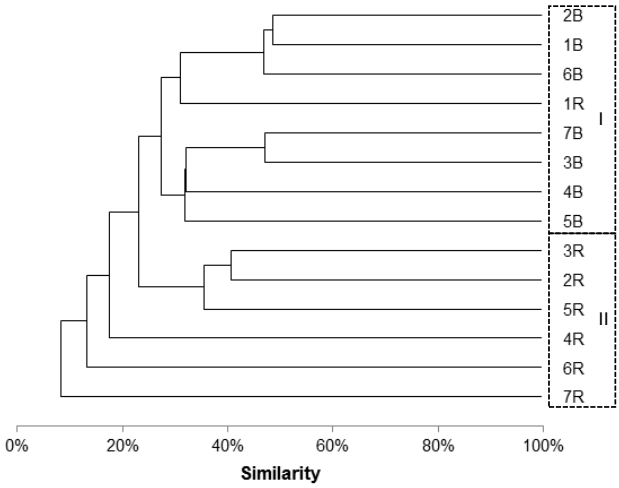

Figure 2. Cluster analysis (CA) of rhizospheric (R) and bulk (B) soils collected along the Midtre-Lovéenbreen glacier chronosequence based on ARISA fingerprints. CA was performed by UPGMA (Unweighted Pair Group Method with Arithmetic Mean) agglomerating method using the Jaccard similarity coefficient. I. bulk soil samples form a separate group comprising rhizosphere of site 1 (4 years out of ice); II. rhizosphere soils from sites 2-7 (18-2000 years out of ice).

The diversity of bacterial communities in desert environments is linked, besides to the environmental factors, also to the diversity of the other BSC biotic components, like fungi and mosses (Belnap & Lange, 2003). The presence of spatially isolated microhabitats in the NP09LOG1 station, associated to BSC of different morphology and taxonomic composition, can hence strongly influence data interpretation.

With the aim to evaluate the specific role of environmental factors in shaping the bacterial community, we focused on the moraines of the Midtre-Lovéenbreen glacier, where a chronosequence had been previously characterized (Hodkinson et al., 2003). The chronosequence comprised sites recently released by ice covering (sites 1-5, 4–100 years out of ice) constituted by protosoil with patchy BSC and plant colonization, and sites with a well developed tundra ecosystem (sites 6-7, 150-2000 years out of ice). It is well recognized that plant cover and diversity play a significant influence on the structure of soil microbiota (Berg & Smalla, 2009). In this study we aimed to consider separately the role of plants and the role of soil development in the primary bacterial succession. We focused on the rhizosphere soil of *Saxifraga oppositifolia*, a vascular plant present along the whole Midtre-Loveénbreen chronosequence (Hodkinson et al., 2003). *S. oppositifolia* rhizosphere in the 7 chronosequence sites provided hence a unique environment for exploring the role of abiotic factor and soil

development stage on bacterial diversity, in an environment where the biotic factor could be considered a constant. ARISA fingerprinting was applied to rhizosphere and bulk soil from the 7 chronosequence sites. Cluster analysis of fingerprints showed that bacterial communities in the bulk soil were different than in the rhizosphere (Figure 2), demonstrating even in this extreme environment the existence of a "rhizosphere effect" (Berg & Smalla, 2009). Only *S. oppositifolia* rhizosphere in site 1, the most recently released from ice cover, harboured a bacterial community that clustered with communities in the bulk soil rather than with the rhizosphere in older sites (Figure 2). These results demonstrated that soil development was the discriminating factor of bacterial community composition in the earlier successional stage, while the rhizosphere effect was significant only after 20 years out of ice covering. Moreover, while no clear trends were detected in bulk soils, rhizosphere communities showed higher similarity between 20 and 100 years of soil development, differentiating in the subsequent successional stages.

The analyses of rhizosphere 16S rRNA clone libraries (data not shown) revealed that specific soil phylogenetic populations responded differently to the soil development process, changing in relative abundance along the chronosequence. Acidobacteria and Verrucomicrobia increased along the chronosequence, positively related with soil respiration and available nutrients, phosphate and nitrogen, respectively. On the contrary, β-proteobacteria and Bacteroidetes decreased along the chronosequence, inversely related to respiration and cation exchange capacity, respectively.

Conclusions

The results demonstrated that, in extreme cold deserts, niches with different soil development stage coexist in the same area. The different niches are subjected to different environmental constraints that lead to the selection of specific pioneer bacterial communities showing distribution patterns in phylogenetic and functional diversity. In the Midtre Loveénbreen glacier moraines, moreover, it was evaluated the specific role of ecosystem evolution in the shaping of bacterial communities, which resulted differently influenced by the interaction with the plant and by (bio)chemical parameters related to soil development stage.

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O3.1

SOCIAL INTERACTIONS IN COMPLEX MICROBIAL COMMUNITIES - SURVIVAL OF THE FITTEST OR THE FRIENDLIEST?

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Introduction

The close association of bacterial cells in biofilms optimizes the conditions for both competitive and cooperative activities among the cells, which in turn indicates that biofilms are ideal for studies of bacterial evolution. Multispecies biofilms are predominant in almost all natural environments, setting the scene for various competitive and cooperative interactions that affect overall functionality and fitness of the individual strains and the community.

Results and discussion

We have developed a model defining cooperation, competition, synergism, antagonism and neutrality within multispecies biofilms, based on the proportion of the species that are present and their ability to form monospecies biofilm.

The model was validated experimentally in control systems of isogenic strains differing only in their ability of biofilm formation as single species. We applied this model to characterize the interactions in multispecies biofilm formation of bacterial consortia isolated from a variety of natural habitats. In brief, strains were co-cultured in microtiter plate wells and biofilm was assessed by a modified version of the Calgary method based on crystal violet retention and quantification. Biofilm formation of single strains and all possible combinations of 2 -7 strains were compared. Synergism in multispecies biofilm formation was observed within all strain collections, ranging from 20 – 80 % of all combinations tested.

In order to further characterize the observed synergistic interaction, we explored a four species community composed of the soil isolates *Stenotrophomonas rhizophila*, *Xanthomonas retroflexus*, *Microbacterium oxydans* and *Paenibacillus amylolyticus*. When co-cultured, these strains produced almost five-fold more biofilm biomass compared to single species biofilms. It was observed that each of the four strains was indispensable for the synergy to occur.

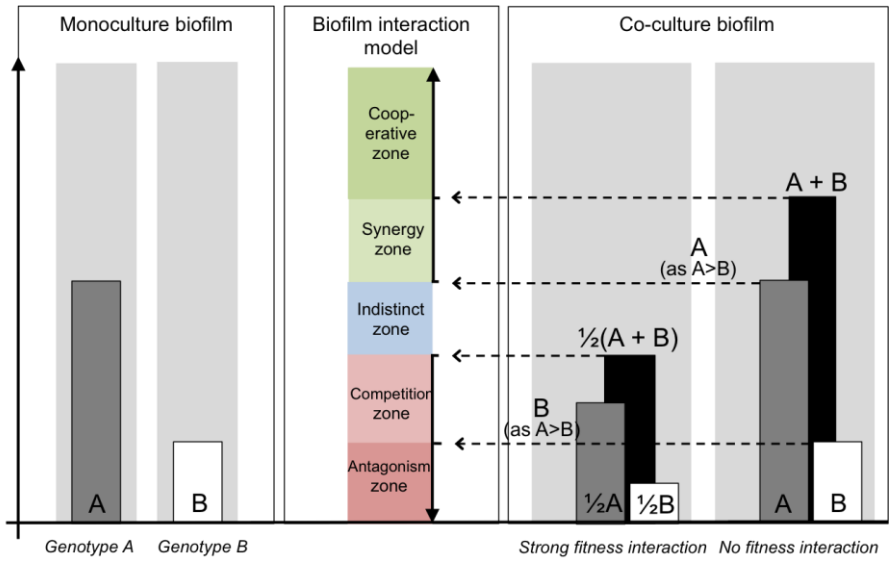

Figure 1. Model used to classify biofilm formation by co-cultures based on monoculture biofilms. *Gray bars:* Mono-culture biofilm formation by genotype A. *White bars:* Monoculture biofilm formation by genotype B. *Black bars:* Null models used to classify co-culture biofilm formation. Cooperative biofilm synergy is inferred when co-culture biofilm formation is larger than the sum of what each strain produced in monoculture. Biofilm synergy is inferred when co-cultures produced more biofilm than the best monoculture biofilm former. Competition is inferred when less biofilm is produced in co-culture than the average produced by monoculture biofilms. Antagonistic biofilm reduction is inferred when co-cultures produced less biofilm than the poorest monoculture biofilm former. As interactions cannot be resolved then biofilm formation of co-cultures is neither synergistic nor reduced an *indistinct zone* was implemented.

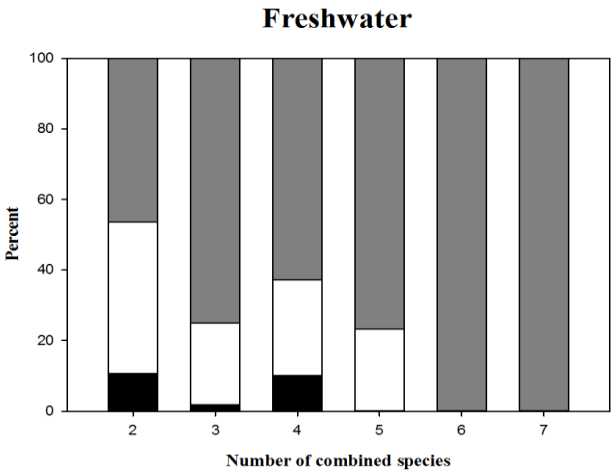

Figure 2. Biofilm formation of 8 species combined in all possible co-culture combinations of 2 to 7 strains after 24 hours. The model shown in Fig. 1 was used to characterize co-culture biofilm formation as synergistic (gray), neutral (white) or competitive (black).

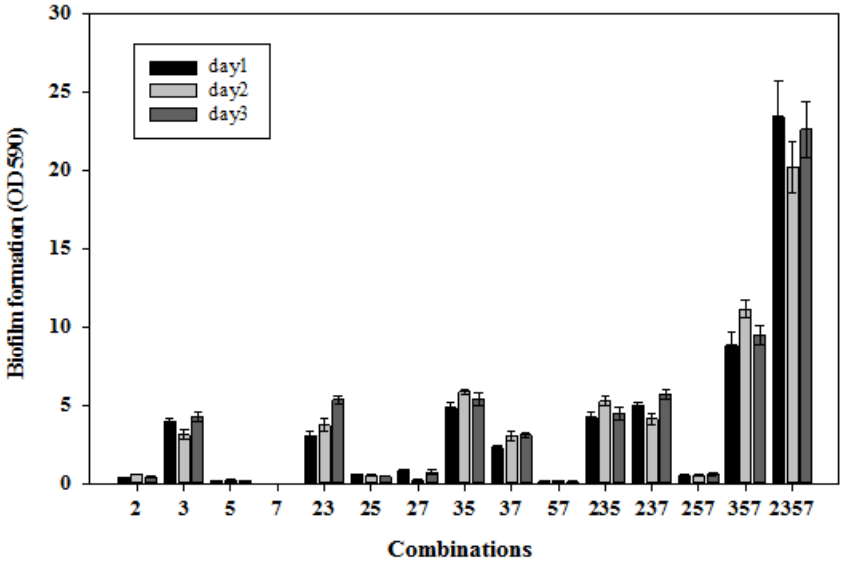

Figure 3. Biofilms formed by four isolates 2 (*Stenotrophomonas rhizophila*), 3 (*Xanthomonas retroflexus*), 5 (*Microbacterium oxydans*) and 7 (*Paenibacillus amylolyticus*) when equal aliquots of the diluted cultures were incubated in all possible combinations of two, three and four isolates. Assays for the detection of synergistic effects were performed three times (day1, day2 and day3) with 4 replicates each time. Bars represent means $\pm$ standard error for four replicates.

Furthermore we have developed a simple assay for screening of antagonistic interactions among bacterial isolates. This approach was used to investigate the number of antagonistic interactions among bacteria co-isolated from the same environmental sample in comparison to interactions between isolates from different environments. A screening of 39 isolates from freshwater and root surfaces showed interesting patterns of antagonism in relations to environment and phylogenetic relatedness.

In conclusion, we present results indicating ubiquity of social interactions in complex bacterial communities.

O3.2

DYNAMIC EVOLUTION DURING STORAGE OF MEAT AND SEAFOOD PRODUCTS TOWARDS THE SELECTION OF CORE AND VARIABLE COMPONENTS OF MICROBIAL SPOILAGE

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Introduction

Meat and seafood products are highly perishable with short shelf life. Microbial spoilage of these products is responsible for product withdrawal from the market leading to important economic losses. Although spoilage is of general economic concern, there is a lack of scientific knowledge on the spoilage microbiota of these products. Indeed, many studies have investigated food spoilage but there are currently limited data available on the microbial composition of non-fermented meat and fish food products using culture-independent approaches. So far, studies based on large-scale sampling have not been carried out and correlation between storage factors and microbial variability is poorly documented. In 2011, the ECOBIOPRO project was launched by a French National Consortium of 10 laboratories and aimed at revisiting the spoilage microbiota of 8 meat and seafood products commonly sold on the market using high-throughput pyrosequencing of the 16S rRNA genes. We undertook this comparative study in hope it could bring significant insight into microbial composition dynamics during food storage.

Material and methods

The V1-V3 region of the 16S rRNA genes was amplified with barcoding tags and pyrosequenced with GS-FLX+ technology. Microbial diversity was evaluated at the species level using a pipeline for taxonomic assignment and compared with UNIFRAC metrics.

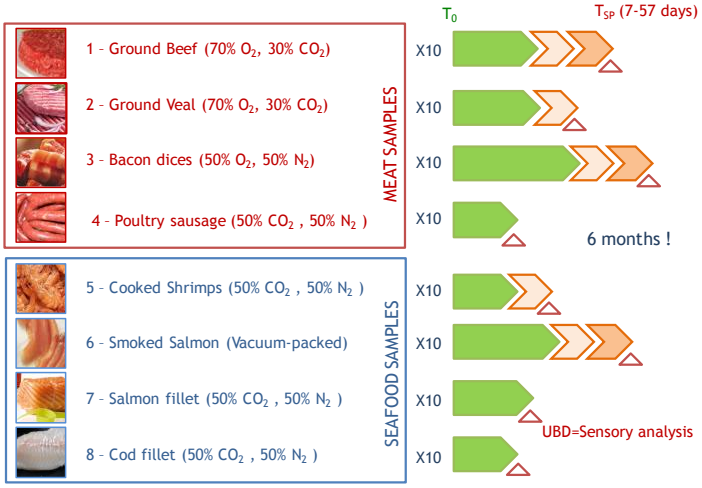

Figure 1. Study design. Between March and October 2011, we collected from commercial suppliers eight different type of freshly produced food products). For each type of food, intra-variability among products was assessed by analyzing 10 different samples over a period of 2 to 8 months. All type of food originated from independent producers in France and food was packed and stored accordingly to the methods commonly applied in the food industry for assessing the product's Use-by-Date. Each set was analysed just after production (Time T_0) and after spoilage (Time T_{SP}) leading to the construction of 160 DNA samples.

Results

We analyzed a total of 2 435 219 bacterial 16S rRNA sequences from 160 T_0 and T_S samples. On average, 16 332 ± 5050 sequences/sample were obtained. This sequencing depth (~4-log scale) provided adequate coverage for assessing the diversity in both initial T_0 population size and spoilage T_{SP} population size. The number of OTU estimated at the species-level using a 97% identity cutoff averaged 189 ± 58 OTUs per sample at T_0 and showed no significant differences between meat and seafood products. A total of 508 operational taxonomic units (OTUs) were characterized for the whole T_0 dataset indicating strong redundancy of OTU's between samples and types of food products. Smoked salmon showed a lower average diversity level of 123 ± 36 OTUs. Each type of product revealed a specific microbiota divided between product-specific OTUs (50% of diversity originating mainly from the animal microbiota), meat- or fish-specific OTUs (35% of diversity) and OTUs highly prevalent in all products (15% of diversity) originating, in the last two cases, from the environment (water and soil/plants).

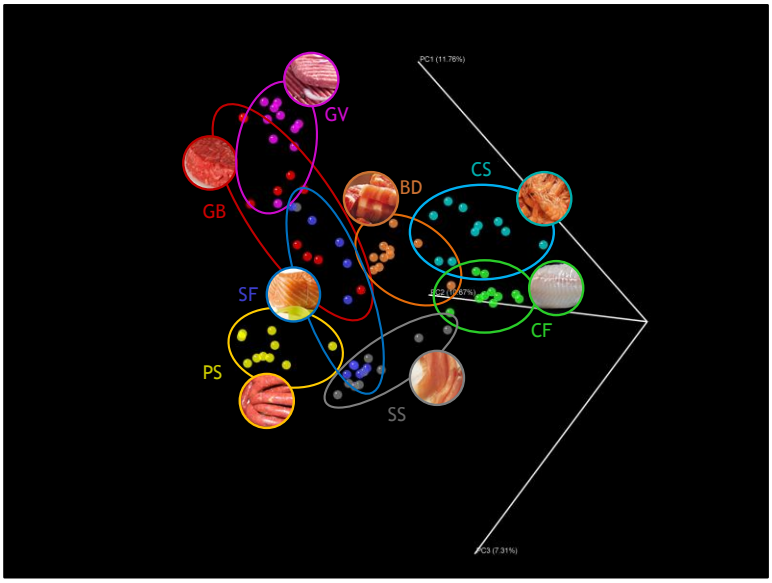

Figure 2. Comparative analysis of food microbiota in fresh products. 3-dimentional PCoA using unweighted UNIFRAc distance. This plot is showing that each type of food products is characterized by a specific microbiota. GB: ground beef; GV: ground veal; BD: bacon dices: PS: poultry sausages; SS: smoked salmon; SF: salmon fillets; CF: cod fillets and CS: cooked shrimps.

At T_{SP} the diversity dropped severely to an average of 30 OTUs in meat products, smoked salmon and shrimps but remained more elevated (80 OTUs) in fresh fishes (cod & salmon fillets). Dominant T_{SP} spoilage microbiota examined at 4-log scale showed, at least, a general 5-fold reduction in OTU's richness versus those of T_0. Comparative analysis between T_0 and T_{SP} revealed that each type of food product is characterized by a set of dominant and subdominant species and that the same OTU may constitute part of the core microbiota in one product and only a variable component in others. Overall, we observed three categories of taxonomic identifications: species already known as spoilers; species newly characterized as potential spoilers and new putative species never described before. However, selectivity patterns setting the spoilage microbiota revealed to be different between the eight food products. Relative abundance of spoilage microbiota in Beef/Veal samples was the most stable during storage, and an identical spoilage microbial community was established in these two products. In contrast, evolution was evidently more dynamic in Bacon Dices and Poultry sausages with large inversions of OTU's relative abundance and a co-evolution towards the selection of similar dominant OTUs. Evolution trajectories of spoilage community in seafood products were more complex than those of meat products. The setting of dominant OTUs was very dynamic

and, in some samples, associated with divergent trajectories. As a general trend of evolution from T_0 microbiota, most of spoilage OTUs from the T_{SP} community arose from T_0 core-community prevalence clusters associated with environmental origin.

Conclusions & Significance

The present comparative study is the first comprehensive culture-independent analysis of microbial communities between spoiled meat and seafood products involving a large sampling survey. We hypothesized first that a large sampling design would help us to overcome the well known inter-sample variability of food microbiota described in many earlier publications. We also estimated that a combined analysis of all 160 meat and seafood samples with a unique protocol (from DNA extraction to 16S pyrosequencing data processing) would increase our correlation analysis and reduce differences between samples which may merely be due to technical issues. Our study is also among the first to quantify that core- or product-specific spoilage relationship occurs at the species level and that higher taxonomic level (genus, family), commonly achieved in 16S rRNA gene amplicon sequencing analysis, may however be insufficient to fully appreciate the ecology of food microbial spoilage. Our exploration allowed us to determine, more precisely, the reservoir of spoilage bacteria and on the impact of processing in the microbial dynamic evolution during food storage.

O3.3

TRANSPOSON MUTAGENESIS OF *LACTOBACILLUS PENTOSUS* C11 IDENTIFIED CRITICAL GENES FOR GROWTH IN OLIVE BRINE

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Introduction

Olive brine is a stressful environment for lactic acid bacteria (LAB) and yeasts generally present in this ecological niche. In fact several variables can affect their growth, such as limited availability of nutrients, salt concentration and the presence of anti-microbial substances like phenolic compounds (Perpetuini et al., 2013). Our previous studies demonstrated that, at the end of fermentation of Italian table olives, bacterial population was clearly dominated by *L. pentosus*. Among the isolated strains, *L. pentosus* C11 showed the best fermentation performances (unpublished data). The mechanisms involved in the ability of this strain to grow in such a hostile environment are not known. In order to establish the main factors inducing LAB growth inhibition in brine and better understand the reason why only few adapted strains are able to grow in this environment *L. pentosus* C11 was mutated by random transposition using the P_{junc}-TpaseIS$_{1223}$–based system (Licandro-Seraut et al., 2012). A library of 6000 mutants was generated and screened for the ability to grow in a new medium called BMS (Brine Medium for Screening)which mimic the stressing conditions commonly encountered by bacteria in brine. Among the 6000 mutants screened, 5 mutants failed to grow. Transposition occurred in two open reading frames and in three transcription terminators, so genes essential for the adaptation and growth of *L. pentosus* C11 in olive brine were identified.

Materials and Methods

Development of Brine Screening Medium (BSM)

Itrana Bianca olive brine, previously characterized for its chemical composition (Servili et al., 2012; Tofalo et al., 2012) was used as a basis for BSM preparation. The brine was two-fold concentrated under vacuum at 52°C, complemented to 20 g/l yeast extract and 20 g/l glucose, pasteurized at 65°C for 45 min (destruction of all

vegetative form) and mixed with an equal volume of 40 g/l sterilized agar in water just before pouring the plates. YG medium (10 g/l yeast extract, 10 g/l glucose) liquid or solid (20g/l agar) was use as non stressing control medium.

Construction of a random transposon mutant library of L. pentosus C11 and analysis of mutants

Mutagenesis was performed using Pjunc-TpaseIS$_{1223}$ system as previously described (Licandro-Seraut et al., 2012). Identification of transposon target sequences was performed with the BLAST software from the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/).

Gene expression (qRT-PCR analysis)

Total RNA from 10 ml of culture was extracted after bead-beating disruption using the Tri-Reagent method. qRT-PCR and calculations of Relative Transcript Levels (RTL) were carried out according to Licandro-Seraut et al. (2008). Genes *tpiA* and *rpoD* were used as internal calibrators for all qRT-PCR analyses.

Results and Discussion

A library of 6000 random mutants (verified by sequencing target of randomly selected mutants) was obtained and store at -80°C. The library was screened by replicating on YG medium (control medium without olive brine) and BSM medium, and 5 mutants unable to grow on BSM were identified. Transposon chromosomal targets were determined by sequencing and comparison of the insertion site sequence with *L. pentosus* IG1 genomic sequence using BLAST database searches (NCBI). The disrupted genes were named from *oba*A to *oba*E, for olive brine adaptation, when no putative function was already attributed. Transposon integrations are indicated in Table 1. Transposition occurred in two open reading frames and in three transcription terminators, so at least 5 genes essential for adaptation and growth of *L. pentosus* C11 in olive brine were identified. In mutant 31B11, the transposon was integrated into the TT of glucose-6-phosphate isomerise (gpi), an ortholog of *lpent*_02771, which encodes glucose-6-phosphate isomerase, whereas in mutant 51D12 it is integrated into the TT of *obaC*, an ortholog of *lpent*_00851, which encodes a putative fatty acid binding protein. The transposon of mutant 25B5 was integrated into a TT that could serve for the two convergent genes *obaA*, an ortholog of *lpent*_01150, which encodes a putative redox-sensitive transcription regulator, and *obaB*, an ortholog of *lpent*_01149, which encodes a putative membrane-bound protease. In mutant 20B10, the transposon was inserted in *obaD*, an ortholog of *lpent*_00392, which encodes a small hypothetical integral membrane protein of unknown function. The *obaD* gene seems to be specific to *L. pentosus*/*L. plantarum* species, since neither ortholog nor even homologs of the ObaD-encoded protein were found in other bacterial species. The mutant 42G1 is disrupted for *enoA1*, an ortholog of *lpent*_01085, which is predicted to encode an enolase (EnoA1), an essential enzyme for

glycolysis. *L. pentosus* C11 is likely to carry another enolase gene which would explain the fact that this disruption is not lethal.

Table 1. Transposon integration sites in the 5 brine sensitive mutants

Mutant	Disrupted locus	Potential function
25B5	*obaA (lpent_01149)* TT/ *obaB (lpent_01150)* TT	membrane-bound protease, CAAX family redox-sensitive transcription regulator
31B11	*gpi (lpent_02771)* TT	glucose-6-phosphate isomerase
51D12	*obaC (lpent _00851)/ lpent _00853*[b]	DegV family protein 30S ribosomal protein S9
20B10	*obaD (lpent_00392)*	integral membrane protein
42G1	*enoA1 (lpent_01085)*	enolase 1

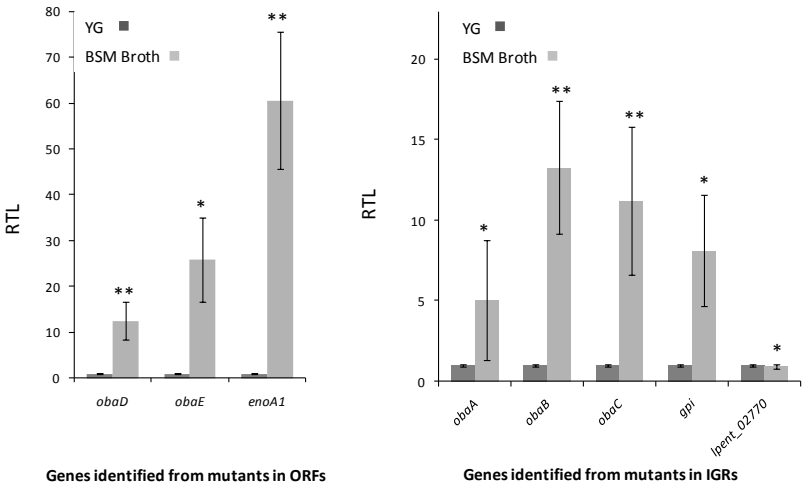

Figure1. Relative transcript levels of *L. pentosus* C11 genes after a 16 h-growth in BSM broth. Transcriptional levels of each gene are expressed in relative fold change, using YG medium as the reference condition (RTL = 1). Four biological repeats were made. Statistical analysis was performed using the unpaired Student's t test, *, $p<0.05$; **, $p<0.005$. Modified from Perpetuini et al. (2013)

Olive brine sensitivity of IGR integrants could be due to polar effects of the transposon on the flanking genes. To check this

hypothesis, qRT-PCR analysis of genes flanking the transposon wasperformed for each mutant, using WT *L. pentosus* as the reference (Fig. 2). Transcriptional analysis of the genes interrupted, or silencedby transposon integration, was carried out on WT *L. pentosus* after16 h of growth in BSM broth, or in YG broth for the reference condition. All genes identified in olive brine-sensitive mutants displayed a significantly higher RTL in BSM broth than in YG broth, with particularly high increases for *enoA1* (60-fold) and *obaE* (35-fold) (Figure 1). This analysis stresses the role of these genes in the resistance to olive brine stress and in olive fermentation adaptability and demonstrates that this adaptation results in an up regulation of *oba* gene transcription in wild type *L. pentosus*.

Conclusion

Five mutants unable to grow in olive brine were isolated, and their analysis led to the identification of at least 5genes essential for the growth of *L. pentosus* C11 under olive fermentation conditions. The identified genes encode proteins involved in carbohydrate metabolism, membrane structure and function, and regulation of gene expression.

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O3.4

ANTAGONISTIC EFFECT BY NATIVE YEASTS AGAINST A TOXIGENIC STRAIN OF *PENICILLIUM NORDICUM* IN DRY CURED MEAT MODEL SYSTEM

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Abstract

Three yeast strains isolated from the surface of Italian typical dry cured hams, previously selected for their ability to inhibit toxigenic mould in culture medium were screened for antagonistic activity against a toxigenic strain of *P.nordicum* and inhibition of ochratoxin A (OTA) biosynthesis in dry cured pork model system. The co-inoculated samples (0.92 ±0.01 a_w) were incubated for 30 days at 18°C. On average, yeast inhibitory activity was effective with a reduction of 2-3 log CFU/cm^2 in the mold growth. In the assay conditions, one strain of *D. hansenii* and *H. burtonii* were the best performing in inhibiting *P.nordicum* growth. OTA accumulation decreased significantly in yeast-added dry cured pork samples, especially in case with strains belonging to *Debaryomyces* spp. These results suggest the potential usefulness of the tested yeast strains as protective cultures in meat matured products.

Introduction

The mycete populations colonizing the surface of dry-cured meat products have been regarded as positively contributing to the chemosensory properties of final outcome (Andrade et al., 2009; Purrinos et al., 2012). Yeast species isolated, mainly belonging to genera *Debaryomyces*, *Candida* and less frequently *Hyphopichia*, are considered non-pathogenic and can grow to high populations in environmental conditions, pH, moisture, a_w and salt typical of dry-cured ham outer layer (Simoncini et al., 2007; Asefa et al., 2009). With regard to moulds isolated from dry-cured meat facilities and products, penicillia are largely dominant; currently, one of the main issues to be faced is *P. nordicum*, a toxigenic contaminant of protein-rich food, which proved to be a specific producer of ochratoxin A (OTA), a mycotoxin classified as a possible human carcinogen (Battilani et al., 2007). Biological control of *P. nordicum* contamination by typical

microbial antagonists has been tested to improve product safety without affecting quality and sensory properties. The aim of this work is to evaluate the ability of selected yeasts, isolated from Italian dry-cured ham, to compete with a toxigenic strain of *P. nordicum* in a dry cured meat model system and to inhibit OTA accumulation.

Material and Methods

Three yeast strains (*D. hansenii* 78, *D. hansenii* 147 and *H. burtonii* 120), selected from a native population during the maturing process of dry cured ham and stored in the collection of Stazione Sperimentale per l'Industria delle Conserve Alimentari (Parma, Italy), were used for the study. Yeast strains were genotyped by sequencing the D1/D2 domain of the 26S RNA encoding gene (Virgili et al, 2012). *Penicillium nordicum* (MPVP 1669), selected for this study, is stored in the fungal collection of the Institute of Entomology and Plant Pathology of the Università Cattolica del Sacro Cuore (UCSC) in Piacenza, Italy. It was isolated from meat products and identified at molecular level; it is a high producer of OTA (Bogs et al., 2006). Meat portions (50 mm in diameter and 10 mm in height) with 0.92 ±0.01 a_w were aseptically removed from pork muscle and processed according to Battilani et al. (2010).

Trial 1. Half of dried pork samples were dipped into ethanol and flamed, to lower naturally occurring microbial populations (mainly yeasts), and the other half were inoculated as such *P. nordicum* strain was single point inoculated on dry-cured ham cores at 3 different concentrations, namely 10^4-10^5-10^6 CFU/ml; then, samples were placed in plastic bags, sealed and incubated at 25°C for 7 days to evaluate mould growth and OTA production.

Trial 2. 100µl of each yeast suspension (10^9 CFU/ml) was uniformly distributed on the dry cured pork meat surface of samples and *P. nordicum* (10^5 CFU/ml) was inoculated in the center. The samples were incubated in the dark at 18°C. Factors involved were: meat portions flaming (flamed or not-flamed) and incubation time (15 and 30 days). The trial was done in triplicate. At the established deadline, dried pork samples were divided into two parts: one half was used for microbiological and the other for OTA analyses. Dichloran Glycerol-Agar plates (DG18, Oxoid) were used for yeast enumerations and Dichloran-Rose Bengal Chloramphenicol Agar plates (DRBC, Oxoid) for penicillia count, after incubation at 25°C for 4-5 days.

OTA production was evaluated at the end of incubation, using the methodology described by Toscani et al. (2007). Data analysis was carried out using the SPSS software package, vers. 19.0. Fungal counts expressed as $\log_{10}$ CFU/cm^2 were analyzed using General Linear Model (GLM); OTA accumulation was analyzed using a non parametric test (χ^2 test).

Results and Discussion

Trial 1. The population of penicillia counted in not flamed samples was found to be lower by two log units than in flamed samples; at the same time, OTA production was much higher in

flamed than in not flamed samples (Table 1).

Table 1. Average counts of yeasts and penicillia (log CFU/cm^2) and OTA content (µg/kg) in dried pork samples inoculated with *P. nordicum* at different inoculum concentrations. All samples were incubated at 25°C for 7 days.

	***P. nordicum* concentration (log CFU/ml)**					
	4		**5**		**6**	
	not flamed	*flamed*	*not flamed*	*flamed*	*not flamed*	*flamed*
Yeasts	6.44	3.0	6.87	3.0	7.08	3.0
Penicillia	6.43	8.30	6.21	8.29	5.57	7.22
OTA	27.3	541	67.6	537	41.9	717

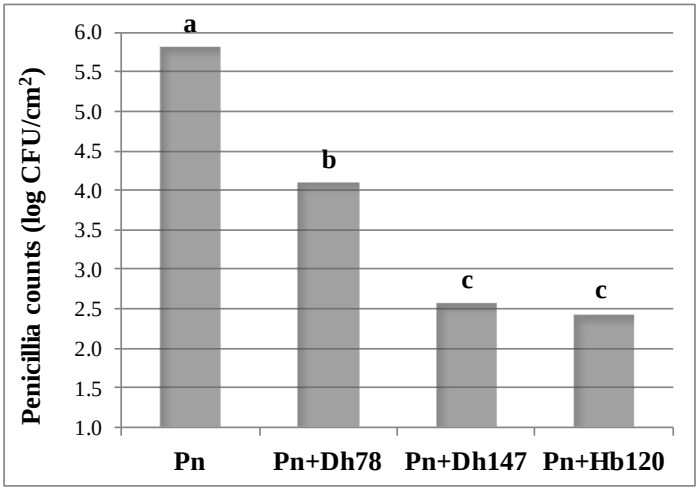

Figure 1. Effect of different applied yeast strain over penicillia population (log CFU/cm^2) in dried pork samples at 18°C. Different letters mean significant differences (LSD test, $P<0.05$).

These findings suggested a possible antagonistic role played by naturally occurring microbial populations (mainly yeasts) against *P. nordicum* (Pn) growth and OTA production.

Trial 2. Yeast strains belonging to *D. hansenii* (Dh) and *H. burtonii* (Hb) species, were co-inoculated with the toxigenic strain of Pn on the dry-cured meat model system. The temperature of the trial was lowered to 18°C, to be representative of the average temperature kept in conditioned rooms during protracted phases of dry-cured

ham processing. After 30 days of incubation, inoculated yeasts proved to be able to reduce Pn growth and to inhibit OTA production. Penicillia were reduced of ≈ 3 log units if compared to samples without co-inoculation, irrespective of flaming treatment. The inhibition of Pn growth and OTA production is an obliged achievement to warrant the safety of dry-cured meat products (Bertuzzi et al., 2013).

In the tested conditions, one strain of Dh and Hb were the worst and the best respectively, in inhibiting penicillia growth (Figure 1).

OTA produced by Pn (non parametric distribution) was evaluated according to percentile distribution (OTA values below and above 75^{th} percentile, corresponding to 5.0 ppb). A comparison was carried out ($\chi 2$ statistic) between Pn and Pn+ yeasts samples percentile distributions. Samples inoculated with Pn, Pn+Dh78, Pn+Dh147 and Pn+Hb120 were above 5.0 ppb OTA production in different percentages ($P< 0.01$), corresponding to 88%, 0%, 0% and 12% respectively.

Conclusions

D.hansenii 147 resulted as the most suitable to reduce *P. nordicum* growth and OTA accumulation. The possibility of using selected typical yeasts, for the biological control of mycete population growing in the outer layer of dry-cured ham over processing, is a current matter of investigation.

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O4.1

DOMINANCE OF *SACCHAROMYCES CEREVISIAE* IN WINE FERMENTATIONS: SECRETION OF ANTIMICROBIAL PEPTIDES AND MICROBIAL INTERACTIONS

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Abstract

In the present work we investigated the antagonistic effect exerted by *S. cerevisiae* against several wine-related microbial species *vis-à-vis* the secretion of antimicrobial peptides (AMPs). AMPs were partially purified from cell-free supernatants and different *S. cerevisiae* strains were screened for its production. The spectrum of action of the AMPs was determined against a broad range of yeasts and bacteria, at enological growth conditions.

Introduction

Saccharomyces cerevisiae plays a primary role in alcoholic fermentation and has a vast worldwide application in the production of wine, beer, bread and fuel-ethanol. Microbial diversity associated with these industrial fermentative processes is huge and originates mainly from the natural microflora of musts. Although several yeasts and bacteria species participate in these fermentative processes, *S. cerevisiae* is the dominant microorganism.

The ability of *S. cerevisiae* to out-compete other microbial species during alcoholic fermentation has been traditionally ascribed to its higher fermentative power and capacity to withstand the increasingly adverse conditions established in the medium as the fermentation progresses, i.e. high levels of ethanol and organic acids, low pH values, scarce oxygen availability and depletion of certain nutrients (Bisson, 1999; Bauer and Pretorius, 2000; Hansen et al., 2001). However, recent studies demonstrated that other factors, such as microbial interactions mediated by cell-cell contact

mechanisms (Nissen and Arneborg, 2003, Nissen et al., 2003; Arneborg et al., 2005) and killer-like toxins (Comitini et al., 2005; Pérez-Nevado et al., 2006; Osborn et al., 2007; Albergaria et al., 2010), might play an important role.

Material and methods

In this work we used the following *S. cerevisiae* strains: CCMI 885 (Culture Collection of Industrial Microorganisms, LNEG, Portugal); ISA 1000 (Instituto Superior de Agronomia, UTL, Portugal), ISA 1011, ISA 1200, ISA 1028, ISA 1029, ISA 1053, ISA 1063, ISA 1065, CBS 101 and ATCC 6269; Non-*Saccharomyces* strains used were: *Hanseniaspora guilliermondii* NCYC 2380; *Kluyveromyces marxianus* PYCC 2671 (Portuguese Yeast Culture Collection, UNL, Portugal); *Kluyveromyces thermotolerans* PYCC 2908; *Torulaspora delbrueckii* PYCC 4478; *Dekkera. bruxellensis* ISA 1649, ISA 1700, ISA 1791, ISA 2104, ISA 2116, ISA 2211; *Oenococcus oeni* ISA 4279 and DSM 2529. Inoculums of all yeast strains were prepared in 50 ml of YEPD medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose) incubated at 30°C and 150 rpm for 16 h (72 h for *D. bruxellensis* strains). Inoculums of *O. oeni* were prepared by transferring 1 ml of stock culture into 9 ml of MRS broth and cultures incubated at 25°C without agitation for 48 h.

AMPs were partially purified from Sc supernatants of alcoholic fermentations performed in synthetic grape juice (SGJ) (as described in Pérez-Nevado et al., 2006) at 25ºC and 80 rpm of agitation for 5-7 days. Cell-free supernatants were first ultrafiltrated through centrifugal filter units (Vivaspin 15R, Sartorius, DE) equipped with 10 kDa membranes and then concentrated (100-fold) with 2 kDa membranes. These peptidic concentrated fractions (2-10 kDa) were then fractionated by gel filtration chromatography, using a Superdex-Peptide column (10/300 GL, GE-Healthcare, UK). 100 µl of each sample were eluted with ammonium acetate (0.1 M) at 0.7 ml/min and all picks obtained were collected, lyophilised and stored until utilisation. All lyophilised fractions were ressuspended in appropriate medium and screened for antimicrobial activity.

Spectrum of action of the bioactive fraction was determined against all strains mentioned above by performing antimicrobial tests (AMT) in 96-wells microplates. Lyophilised AMPs were resuspended in 100 µl of YEPD (or MRS for *O. oeni*) medium (with 30 g/l of ethanol and pH 3.5) to a final protein concentration of 1 mg/ml. Control essays were performed for each strain using the respective medium without addition of AMPs. Cultures were inoculated with 10^5 cells/ml for yeasts and 10^6 cells/ml for bacteria. Microplates were incubated in a thermo-Shaker (Infors HT, Bottmingen, DE) at 30°C and 700 rpm for yeasts and at 25ºC without agitation for *O. oeni*. Growth was followed by absorbance at 590 nm in a microplate reader (Dinex Technologies Inc., USA) and by

colonies forming units (CFU). All AMTs were performed in triplicates.

Results and discussion

In previous work we found that Sc CCMI 885 produces a peptidic fraction (2-10 kDa) during alcoholic fermentation that is active against some wine-related yeast (Albergaria et al., 2010). This peptidic fraction was fractionated by gel filtration chromatography and the picks obtained were tested for antimicrobial activity. Results showed that only one fraction exhibited strong antimicrobial activity and thus this fraction was selected to be further characterised. Here we show that several other Sc strains exhibiting antagonistic effect against non-*Saccharomyces* strains also secrete these AMPs during alcoholic fermentation.

The spectrum of action of the AMPs present in the bioactive fraction was determined and results demonstrated (Fig. 1) they are active against a wide variety of microorganisms associated with wine fermentations, although the sensitivity of these microbial species towards the AMPs is strain-specific, as shown by the results obtained for different strains of *D. bruxellensis* and *O. oeni* (Figure 1).

Table 1. Death rate of *H. guilliermondii* (Hg) during alcoholic fermentation (AF) performed in co-cultivation with different Sc strains (inoculated with 10^5 cells/ml for each species) and the relative pick intensity (measured by absorbance) of the bioactive fraction detected by gel filtration chromatography in those AF supernatants.

Strain of Sc	AF time (h) of initial Hg death	AF time (h) till total death of Hg	Detection of the bioactive fraction by gel filtration
ISA 1000	48	96	++
ISA 1200	24	168	+
ISA 1063	24	96	++
ISA 1028	24	72	+++
ISA 1029	48	168	+
ISA 1046	24	72	++
ISA 1053	24	72	+++
CBS 101	24	168	+
CCMI 885	24	72	+++

+, ++; +++ is the relative pick intensity (measured by absorbance at 280 nm) of the bioactive fraction in the respective supernatants.

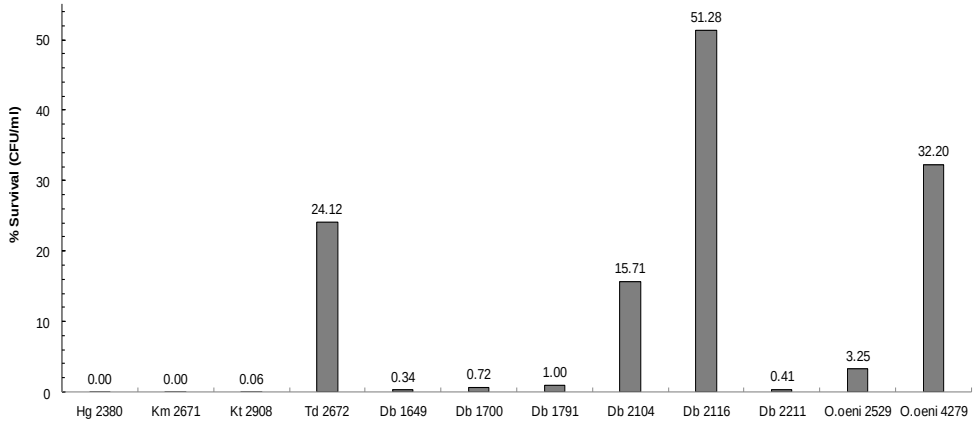

Figure 1: Survival of NS yeasts and *O.oeni* in the AMT performed with YEPD (or MRS) medium (pH 3.5) containing 1 mg/ml of the AMPs. The percentage of viable cells (relative to the initial cell density) in the cultures was determined after 14 h for *H. guilliermondii* (Hg), 48 h for *K. marxianus* (Km), *K. thermotolerans* (Kt) and *T. delbrueckii* (Td), 102 h for all *D. Bruxellensis* (Db) strains and 72 h for *O. oeni* strains.

Conclusions

Our work shows that dominance of the wine habitat by *S. cerevisiae* strains is, at least to some extent, mediated by the production of AMPs.

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O4.2

TRANSCRIPTOMIC APPROACH FOR THE IDENTIFICATION OF GENES INVOLVED IN THE DOMINANCE PHENOMENON OF *SACCHAROMYCES CEREVISIAE* STRAINS

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Introduction

Enological starter selection of autochthonous strains is a long process including molecular identifications and characterizations, physiological tests, microfermentations and other tests that research specific behaviors. *Saccharomyces cerevisiae* is the yeast commonly used for wine fermentation and is the object of continuous research in food technology and biotechnology. In this study we wanted to investigate a behavior that we want to include as an important behavior for future starter selections. This research investigated dominance as competition between different strains of *S. cerevisiae* in the same fermenting must. In the winery environment, sometimes, natural contamination could represent an important menace for the fermentation and compete with starters if present in important concentration. This study started investigating how two different strains of *S. cerevisiae* compete by revealing their presence by molecular methods. In a previous study (Perrone et al., 2013) we discover that in 35% of the cases one of the two strains totally overlaps the other. Strains used for the dominance tests were previously selected as suitable as starter and everyone of them was able to conduct the fermentation.

Pairs for which dominance was observed were also investigated for further tests. A bioreactor was setup, in which cells of different strains were kept separated but in communication sharing the same fermenting must. Surprisingly, no one of the pairs for which dominance was observed confirmed the same behavior in bioreactor fermentation. As well, the same cell density for the two compartment was reached. By this method we understood that strains feel each other only in case of cell to cell contact. This theory was also described by Nissen and Arneborg, 2003.

With this knowledge we performed further studies based on yeast transformation, flow cytometry separation and RNA-seq in order to get a better insight in dominance phenomenon and understand which is the genetic basis of the phenomenon.

Materials and methods

Two strains of *S. cerevisiae*, one dominant and one recessive, were genetically engineered in order to constitutively express the green fluorescence protein (GFP) or the eSapphire protein. The various steps of genetic engineering were:

- Promoter insertion through manipulation in a Ura- strain of *S. cerevisiae*;
- Purification of the recombinant plasmid;
- Transformation and amplification in *Escherichia coli*;
- Purification from *E. coli*;
- Transformation of autochthonous strains of *S. cerevisiae*.

A plasmid containing the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter upstream the region coding for GFP/eSapphire was obtained by recombination. Dominant and non dominant strains were transformed by inserting in the genome a cassette amplified from the recombinant plasmid.

Labelled and wild strains were subjected to fitness tests in order to verify that no differences were statistically observable between dominant and non dominant strain and between mutants and wild strains as described by Salvadò et al. (2011). Growth was monitored at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany). Measurements were made every 30 minutes for 3 days after a pre-shaking of 30 s. The wells of the microplate were filled with 0.01 ml of inoculum and 0.49 ml of natural must, to always ensure an initial OD of approximately 0.05. The inocula were always above the detection limit of the apparatus, which was determined by comparison with a previously established calibration curve. Student's T test (Statistica 7.0 software) was used to check for significant differences between mutants and wild strains. The dependent variables introduced for the analysis were the parameters obtained from the modelling (D, μ_{max}, λ). Furthermore, the same approach was applied to evaluate the growth of dominant/non dominant strains in mixed fermentation. For this purpose the POLARStar Galaxy Microplate Reader (BMG Labtech, Offenburg, Germany) was used and data were collected upon excitation at 485 nm with an emission filter at 520 nm. Data were fitted following the same protocol as described before.

With the final aim to extract RNA from dominant and non dominant strains in competition, fermentations were setup as follows:

- single fermentation of GFP-labelled dominant strain;
- single fermentation of GFP-labelled non- dominant strain;
- mixed fermentation of GFP-labelled dominant strain/Sapphire-labelled non-dominant strain;
- mixed fermentation of GFP-labelled non-dominant strain/Sapphire-labelled dominant strain.

Fermentations were performed in triplicate. Dominant and non-dominant strains were inoculated reaching the same cell density (OD ≅ 0.1) in 10 ml of Nebbiolo natural must keeping flasks in shaking at 25°C. Samples of 1 ml

were taken at 3 points of the fermentation on the basis of the results arising from competition kinetics curves. The first sample was taken a the beginning of the exponential growth phase, the second sample was taken in the middle of the exponential growth phase and the last sampling point was at the beginning of the stationary phase. Samples were rapidly centrifuged to eliminate must fraction, RNA*later* (Ambion, Life Technologies, Milan, Italy) was added, immediately frozen in liquid nitrogen and kept at -80°C until flow cytometry separation. Cells were sorted on the basis of labels according to fluorescence intensities, collected in tubes containing PBS buffer and directly put in dry ice to flash freeze at the exit of the cytometer following the method described by Achilles et al, 2006. This precaution was adopted to avoid the expression of stress genes due to the low temperature. Equipment did not permit the freezing through the use of liquid nitrogen as collect devices do not resist to the required temperature (-176°C). Also, evaporating gases could affect the drop flow in exit from the cytometer changing its direction outside collection tubes. MoFlo (Dako cytomation, Glostrup, Denmark) cell sorter was used as cytometer. It was equipped with two lasers: 488nm (for GFP excitation) and 345 nm (for Sapphire excitation). For GFP and Sapphire FL7 570/20 and FL1 530/20 channels were respectively used. Separation was performed with a sorting rate up to 20000 events per second for the time necessary to reach between 500000 and 1000000 cells. Frozen samples were kept at -80°C and thawed in ice for the subsequent RNA extraction using the RNA Mini Kit (Qiagen). To check RNA quality, samples were analysed through Bioanalyser and best samples were subjected to total RNA sequencing. RNA extraction and sequencing were performed for each of the biological triplicates at each sampling point.

Results and discussion

Data of fitness activity were compared by statistical analysis (student T test) in order to check differences between dominant and non dominant strain (i. e. 11 and 12 strains). From T test applied to growth curve parameters, no differences in lag, exponential and stationary phase were found. Moreover, the results of fitness analysis performed by SPECTROstar showed no changes in the behaviour of the transformants compared to the wild type. Using the same method dominant and recessive population kinetics were determined in mixed strain fermentations and student T test was applied to fluorescence data.

Differences were appreciable mostly between curve parameters from strain 11 in single and in mixed culture as it decreased sensibly its growth rate and final fluorescence reached in the case of the mixed culture. Strain 12 showed no significant differences in its growth characteristics in single and mixed culture (Figure 1).

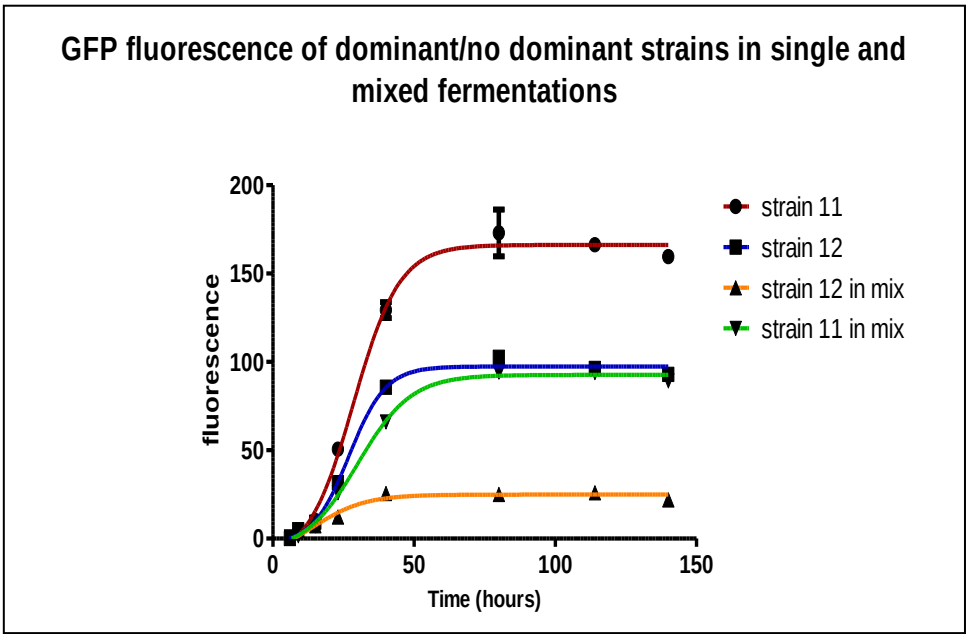

Figure 1. Growth curves from dominant and non dominant strains in single and mixed culture. curve named 'strain 11' shows the growth of non dominant strain in single culture and curve named 'strain 11 in mix' shows the growth of non dominant strain in mixed culture. Similarly, curves named 'strain 12' and 'strain 12 in mix' show respectively growth of dominant strain in single and mixed culture.

From total RNA sequencing it was possible to identify genes involved in the dominance phenomenon through a statistical analysis. Data from the RNA sequencing are currently in elaboration in order to determine genes for which differences can be observed between dominant and non dominant strain or between the same strain in single and mixed fermentation. First, expression of SSU1, encoding for a plasma membrane sulfite pump involved in sulfite metabolism and required for efficient sulfite efflux, seems to be increased in dominant strain, potentially giving the strain a major competitiveness in matrixes like grape must. Beside, for non dominant strain, several proteins involved in calmodulin metabolism are more expressed. Calmodulin accumulation is related to two different kind of stresses of the cells: electrolytes and minor component disequilibrium and stresses related to alpha-mating factor for which the cell can respond to stimuli belonging to the other strain arresting its growth in order to prepare cells to the mating. Next aims are to recognize which of these differences could be the one actually related to the dominance phenomenon by planning new trials.

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O4.3

TORULASPORA DELBRUECKII - SACCHAROMYCES CEREVISIAE AROMATIC INTERACTIONS IN SAUVIGNON BLANC WINE

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Introduction

The alcoholic fermentation (AF) of grape must is a complex microbial process, involving sequential development of various yeast communities. Non-*Saccharomyces* species start the fermentation until nutrient depletion, the increasing ethanol content and the heat production fermentation (Salvadó et al., 2011; Goddard, 2008) gradually eliminate the less tolerant species, thus favouring the development of *S.cerevisiae* which completes the fermentation (Heard and Fleet, 1985). Many researchers have investigated the specific metabolisms of the various non-*Saccharomyces* yeast species and their potential applications in the wine industry.

In this context, *Torulaspora delbrueckii*, one of the non *Saccharomyces* yeast species available on the market, has been described as having a positive impact on the organoleptic quality thanks to its low production of off-flavour compounds like acetic acid, ethyl acetate, acetaldehyde, hydrogen sulphide and volatile phenols (Renault et al., 2009). According to several authors, *T.delbrueckii* species is also able to release significant levels of 3-sulfanylhexan-1-ol (3SH) from a specific non-volatile S-cysteine conjugate, without releasing 4-methyl-4-sulfanylpentan-2-one (4MSP). The capacity of *T.delbrueckii* to modulate the esters productions has also been demonstrated by several authors.

Nevertheless, despite increasing interests to associate *S.cerevisiae* and *T.delbrueckii* in wine applications, few studies have to date focused on the aromatic potential of mixed cultures. Moreover, if the majority of authors demonstrated that it is possible to improve the aromatic properties of wines, reducing levels of off-flavours compounds like volatile acidity and acetaldehyde, the impact of mixed cultures on esters and thiols production are more confused and sometime in contradiction.

In this work, we compared the aromatic profile of Sauvignon Blanc wines fermented with pure and mixed cultures of *T.delbrueckii* and *S.cerevisiae*. The kinetics of 32 esters and 3 volatile thiols (3SH, 4MSP and A3SH) and their related precursors were analyzed.

The CO_2 production rate and growth evolution of both species were also monitored.

Material and methods

Two commercial yeast strains (Laffort Oenologie, France) were used in this study: *S. cerevisiae* Zymaflore® X5 and *T. delbrueckii* Zymaflore® Alpha TD N. SACCH.. Pure and mixed cultures were inoculated with 1.10^7 viable cells/mL for *T. delbrueckii* and 2.10^6 viable cells/mL for *S. cerevisiae*. Two types of mixed cultures were carried out: simultaneous modality where *T. delbrueckii* and *S. cerevisiae* were inoculated at the same time and sequential modality where *T. delbrueckii* was inoculated 24h before *S. cerevisiae* yeast. Yeast growth was determined by plate counting on 2 different agar media.

Fermentations were carried out in triplicate at 24°C in Sauvignon Blanc grape must containing 203 g/L sugar and an assimilable nitrogen concentration of 210 mg/L. The amount of CO_2 release (g/L) was determined by automatic measurement of fermentor weight loss.

Ester concentration was determined using a head space solid phase microextraction (HS-SPME) followed by gas chromatography-mass spectrometry (GC-MS) as described by Antalick et al. (2010). This method allowed the quantification of 32 apolar esters in wine.

Volatile thiols quantification was performed by the laboratory SARCO (Bordeaux, France) by gas chromatography–mass spectrometry according to method described by Tominaga et al. (2000). Thiol precursors concentrations were performed using the method described by Luisier et al. (2008) modified to assay all forms of precursor.

Results and discussion

All the cultures achieved the fermentation (residual sugar < 2 g/L) except for the pure *T. delbrueckii* culture which expectedly presented a residual sugar concentration above 100 g/L for an ethanol content of 6.2 %/vol. (Table 1). Fermentation kinetics varied markedly from one culture to another (not shown). The assay involving the inoculation with *S.cerevisiae* alone showed the highest fermentation rate and the shortest fermentation duration.

The maximum population (Xmax) reached during alcoholic fermentation by *T. delbrueckii* and *S. cerevisiae* was significantly higher when inoculated alone than in the presence of the other species. Both species hence had an influence on each other's development. It is interesting to note that the Xmax of *T. delbrueckii* in sequential mixed culture was significantly higher ($6.1.10^7$ viable cells/mL) than in simultaneous assay ($4.3.10^7$ viable cells/mL). Whereas, for *S. cerevisiae*, the Xmax were respectively of $4.5.10^7$ and $2.4.10^7$ viable cells/mL.

TABLE 1: Ethanol, fermentation duration (F.D), growth cell, esters and volatile thiols concentrations of pure and mixed fermentations

	Ethanol (vol.%)	F.D (h)	Xmax (viable cells/mL)		Esters (µg/L)			Volatile thiols (µg/L)		
			T. delbrueckii	*S. cerevisiae*	Main esters	Minor esters	Sum	4MSP	3SH	A3SH
T. delbrueckii pure culture	6.2 ± 0.3	350 ± 7*	$8.1 \cdot 10^7 \pm 2.8 \cdot 10^6$	/	914.62 ± 104.37	271.16 ± 2.94	1185.79 ± 107.32	ND	623 ± 103	14.0 ± 2.6
S. cerevisiae pure culture	11.8 ± 0.4	334 ± 3	/	$7.6 \cdot 10^7 \pm 1.8 \cdot 10^6$	6107.29 ± 369.02	194.84 ± 22.49	6302.13 ± 391.52	33.5 ± 9.9	303 ± 141	83.0 ± 9.6
Simultaneous mixed culture	12.4 ± 0.4	390 ± 2	$4.3 \cdot 10^7 \pm 3.5 \cdot 10^6$	$4.5 \cdot 10^7 \pm 2.3 \cdot 10^6$	9495.95 ± 336.30	354.31 ± 29.97	9850.27 ± 366.27	ND	362 ± 97	79.3 ± 20.2
Sequential mixed culture	11.9 ± 0.8	473 ± 7	$6.1 \cdot 10^7 \pm 7.1 \cdot 10^6$	$2.4 \cdot 10^7 \pm 7.1 \cdot 10^5$	6471.56 ± 286.63	459.57 ± 35.56	6931.14 ± 322.20	7.5 ± 0.6	1312 ± 324	217.5 ± 34.6

ND: no detected; * stuck fermentation

In this study, esters were classified according their abundance in *S. cerevisiae* wine rather than their chemical family: 7 esters present at highest concentrations (ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, isoamyl acetate, hexyl acetate and phenylethyl acetate) were named "major esters" and the 25 others esters measured in this study were named "minor esters". Esters were estimated at the end of alcoholic fermentation. Our results (Table 1) revealed that with both mixed inoculations, the total amount of esters increased in comparison to *S. cerevisiae* alone (up to 55%). Among the 25 esters monitored, 3 were particularly interesting as they could be considered as aromatic markers of *T. delbrueckii* during mixed inoculation (ethyl propionate, ethyl isobutyrate and ethyl dihydrocinnamate). Indeed, these compounds were produced in low amounts in *S. cerevisiae* pure cultures and were highly correlated to *T. delbrueckii* population size in mixed cultures. It is important to note that these compounds, although produced at low levels, contribute to enhance the complexity and fruitiness of wines (G.Lytra, Thesis of Bordeaux Segalen University 2010).

The concentrations of volatile thiols (4-mercapto-4-sulfanylpentan-2-one (4MSP), 3-sulfanylhexane-1-ol (3SH) and 3-mercaptohexyl acetate (A3SH)) and their precursors were estimated throughout the fermentation process. We confirmed the potential of the *T. delbrueckii* species to reveal 3SH and not 4MSP, as already described by Sadoudi et al.(2012) and Zott et al.(2011). The sequential culture increased the 3SH production (1312 ng/L) in comparison to S.*cerevisiae* pure culture (303 ng/L) in particular at the last stage of AF (Figure 1) suggesting a positive interaction between the 2 species. This phenomenon was confirmed by the amount of precursors assayed during fermentation.

Conclusion

Resulting from this study, it appears that *T. delbrueckii* produced, in pure fermentation, less overall esters than *S. cerevisiae* confirming previous investigations. However, some minor esters, and especially ethyl propanoate, ethyl isobutyrate and ethyl

dihydrocinnamate seem to be preferentially produced by this species and impact the mixed cultures. 3SH production kinetic in the sequentially inoculated assay was similar to that of the pure *T. delbrueckii* culture, except that the final concentration was much higher as it drastically increased in the last step of the fermentation. This work highlights synergic interactions between the 2 species which could have a significant impact on the wine aroma profile.

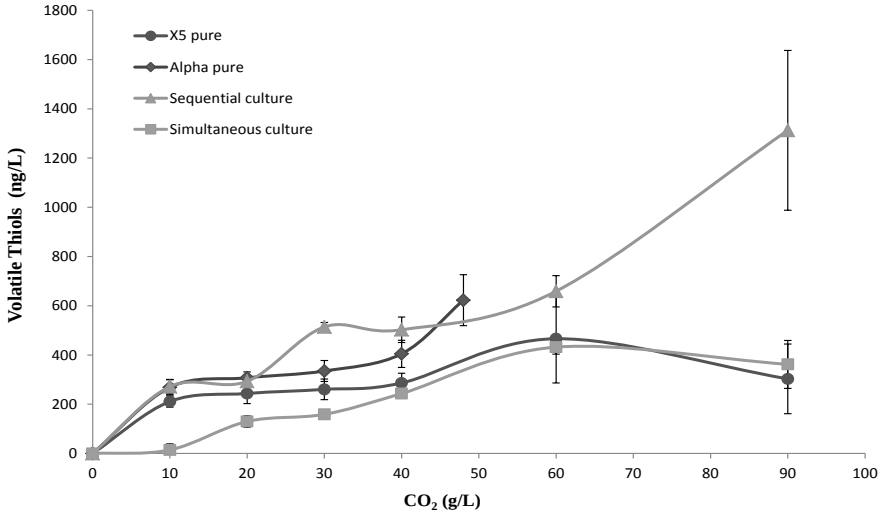

Figure 1. 3SH(3-sulfanylhexane-1-ol) productions in fonction of CO_2 release.

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O4.4

EFFECTS OF THE PEPTIDE PHEROMONE PLANTARICIN A AND COCULTIVATION WITH *LACTOBACILLUS SANFRANCISCENSIS* DPPMA174 ON THE EXOPROTEOME AND THE ADHESION CAPACITY OF *LACTOBACILLUS PLANTARUM* DC400

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Introduction

Exoproteome refers to proteins that are anchored to the membrane (GO:0046658), are intrinsic to the external side of the plasma membrane (GO:0031233) and cell wall (GO: 0005618), and are released into the extracellular milieu (GO: 0005576) from the bacterial cell surface (Zhou et al., 2010). Overall, the bacterial exoproteome affects processes such as recognition, binding, degradation, and uptake of extracellular complex nutrients, signal transduction, environmental communication and attachment to specific sites or surfaces (Zhou et al., 2010). Lactobacilli have a key role in the food industry. Studies on the role of the exoproteome in cell-cell communication or quorum-sensing (QS) mechanisms and, more in general, under stressful conditions, such as cocultivation with competing bacteria, may give new insights into the mechanisms of bacterial adaptation. Notwithstanding other regulatory factors, QS-mediated mechanisms, such as the peptide pheromone plantaricin A (PlnA) (Di Cagno et al., 2010), might play a pivotal role in the regulation of the microbial interactions in food (e.g., sourdoughs) and human (intestine) ecosystems. This study aimed at investigating the exoproteome of *Lactobacillus plantarum* DC400 when cultivated in the presence of the pheromone PlnA or cocultivated with other lactobacilli. A systematic proteomic approach, which included the separation of extracellular and cell wall-associated proteins by two-dimensional gel electrophoresis (2-DE) and protein identification by matrix-assisted laser desorption ionization-time of flight tandem mass spectrometry (MALDI-TOF-MS/MS) and multidimensional liquid chromatography (MDLC) coupled to nano-electrospray ionizationion trap tandem mass spectrometry (nano-ESI-MS/MS) was used. The capacities of *L. plantarum* DC400 to form biofilm and to adhere to Caco-2 human colon carcinoma cell line were also assayed to better define the influence of the exoproteome on QS-related phenotypes.

Material and methods

L. plantarum DC400 and DPPMA20 and *Lactobacillus sanfranciscensis* DPPMA174 were previously identified from Italian sourdoughs by 16S rRNA gene sequence analysis (Di Cagno et al., 2010). *L. plantarum* DC400 was grown into modified chemically defined medium (CDM) in monoculture, in CDM which was supplemented with 2.5 µg/ml of chemically synthesized PlnA (NeoMPS PolyPeptide Laboratories, S.A., Strasbourg, France), or in CDM under coculture with *L. plantarum* DPPMA20 or *L. sanfranciscensis* DPPMA174. The concentration of chemically synthesized PlnA used was based on previous findings (Di Cagno et al., 2010). For 2-DE analysis, cultures were centrifuged and both cell-free supernatants (CFSs) and cell pellets were used. CFS was freeze-drying, filtered, concentrated, dissolved in denaturing buffer and used to map the extracellular proteins. Cell wall-associated proteins were obtained as described by Izquierdo et al. (2009) with some modifications. Two-DE and spot detection were performed as described by De Angelis et al. (2001). Protein identification was carried out by MALDI-TOF-MS/MS and MDLC coupled to nano-ESI-MS/MS. Proteins were identified using an MS/MS ion search of the Mascot search. Database searches were also done with the peptide masses against the non-redundant NCBI database using the search program ProFound (http://www.prowl.rockefeller.edu/cgibin/ProFound) from Rockefeller University and ProteoMetrics. Cells of *L. plantarum* DC400 grown under different culture conditions were used to assay the capacities to form *in vitro* biofilm, to adhere at the Caco-2 human colon carcinoma cell line and to compete with potential gastrointestinal pathogens for adherence, as described by Ramiah et al. (2008), with minor modifications.

Results and discussion

The highest similarity of the 2-DE maps was found between DC400 cells cultivated in monoculture and in coculture with strain DPPMA20. Almost all extracellular proteins (25 spots) and cell wall-associated proteins (42 spots), which showed decreased or increased levels of synthesis during growth in CDM supplemented with PlnA and/or in coculture with strain DPPMA20 or DPPMA174 were identified. On the basis of the sequences in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, changes to the exoproteome concerned proteins involved in QS, transport system, stress response, carbohydrate metabolism and glycolysis, oxidation/reduction processes, proteolytic system, amino acid metabolism, cell wall and catabolic processes, and cell shape, growth, and division (Fig. 1).

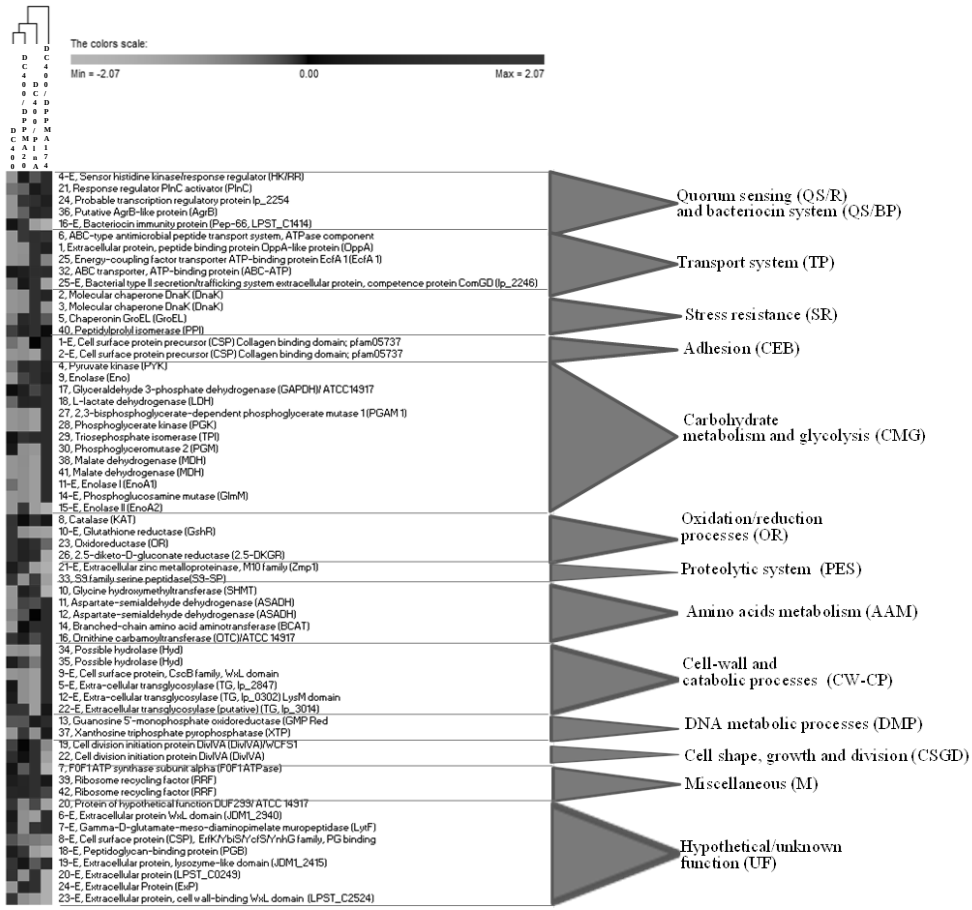

Figure 1. PermutMatrixEN analysis of the amount of proteins of *Lactobacillus plantarum* DC400 cells grown until the early-stationary phase (16 h) of growth was reached. Mono-cultures on modified chemically defined medium (CDM) (DC400) or on CDM supplemented with 2.5 µg/ml of plantaricin A (DC400/PlnA); and co-cultures with *L. plantarum* DPPMA20 (DC400/DPPMA20) or *Lactobacillus sanfranciscensis* DPPMA174 (DC400/DPPMA174). Changes in the protein amount (average of three replicates) are represented in grey scale, with black and grey indicating the highest and lowest values of the standardized data, respectively, for each protein in different culture conditions. "-E", extracellular proteins.

Compared to monoculture, the exoproteome of *L. plantarum* DC400 cultivated on CDM supplemented with PlnA or cocultured with other lactobacilli showed increases in the levels of synthesis of QS and bacteriocin-related proteins. Proteins involved in the general ABC transport

and peptide uptake (OppA) were induced in the same conditions. The ABC transporter Opp/Aml of *Streptococcus mutans* is an importer of the *sigX*-inducing peptide (XIP) pheromone, which is involved in peptide-mediated QS systems (ComCDE and ComRS) (Li and Tian, 2012). In addition, the capacity to form biofilms from streptococci is mediated via a QS system, which is responsible for developing competence proteins for natural transformation (Petersen et al., 2004). In this study, the level of the competence protein ComGD, which is responsible for exogenous DNA uptake across the bacterial cytoplasmic membrane, increased during cultivation of *L. plantarum* DC400 with PlnA. The cultivation of *L. plantarum* DC400 with PlnA or, especially, with *L. sanfranciscensis* DPPMA174 increased the levels of moonlighting proteins, which are responsible for stress resistance, adherence, and immunomodulation (e.g., GroEL and/or DnaK). Moonlighting proteins are highly conserved proteins from cytoplasm that, when expressed at the level of the bacterial cell wall, acquire the moonlighting function, which is different from that performed at the cytosolic level (Gupta et al., 2011). This study showed the presence of the above described chaperones at the exoproteome level and suggested the direct role of DnaK and GroEL in QS circuitries of *L. plantarum*. The exoproteome levels of some carbohydrate metabolism and glycolysis proteins was affected by cultivation with PlnA and, especially, with *L. sanfranciscensis* DPPMA174. Some of these proteins express moonlighting activities (Pessione, 2012). Proteins presumptively responsible for adhesion were found at the highest levels during cultivation with PlnA and DPPMA174. According to the proteomic results, the capacity to form biofilms, to adhere to Caco-2 cells and to prevent the adhesion of potential intestinal pathogens was found at the highest levels under cultivation with PlnA and, especially, with *L. sanfranciscensis* DPPMA174.

Conclusions

Bacterial performance is mostly the consequence of very complex community interactions. In such a context, taking *L. plantarum* as a model microorganism, this study shows how the adhesion and competition of *L. plantarum* DC400 are also mediated via the peptide pheromone PlnA and cocultivation with other species of lactobacilli which inhabit the same ecosystem. These phenotypic traits are strictly related to changes in protein synthesis and moonlighting activity of chaperones and enzymes, which are mainly responsible for carbohydrate metabolism and glycolysis.

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O5.1

BACTERIOCIN PRODUCTION AND ITS IMPACT ON GUT MICROBIAL DIVERSITY

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Introduction

As the scientific community continues to develop an ever-greater understanding of the composition and function of the human gut microbiota, and the role of specific microbial populations in health and disease, attention will turn to the tools that are at our disposal with respect to altering these microbes in a beneficial way. The use of antimicrobial agents has the potential to make an important contribution in this regard. However, care needs to be taken as it is evident that the administration of broad spectrum antibiotics can inflict significant collateral damage on commensal gut microbes. We have investigated the extent of this collateral damage through studies in which gut populations have been exposed to broad spectrum antibiotics. We have contrasted these impacts with those induced by broad and narrow spectrum bacteriocins (Cotter et al., 2005; Cotter et al., 2013), which are applied in a purified form or are produced *in situ* by a bacteriocin-producing probiotic. The results from these studies highlight the significant potential of bacteriocins as tools to alter the composition of the gut microbiota in a beneficial way.

Material and methods

A variety of different methods including antimicrobial activity assays, peptide purification, mass spectrometry, N-terminal peptide sequencing, comparative genome hybridisation, genome sequencing, high-throughput DNA sequencing of 16S amplicons and murine studies were utilised during the course of the studies described below.

Results and discussion

In recent years our group has been involved in many studies to identify novel producers of bacteriocins from the gut. During this period such bacteriocins of note have included Salivaricin T, salivaricin P, bactofencin and thuricin CD. Salivaricin T is a two peptide bacteriocin *Lactobacillus salivarius* 6488 that resembles Thermophilin 13 (O'Shea et al., 2011). Salivaricin P is also a two peptide bacteriocin produced by *L. salivarius* DPC6005 (O'Shea et al., 2011), which closely resembles abp118. Abp118 is

notable by virtue of the fact that its production has been established as being critical with respect to the ability of the corresponding producer to control *Listeria monocytogenes* infection in mice (Corr et al., 2007). Bactofencin A is a particularly unusual bacteriocin that is produced by another *L. salivarius* DPC6502. Array comparative genome hybridisations with this strain highlighted the absence of genes encoding known salivaricins and, thus, genome sequencing was employed to identify the antimicrobial in question. In parallel, peptide purification and N-terminal sequencing revealed the following sequence: KRKxHRxRVYNNGMPTGMYRYM. Screening of the DPC6502 genome sequence revealed the associated gene cluster. This consisted of 4 genes, designated *bfnABCD*. BfnA is the antimicrobial peptide, BfnB is a homologue of DltB, i.e. a transmembrane protein responsible for the transfer of activated D-alanine across the cytoplasmic membrane which is indispensable for the D-alanyl esterification of teichoic acids, while BfnC is a putative bacteriocin ABC-transporter and BfnD is bacteriocin transport accessory protein (O'Shea et al., 2013). Finally, Thuricin CD is a representative of novel a family of post-translationally modified bacteriocins known as the sactibiotics (so know because of the distinctive sulphur to α-carbon linkages in the associated peptides). Thuricin CD is a two peptide antimicrobial that was identified following screening of gut samples to identify strains that exhibit antimicrobial activity against *Clostridium difficile*. The thuricin CD producer, *Bacillus thuringiensis* DPC6431, was noted on the basis of its narrow spectrum activity against the pathogen (Rea et al., 2010).

In addition to identifying novel gut-associated bacteriocin producers, we are also interested in assessing the impact of bacteriocin producers or bacteriocins on the diversity of the gut microbiota. These can be subdivided into those in which an *in vitro* distal colon model was employed and those in which mouse models were utilised.

In the former case, the impact of exposing the gut microbiota to the lantibiotic Lacticin 3147, Thuricin CD and the antibiotics metronidazole and vancomycin was compared while, at the same time, assessing the ability of the antimicrobials to control a *Clostridium difficile* population that had been spiked into the sample. It was established that while all 4 compounds reduced pathogen levels, Lacticin 3147, metronidazole and vancomycin all inflicted considerable collateral damage on the gut microbiota (as assessed through culture independent high throughput sequencing based analyses). In contrast, Thuricin CD displayed remarkable target specificity, suggesting that its application is less likely to induce undesirable effects arising from damage inflicted on the commensal gut community (Rea et al., 2011).

In the latter case, a diet induced obese mouse model was employed to determine if the modulation of the gut microbiota by antimicrobials had the potential to minimise weight gain. This research was prompted by

investigations highlighting the contribution of gut microbes to weight gain. In this instance an abp118 producing probiotic (*Lactobacillus salivarius* UCC118), a non bacteriocin producing isogenic derivatives thereof and vanocmycin were employed for intervention purposes. It was established that both antimicrobials impacted significantly on weight gain, though, in the case of the abp118 producer, temporarily. More notably, with respect to gut microbial composition, it was again established that vancomycin dramatically changed the composition of the gut microbiota whereas the impact of abp118 were more subtle (Murphy et al., 3013).

Given the ever increasing evidence of the importance of the gut microbiota with respect to health and disease, these results highlight the merits of utilising narrow spectrum antimicrobials that have the potential to target specific pathogens or pathobionts without damaging other gut microbes or, theoretically, could be used to confirm the health promoting roles of specific populations through targeting of these microorganisms in animal studies.

Conclusions

The gastrointestinal tract is a rich reservoir of bacteriocin producers that can be used to, among other things, modulate the composition of the gut microbiota in a beneficial way.

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O5.2

THE OVEREXPRESSION OF *Pmrb* REDUCE THE SENSITIVITY OF *STREPTOCOCCUS THERMOPHILUS* TOWARDS SEVERAL ANTIMICROBIAL MOLECULES

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Introduction

Both bacterial and eukaryotic cells are tipically equipped with different type of cytoplasmic membrane transport systems involved in several vital roles. Indeed, such carriers transport nutrients and ions into the cell, they excrete waste products and toxic compounds from the cell, and are involved in maintenance of cellular homeostasis (Simm et al., 2012; Paulsen et al., 1996). Moreover, some of these transport/efflux systems have been demonstrated to play an important role in bacterial and eukaryotic cells by conferring resistance to toxic compounds. Export systems which can handle a wide range of structurally dissimilar substrate are defined as multidrug exporters or multidrug resistance (MDR) efflux pumps (Paulsen et al., 1996; Floyd et al., 2010). About 25% of all known membrane transport proteins in prokaryotes belong to the major facilitator superfamily (MFS) (Saier et al., 1999), the largest and most diverse superfamily of secondary active transporters (Reddy et al., 2012). *Streptococcus thermophilus* is a lactic acid bacterium widely used for the preparation of several dairy products such as fermented milks, yogurt, and cheeses (Hols et al., 2005). In the study reported here, we focused our attention on *pmrB* gene of *S. thermophilus* DSM 20617^T, encoding for a putative efflux pump belonging to the MFS potentially involved in the reduction of toxicity towards cytotoxic compounds. For understanding of the role and the activity of PmrB in *S. thermophilus* DSM20617^T, we over-expressed *pmrB* gene and we evaluated its effects on the sensitivity against several antimicrobial compounds. Moreover, we address the substrates spectrum of the above efflux pump by means of high-throughput technology as Phenotype Microarray. For the activity characterization of PmrB, we exploit the ability recombinants *S. thermophilus* to efflux

ethidium bromide (EB), which inside the cytoplasm intercalates with double-stranded nucleic acid thus determining its fluorescence increase when properly excited. Then, being EB a substrate for a variety of membrane pumps, it spontaneously diffuses across the membrane thus decreasing the overall measurable fluorescence (Czechowska et al., 2011; Lubelski et al., 2007). Moreover, the effect of *pmrB* overexpression on tetracycline and EB sensitivity has been evaluated in a strain naturally devoid of this efflux pump.

Materials and methods

Wild-type *S. thermophilus* DSM 20617^T and MIMST24 were maintained in M17 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 20 g/L of lactose at 37°C. The recombinant strains MIM27 and MIM28 (*pmrB*-overexpressing recombinant strain), MIM20 and MIM21 (harbouring the empty vector pMIT5) were cultivated in the same medium with chloramphenicol at a final concentration of 4 µg/ml. Recombinat strains MIM20 and MIM27 derived from strain DSM 20617 while recombinant strains MIM21 and MIM28 derived from strain MIMST24. *Escherichia coli* strain harbouring plasmid pNZ8048, pMIT5 or pMIT5-*pmrB* were routinely maintained in aerobic conditions in Luria broth at 37°C supplemented with 25 µg/ml chloramphenicol. *pmrB* was amplified and ligated into pMIT5 (originated by pNZ8048 by replacing the nisA promoter with a strong phage T5 promoter and lac operator) in One ShotR MatchTM-T1R Chemically competent *E. coli* (Invitrogen) by heat-shock. *S. thermophilus* DSM 20617^T or MIM24 electrocompetent cells were prepared and transformed as previously indicated (Arioli et al., 2007) either with pMIT5 and pMIT5-*pmrB*. The cells were selected by plating onto M17 agar containing 4 µg/ml of chloramphenicol after incubation of 48 h at 37°C in anaerobiosis. The effect of *pmrB* over-expression on the MICs of MIM20 and MIM27 against EB, chlorexidine (CH), or tetracycline (TET) was determined using a semi-automated microdilution method performed in a 384 wells microtiter plate as reported by Arioli et al. (2013). For EB efflux assay, the cells MIM20 and MIM27 were grown until the mid-log phase ($O.D._{600nm}$ of 0.5). Cells were washed and resuspended in sterile PBS (pH 7), in presence of 0.5 µg/ml of EB with and without two commonly-employed efflux pump inhibitors: 0.1 mM of the protonophore CCCP or 2 µM of reserpine, both purchased from Sigma Aldrich (Italy). This effectively allowed the loading of the cells with EB after an incubation time of 10 min at 37°C. Then the cells were pelleted and resuspended in PBS. To induce efflux activity, an energy source was given to the cells (1% lactose, wt/vol, and urea 1 mM) (Arioli et al., 2010), whereby cellular metabolism could be initiated thus creating an electrochemical gradient across the plasma membrane necessary for the efflux pump activity (Floyd et al., 2010). Then the decreasing fluorescence was measured for 100 min by means of a fluorescence

spectrophotometer (Victor3, PerkinElmer, Italy). The monitoring of EB efflux was also assessed by flow cytometry using a Apogee flow cytometer (Apogee, UK) staining the cells were stained with SYBR-green I and EB. Phenotype microarray analysis was performed for strains MIM20 and MIM27 using PM11-PM20 as previously described (Decorosi et al., 2011).

Results and discussion

The 1,170-nucleotide-long *pmrB* encodes a putative protein of 389 aminoacids identified in the whole genome of only some strains of *S. thermophilus* (CNRZ1066, LMD-9, JIM 8232 and MN-*ZLW-002*) and in *S. macedonicus* ACA-DC 198 displaying a nucleotide and aminoacidic identities of 100% and 99%, respectively. Alignment of *pmrB* with putative tetracycline resistance proteins of other Gram-positive bacteria revealed highly conserved motifs characteristic of the MFS family. Moreover, a PCR survey carried out on *pmrB* gene in a collection of 100 *S. thermophilus* strains of different isolation sources revealed that this gene is present in 34% of the analyzed population.

The strain MIM20 and MIM27 were analyzed for chemical sensitivity towards hundreds compounds, each at four concentrations using the Phenotype Microarray approach. Interestingly, MIM27, compared to MIM20, showed an increased metabolic activity in presence of several cytotoxic compounds, among them, CH and TET. The MICs evaluation was performed in M17 in presence of 0.5% and 2%, that is, low and high carbon source availability, respectively. The results obtained highlighted a strong correlation between the carbon source concentration and the antimicrobial compound sensitivity. In all tested conditions, the recombinant strains over-expressing *pmrB* had reduced sensitivity compared to the recombinant strains harboring the empty pMIT5 vector (Table 1). In specific, the most significant differences between the strains were detected in presence of TET and EB.

The effect of the over-expression of *pmrB* on the ability of the cell to pump out the EB was evaluated by analyzing the kinetics of EB efflux. The on-line visualization of EB efflux activity was assessed on the overall bacterial population by standard fluorometer and at single cell level by flow cytometry (Figure 1). The results obtained revealed a maximum of decrease in fluorescence (about 36%) after 40 min at 37°C in MIM27 compared to that for the MIM20 control cells (about 20%), suggesting an active extrusion of EB from the cells over-expressing *pmrB*. After 75 min of incubation, the decrease of fluorescence of MIM20 cells was similar to that of MIM27 cells; this delay is probably due to the time necessary for the induction of *pmrB* expression.

Following the addition of the protonophore CCCP (Figure 1A) or reserpine (data not shown), the fluorescence of both the control and the mutant cells was higher compared to the control without the efflux pump inhibitors due to the collapse of the

proton gradient across the membrane. This phenomenon provides indirect evidence that suggests H^+-dependent activity of PmrA. The data obtained revealed that the MIM27 population is extremely homogeneous, otherwise the MIM20 population is composed by 2 subpopulations, identified on the basis of the cell ability to efflux EB. The two population observed for MIM20 strains were also identified for MIM27 when cells were treated with CCCP (Figure 1B), thus suggesting that the PmrB activity is only partially locked by the presence of the inhibitor in the overall population over-expressing *pmrB*. On the other hand, in MIM20, in absence of CCCP, the presence of two subpopulations underlined a transcriptional bistability of *tetA*.

Table 1. Effect of *pmrB* overexpression and lactose concentration on *S. thermophilus* sensitivity against several antimicrobial compounds.

Strain	MIC (µg/ml) for TET		MIC (µg/ml) for CH		MIC (µg/ml) for EB	
	5g/l lactose	20g/l lactose	5g/l lactose	20g/l lactose	5g/l lactose	20g/l lactose
MIM20	0.125	0.5	0.25	0.5	0.25	1
MIM27	0.25	1	0.5	0.5	0.5	2
MIM21	0.125	0.5	0.125	0.25	0.125	0.5
MIM28	0.5	2	0.125	0.25	1	2

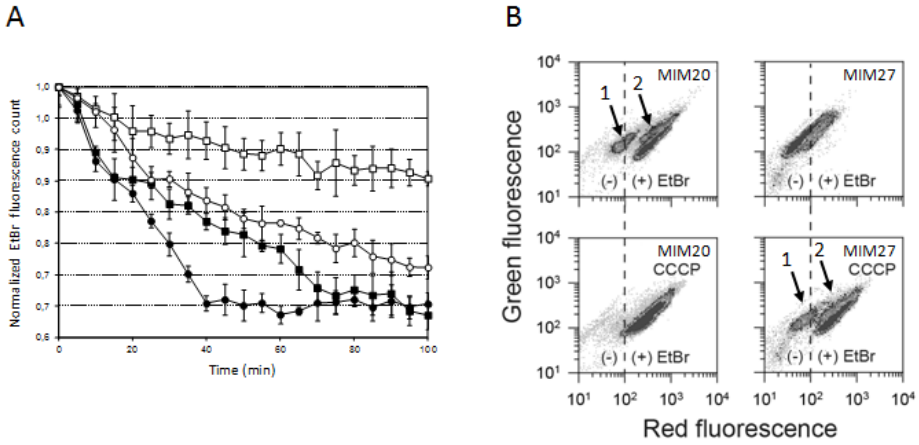

Figure 1. Monitoring of EB efflux by standard fluorometer and by flow cytometry in *S. thermophilus* cells. A) Monitoring of EB efflux in *S. thermophilus* cells by standard fluorometer. Data points indicate average geometric mean of three replicates ± one SD calculated from three independent biological assays for each time point. (square) wild-type. (circle) MIM27. White symbols represent data obtained using cells treated with the membrane protonophore CCCP (100 µM). B) Diagrams presents density plots of ^{10}log-^{10}log transformed SYBR green I versus ethidium bromide fluorescence of individual cells in samples taken after 50 min of EB efflux in *S. thermophilus* MIM27 overexpressing the efflux pump *pmrB* gene, and its reference strain MIM20. The arrows indicate the two subpopulations of the

MIM20 (1 and 2) having different ethidium bromide efflux efficiency. Cells prepared as described above but treated with the membrane protonophore CCCP were used as a control of reduced efflux efficiency.

Conclusions

In conclusion, this study characterized for the first time an efflux pump, *pmrB*, in the dairy species *S. thermophilus* underlining its role in cell detoxification from several cytotoxic compounds, among them tetracycline and ethidium bromide. The sequence analysis of *pmrB* revealed that this gene was exchanged among *S. thermophilus* and *S. macedonicus* by horizontal gene transfer. It remains to define if *pmrB* confers an ecological fitness to *S. thermophilus* in milk environment, thereby justifying the diffusion of this efflux pump within the species.

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O5.3

LACTIC STARTER ENSURE SAFETY IN LOW SODIUM FERMENTED VEGETABLES; A HURDLE TECHNOLOGY

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Abstract
Sodium chloride intake via foods is a worldwide concern and many countries have elaborated sodium reduction strategies. However, certain groups of unpasteurized foods like fermented vegetables can present a risk in regards to the safety. This study presents a challenge test with a clostridial cocktail in spontaneous and starter fermentations of sauerkraut with various sodium chloride concentrations (1.0, 1.5 and 2.0%). Results have shown that a reduction in sodium may jeopardize the stability and the safety of fermented vegetables, particularly in spontaneous fermentation. Even though all conditions were able to avoid the growth of *Clostridium*, an increase of the final pH was allowing it and therefore, any hurdles like sodium chloride have to be considered seriously to keep fermented vegetables safe.

Introduction

Risk mitigation associated to sodium reduction strategies has raised a few concerns in regards to potential botulism outbreaks in unpasteurized foods where sodium chloride is part of the processing like fermentation. Fermentation is the perfect example of hurdle technologies where organic acids, hydrogen peroxide, pH, diacetyl, bacteriocins, sodium chloride and others barriers are generated during the process and act in synergy to result in a large spectrum of bactericidal activity. The FAO has identified "fermented foods" being generally safe for consumers. Vegetables, in particular, possess a "clean record" in terms of outbreaks. Therefore, many countries allow producers to commercialize unpasteurized fermented vegetables. However, the worldwide sodium reduction trend could impair this safety record as one of its roles is to favor lactic bacteria and to compete food-borne pathogens. In regards to this, the FDA (2010) has raised concerns for botulism risk associated with the sodium reduction in this particular food group where incomplete fermentations could allow an increase in pH and therefore permit clostridial growth. A previous study has indicated that a slight sodium reduction from 2.5% to 2.0% (w/v) in a fermented cabbage juice media was allowing the survival and growth of *Clostridium botulinum* 62-A when pH was raised at 6.0 while 2.5% NaCl prevented the growth (Savard et al., 2011). In the present study, we have challenged the sodium

reduction in spontaneous and starter fermentation of sauerkraut with a cocktail of three clostridial strains.

Material and methods

Three sodium chloride concentrations have been added to shredded cabbage in four separate trials (brines giving a final sodium concentration of 2.0%, 1.5% and 1.0%). Three fermentation conditions have been compared. Set 1 (control) was non-contaminated sauerkrauts fermented spontaneously or with a commercial starter (BLAC 1, CBFC Inc, Canada) inoculated at 6.5 log CFU/g. Sets 2 and 3 were contaminated with 4 log CFU/ml of a cocktail of *Clostridium botulinum* spores (strains 62-A, PC0101AJ0 and 13983B) isolated from outbreaks in vegetables (Austin, J., Health Canada) and were spontaneously fermented or inoculated with the BLAC starter, respectively. All sets were manually mixed over 2 minutes and distributed in 2 liters pails. Trials were incubated for 7 days at 19°C and transferred at 4°C for a curing phase of 6 months. HPLC analysis (sugars and acids), pH and microbial count on MRS x-Gal and MSE for lactic acid bacteria, YM-tetracyclin-chloramphenicol for yeasts and Trypticase Peptone-polymixin-kanamycin for clostridial cells, have been followed during fermentation and curing phases. Results are the mean of 4 independent trials in duplicate.

Results and discussion

As reported by Gardner and collaborators (2001), the use of a starter not only increases the speed of fermentations but also allows to reach a lower final pH and to standardize the process from one batch to another. The results of this study clearly confirm that fermentations with the BLAC starter are much faster than spontaneous fermentations (delta pH of 1.5 units in the first 24 hours) and result in a lower final pH (0.5 units lower) as illustrated in figure 1. Those characteristics increase the safety of fermented vegetables in comparison to spontaneous fermentations as a pH 4.0 is reached in the first day with the production of lactic and acetic acids which, combined to the pH, are bactericidal for most of the food-borne pathogens (vegetative cells) naturally present at the surface, cracks and stomas of vegetables. Overall, the sodium reduction does not have a significant influence on acidification profiles even though a clear trend can be seen on spontaneous fermentation as the concentration reduction from 2.0% to 1.0% seems to reduce the pH drop in the first day as well as the final pH while fermentations with BLAC starter do not show any influences of the sodium chloride concentrations.

Chemical profiles of fermentations are presented in Table 1. The final concentrations of organic acids, lactic and acetic, produced during the fermentation are also not significantly influenced by the sodium chloride reduction independently of the use of starter or not. However, the concentration of sodium seems to modulate the acids productions during the first 7 days of spontaneous fermentations probably linked to the osmosis phenomenon of sugars diffusion resulting from the sodium

chloride even though this effect disappears after 7 days. However, the sodium concentration shows a significant modulation of the fructose-mannitol bioconversion in fermentation with BLAC starter as the strain *Leuconostoc mesenteroides*, responsible of the bioconversion, disappeared faster than the one in spontaneous fermentations (data not shown) as a result of the faster acidification and therefore has an impact on organoleptic qualities. Spontaneous fermentations have shown too much variability between trials (SEM of 0.2%) to extract any significant conclusions on the fructose-mannitol bioconversion profiles as it shows a decrease in both NaCl concentrations between days 48 and 90. Spontaneous variability and modulation of the bioconversion in the starter fermentations may increase the risk of secondary fermentations by yeasts resulting in a raise of the pH as reported by Savard et al. (2000).

The challenge test with the Clostridial spores cocktail inoculated at 4 log CFU/ml did not show any influences of sodium chloride concentrations in the tested ranges (2.0% to 1.0%) whatever a starter was used or not as the same level of spores (4 log CFU/ml) have been found after the 6 months period. It was then decided to mimic a secondary fermentation by yeasts by raising the pH to 6.0 with NaOH 1N. This raise has allowed the growth of Clostridial spores in "altered" sauerkrauts with all sodium concentrations (1.0 to 2.0%) in comparison to a previous study where NaCl at 2.5% was able to inhibit the growth at the same pH (Savard et al., 2011).

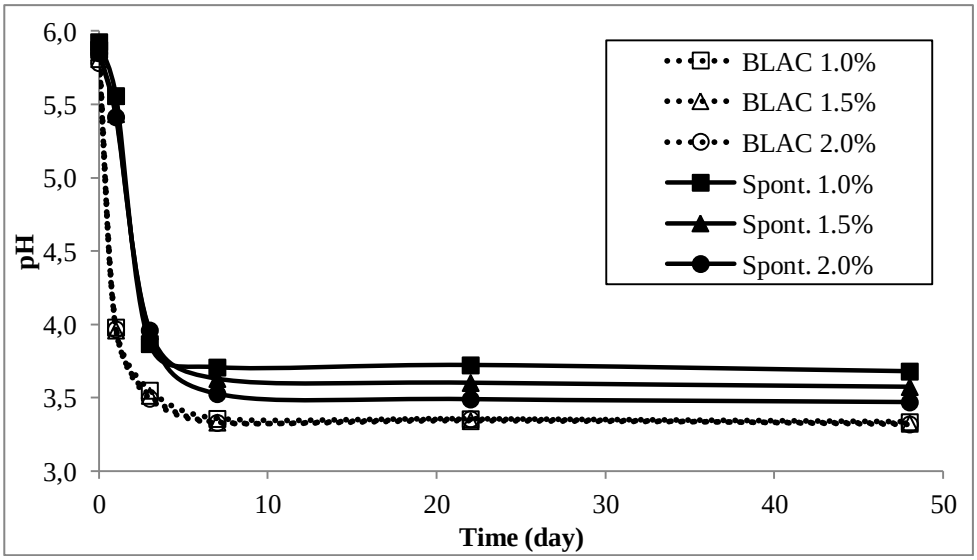

Figure 1. Acidification curves of sauerkrauts at different sodium chloride concentrations (1.0%, 1.5% and 2.0%) in spontaneous (Spont.) and starter (BLAC) fermentations. Results are the mean of 4 trials in duplicate. SEM and results from 3 and 6 months has been removed for more clarity.

Table 1. HPLC results[1] of sugars and organic acids reported as percentage (g/100 ml) followed over 90 days.

	Day 0				Day 1				Day 3			
	1% NaCl		2% NaCl		1% NaCl		2% NaCl		1% NaCl		2% NaCl	
	BLAC	Spont.	BLAC	Spont.	BLAC	Spont.	BLAC	Spont.	BLAC	Spont.	BLAC	Spont.
Fructose	0.38	0.40	0.33	0.30	0.25	0.41	0.09	0.33	0.26	0.54	0.62	0.44
Mannitol	0.00	0.00	0.00	0.00	0.53	0.00	0.24	0.00	1.19	0.93	0.87	0.58
Lactic acid	0.00	0.00	0.00	0.00	0.20	0.00	0.11	0.00	0.83	0.30	0.82	0.16
Acetic acid	0.00	0.00	0.00	0.00	0.08	0.00	0.04	0.00	0.25	0.19	0.19	0.11
	Day 7				**Day 48**				**Day 90**			
	1% NaCl		2% NaCl		1% NaCl		2% NaCl		1% NaCl		2% NaCl	
	BLAC	Spont.	BLAC	Spont.	BLAC	Spont.	BLAC	Spont.	BLAC	Spont.	BLAC	Spont.
Fructose	0.18	0.11	0.54	0.14	0.15	0.13	0.50	0.17	0.13	0.12	0.41	0.16
Mannitol	1.20	1.28	0.90	1.39	1.12	1.30	0.90	1.34	1.04	1.14	0.79	1.15
Lactic acid	1.18	0.44	1.10	0.61	1.09	0.51	1.05	0.55	1.01	0.48	0.93	0.47
Acetic acid	0.25	0.26	0.19	0.29	0.23	0.26	0.19	0.26	0.21	0.23	0.17	0.22

1. Results are the means of 4 independent trials. SEM and results obtained at 6 months have been removed for more clarity.

Conclusions

When analysed in terms of the processing, sodium reduction looks to have low impacts on fermentative microbial flora, final pH and organic acids even though clear trends can be observed in this study in the tested range (1.0% to 2.0%). Our results tend to demonstrate that the sodium chloride reduction in fermented vegetables can jeopardize the overall stability of unpasteurized final products as reported by Taormina (2010). The *Clostridium botulinum* cocktail was not influenced in the tested range of NaCl reductions and only the pH seems to avoid the spore growth in fermented vegetables. Therefore, the use of a specific starter may ensure a better safety of the product in the absence of thermal treatments as it allows to standardize the products, to reach a lower pH and to compete indigenous yeasts flora which could raise the pH following a secondary fermentation.

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O5.4

INHIBITION OF *LISTERIA MONOCYTOGENES* BY THE BIOPROTECTIVE STRAIN *LACTOCOCCUS PISCIUM* CNCM I-4031: A CELL-TO-CELL CONTACT DEPENDANT SYSTEM ?

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Lactococcus piscium CNCM I-4031 is a psychrotrophic lactic acid bacteria isolated from salmon steak. This strain is well adapted to chilled temperatures, with a minimum growth at - 2°C, an optimum at 26°C and no growth at temperature higher than 28°C (Leroi et al., 2012). *L. piscium* is able to prevent the off-odours formation in naturally contaminated peeled and cooked shrimp packed under modified atmosphere stored at 8°C, and to guaranty a sensory shelf-life of four weeks instead of twelve days (Matamoros et al., 2009). This has been attributed to the inhibition of the spoiling specie *Brochothrix thermosphacta* (Fall et al., 2010a, 2012). *L. piscium* is also able to inhibit the growth of *Listeria monocytogenes* in shrimp by 4 log. The growth is totally stopped when *L. piscium* reaches its maximum cell concentration (10^8 CFU/g) suggesting a "Jameson" effect (Fall, 2010b). The predictive model Seafood Spoilage and Safety Predictor shows that the pH decrease (from 6.6 to 5.9) and the lactic acid production (90 mM) observed *in situ*, as a result of *L. piscium* metabolism, cannot not explain the inhibition.

A chemically defined culture medium (MSMA) that reproduces the inhibition was developed to study those interactions. Addition of lactic acid in the buffered MSMA at the concentration produced by *L. piscium*did not prevent *L. monocytogenes* growth. *L. piscium* does not excrete nor produce internal inhibitory molecules such as bacteriocin or H_2O_2. The consumption of glucose, amino-acids, vitamins, nucleic acids, Fe and Mg by *L. piscium* was measured by HPLC and a global metabolomic approach. After 24 h of co-culture, when inhibition of *L. monocytogenes* by *L. piscium* occurred, supplementation of the medium with those compounds did not restore the growth of *L. monocytogenes*, showing that the inhibition was not due to competition for those nutrients. A competition for glucose was observed but did not entirely explain the inhibition. It is noteworthy that inhibition was observed in co-culture only. No effect was observed when

cultures were separated by a 0.45 µm membrane or when *L. monocytogenes* was cultivated in MSMA pre-fermented by *L. piscium*, indicating a possible cell-to-cell contact dependant system. Preliminary experiments evidenced the presence of the *luxS* gene in *L. piscium* and the production by the strain of autoinducers-2 like molecules involved in the quorum sensing. Further studies will be conducted to determine if these compounds could play a role in the inhibition.

Keywords: inhibition, quorum sensing, competition, *Lactococcus piscium*, *Listeria monocytogenes*

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O6.1

USING GENOME DATA AND RNA-SEQ TO UNRAVEL INTERACTIONS BETWEEN *BIFIDOBACTERIUM THERMOPHILUM* AND *SALMONELLA* SPEC.

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Introduction

Bifidobacteria are Gram-positive anaerobic rods with a high G+C content belonging to the phylum actinomycetes. They can be distinguished physiologically from other members of the actinomycetes by the composition of their cell walls, which is more similar to that of lactobacilli (Kandler & Lauer, 1974). Bifidobacteria are frequently encountered in the GI-tract of humans, being predominant in the GI-tract of newborns and persist at lower levels in adults (Vaughan et al., 2005). Bifidobacteria are believed to play a pivotal role in gut health and many strains confer beneficial effects on the host (Klijn et al., 2005). Some strains of bifidobacteria are therefore used as food-additive in probiotic products. The global market of probiotics is still growing and is estimated to reach a value of 24 billion USD in 2017 (marketsandmarkets, 2013). As such, bifidobacteria are clearly of great economic and health importance. Their biology is, however, still little understood. This is partly due to their limited genetic accessibility and although protocols for transformation have been published, reports on genetically modified strains are rare (Brancaccio et al., 2013).

Bifidobacterial colonization of the GI-tract and their interactions with other microbes residing in it, has been studied extensively in the past decade (reviewed by Gonzalez-Rodriguez et al., 2013). Factors and proteins involved in colonization include, among many others, pili (O'Connell Motherway et al., 2011) and the surface lipoprotein BopA (Guglielmetti et al., 2008). Genes encoding for pili formation are apparent highly conserved in all *Bifidobacterium* spec, whereas a Blast search reveals that BopA homologous are only found in *B. breve*, *B. bifidum* and *B. longum*. Such heterogeneity with the genus *Bifidobacterium* suggests that many different factors involved in colonization and interaction with other microbes exist in different species. As the genetic accessibility of *Bifidobacterium* spec is poor, with a few exception, -omics approaches like comparative genomics and RNAseq are the most suitable methods to identify new colonization factors.

Bifidobacterium thermophilum RBL67

B. thermophilum belongs to the "*B. boum*"-group of bifidobacteria (Turroni et al., 2011), a group that has not been studied extensively yet. *B. thermophilum* strains have been isolated from bovine rumen, calf feces, sewage and piglet feces, whereas strain RBL67 was isolated from baby feces (von Ah et al., 2007). RBL67 is moderately tolerant to oxygen and reaches high cell numbers in fermentation. Furthermore, cells-agglomerates are formed at higher densities in non-pH-controlled fermentations, but not in fermentation with controlled pH at 7. These agglomerates might be responsible for micro colony formation, whose formation is important for survival in the GI-tract. Indeed, micro colonies formed by bifidobacteria have been observed in the GI-tract (Macfarlane et al., 2004).

RBL67 is active against *Salmonella enterica* subsp. *enterica* serovar Typhimurium establishment in a colonic fermentation and protects HT29-MTX cells against *Salmonella* infection (Zihler et al., 2011). Furthermore, RBL67 increased the life span of *Caenorhabditis elegans* during *Salmonella* exposure and reduced the severity of rotavirus-associated diarrhea in suckling mice (Zihler et al., 2011, Gagnon, 2008). Its protective and antimicrobial effects, growth characteristics, and robustness make RBL67 a promising microbe for enhancing gastrointestinal health.

Complete genome sequence of RBL67

The genome of *B. thermophilum* RBL67 consists of a single 2,291,643-bp circular molecule with a G+C content of 60.1% (Jans et al., 2013). The genome encodes 1,845 coding sequences (CDS), 47 tRNA genes and 4 copies of rRNA genes. The genome of RBL67 was compared to the virulence database VFDB (Chen et al., 2012) and 93 genes putatively involved in virulence were identified. Homologous of virulence factors in commensal microorganism are frequently niche factors (Hill, 2012) and therefore an additional selection was performed. First, the presence of the putative virulent genes products in 17 other bifidobacterial genomes was assessed and proteins found in at least 5 other bifidobacteria were considered as niche factors. This reduced the number of virulence genes from 93 to 30. These 30 candidates were further checked for correct annotation, for example the virulence factor "Iron ABC transporter" gave also a hit with "Methionine ABC transporter". This cleaned up the list and finally 16 candidates were identified (Table 1). The remaining list contains mainly genes without a clear relation to virulence. Moreover, the set of putative virulence factors in RBL67 is low and consumption of large amounts of RBL67 can therefore be considered as safe.

However, an internalin like protein was identified as virulent factor (bt_0413). Internalins are listerial surface proteins that adhere to cadherins and guide infection of epithelial cells. The RBL67 internalin-like protein possesses a LTA

binding GW-domains and leucin rich regions, but no cell wall binding LPxTG motif. Further analyses revealed that approximate 130 amino acids at the C-terminal of the protein has virtual 100% homology with 21 other proteins in RBL67. Some of these 21 proteins are putatively involved in the extracellular biology of the bacterium, like a murein transglycosylase, a transpeptidase, and the internalin. This suggests that the conserved 130 amino acids region is associated extracellular functions.

Table 1. Genes putatively involved in Virulence.

Function	E-score
pyruvate kinase I	5.30E-85
twitching motility protein PilT	3.80E-80
InlA - Internalin A	6.40E-36
Aldehyde dehydrogenase	5.60E-25
helicase, putative	4.10E-36
feoB - ferrous iron transporter B	2.00E-73
putative FMN-dependent alpha-hydroxy acid dehydrogenase	1.10E-36
aggregation substance Asa1	2.00E-22
iron(III) ABC transporter, ATP-binding protein	3.50E-25
general secretion pathway protein E	2E-100
IS100 transposase; transposase ORFA	1.00E-38
capsular polysaccharide biosynthesis protein Cps4H	4.70E-35
InlA - Internalin A	2.00E-31
AatC ATB binding protein of ABC transporter	5.00E-24
iron(III) ABC transporter, ATP-binding protein	1.50E-22
glf - UDP-galactopyranose mutase	2.10E-78

Beside the pili-formation machinery already identified in *B. breve* (O'Connell Motherway et al., 2011), a second set of pili genes was identified in RBL67. Further, a high number of pili-building block (PilA) encoding genes were found, suggesting that RBL67 is able to produce a number of different structured pili on its surface. Homologous of the corresponding genes were found only in *B. dentium, B. catenulatum* and *B. pseudocatenulatum*, suggesting again many different types of colonization factors in the genus *Bifidobacterium* and different modes of interactions.

RNAseq of a Co-Culture of RBL67 with *Salmonella*

To unravel the interaction of RBL67 with *Salmonella enterica* subsp. *enterica* serovar Typhimurium N15, both strains were grown in a co-culture and the global transcriptional response compared to mono-cultures was determined using RNAseq. The genes were divided into 3 groups: 1) genes highly expressed in both co- and mono-culture 2) genes highly expressed in the monoculture only, and 3) genes highly expressed in the co-culture only. The first group consists of a list of 188

genes and provides insight in the biology of RBL67 during growth. The group contains genes involved in cell growth, DNA metabolism and primary metabolism. Also the synthesis pathways of some amino acid including branched chain amino acids, lysine and glutamine were highly expressed, which agrees with early observations that bifidobacteria excrete amino acids into the medium (Matteuzzi, 1978). In the co-culture, transporters for amino acids were higher expressed, whereas synthesis pathways were down regulated. The regulation of these pathways suggests that RBL67 provides the medium with amino acids, but turns to another strategy in the presence of competing microbes. Additionally, genes involved in the peptidoglycans biosynthesis were higher expressed in the presence of *Salmonella*, suggesting changes in the cell wall. Peptidoglycans from *B. thermophilum* protect mice against *E. coli* infection (Sasaki, 1994). Also the internalin-like protein bt_0413 was higher expressed in the co-culture, suggesting involvement of the protein in interactions of RBL67 with other microbes.

Conclusion

The genome of RBL67 reveals the presence of putative colonization factors of which some are unique for the strain, others are shared with only a few and some are found in many *Bifidobacterium* spec.

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O6.2

CONFOCAL MICROSCOPY AS A TOOL TO VALIDATE PYROSEQUENCING RESULTS AND TO ASSESS INTERACTIONS WITHIN LETTUCE ROOT MICROBIOME

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Introduction

Plants in nature are a puzzle of microhabitats for ubiquitous bacterial communities, below and above ground. Many of these plant-associated bacteria are highly adapted to these habitats as several studies in the past decade have shown (Berg & Smalla, 2009). The impact of the microbiome for plant health has been increasingly recognized as plants may modulate their rhizosphere communities to their own benefit by selecting beneficial microbes. The significance of specifically adapted rhizosphere communities as "health insurance" for commercial plants can hardly be overestimated, especially in times of rapid climatic change and increasing stress on agriculture. It is thus of utmost importance to extend our knowledge about the factors contributing to the plant root bacterial community specificity and to understand correlations and interactions within plant microbiomes from a perspective of sustainability. A method to detect interactions among bacteria within natural microbiomes is based on the correlation of their occurrence patterns across different samples from the same environment (for example soils, human body, etc.; Barberán et al., 2012). The occurrence pattern is estimated by the deep sequencing of marker genes, the most common being the 16S rRNA gene. Unfortunately, it is not possible to unravel the origin of such correlations, which could either be a real interaction (i.e. syntrophy) or mere habitat sharing due to similar ecological traits. Therefore, a different approach is needed to discriminate between such relationships, thus distinguishing real interactions among environmental bacteria.

In this study, we investigated the specificity of the root microbiome to lettuce (*L. sativa* L.), using deep sequencing of 16S rRNA gene amplicon libraries. Using the information from 8 different cultivars, we established a significant understanding of the lettuce root core microbiome. Furthermore, we calculated correlations among OTUs in order to identify potential interactions among bacteria. Here, for the first time,

we used specific fluorescence *in situ* hybridization and confocal microscopy to unravel, by direct visualization, the nature of such correlations thus validating or confuting the pyrosequencing results.

Materials and methods

Root samples of 24 lettuce plants (*Lactuca sativa* L.) belonging to 8 different cultivars (2 convars, 4 subspecies) and of the wild ancestor *Lactuca serriola* L. were provided by Archae Noah (Krems, Austria): a non-profit association devoted to the conservation of plant cultivars in Europe. This unique location allowed us to investigate the specificity of the microbiome at different plant taxonomic levels (species→ convar→ subspecies→ cultivar) under real field conditions and completely leveled environmental factors.

The metagenomic DNA of three replicates per cultivar was extracted from ~5 g of root material. The 16S rRNA genes were amplified with Multiplex Identifier (MID) tagged universal primers, and purified PCR products were pooled and sequenced on a Roche GS FLX+ 454 Titanium platform.

Pyrosequencing data was processed with the software Qiime 6.0 (Caporaso et al., 2010), and sequences were length and quality filtered and denoised; chimeras and plastidic/mitochondrial sequences were removed. Four different OTU tables were created with similarity levels of 90, 95, 97 and 100%. Beta diversity and core microbiome (OTUs occurring in all *L. sativa*) were computed on a normalized dataset. Spearman correlations between abundant OTUs (>0.5% of the total microbiome) were calculated on the basis of occurrence patterns, to identify potential interactions, and only strong correlations ($R>0.6$ or <-0.6, and $p<0.01$) were considered and visualized using network analysis with Cytoscape.

FISH was performed according to Cardinale et al. (2008). FISH probes were applied in various combinations, to understand the nature of the correlations detected by correlation analysis (i.e. to discriminate between real interactions and habitat sharing). FISH-CLSM was also used to validate pyrpsequencing−assessed relative abundance of bacterial taxa. FISH stained samples were observed with a Leica TCS SPE confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany) and the images were processed with the software Imaris 7.3 (Bitplane, Zurich, Switzerland).

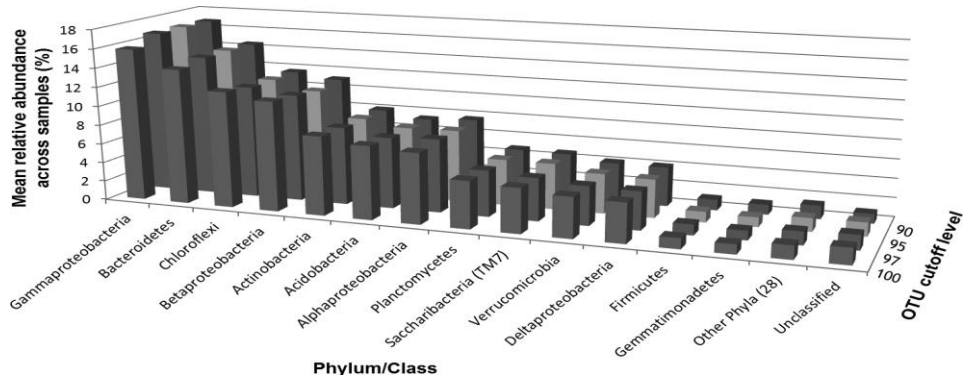

Figure 1. Taxonomic structure of the lettuce root microbiome as assessed by 454-pyrosequencing of the 16SrRNA genes

Results and discussion

A total of 216,466 high quality reads (an average of about 4000±1690 sequences per sample) were grouped into OTUs at 90, 95, 97 and 100 % cutoff level. Thirty-eight bacterial Phyla were detected, and the most abundant was Proteobacteria (~40.0%), followed by Bacteroidetes and, surprisingly, Chloroflexi. Gammaproteobacteria was the most abundant class (Fig. 1).

Microbiomes were specific to lettuce from species to cultivar level (UniFrac weighted pairwise distances), but specificity was highest at subspecies and cultivar level. Sixty-eight OTUs were found in all *L. sativa* samples (core microbiome), which included members of Gammaproteobacteria (15), Bacteroidetes (10), Actinobacteria (7), Alphaproteobacteria (7), Acidobacteria (6), Betaproteobacteria (6), Chloroflexi (6), Verrucomicrobia (5), Deltaproteobacteria (2), Saccharibacteria (2), Firmicutes (1) and Planctomycetes (1).Correlation analysis resulted in 25 positive and 1 negative correlations (Fig. 2). The network appeared to be composed of fully-networked subunits (such as Ps-Ps-Fs-Spm and Xa-Xa-An-Spb) connected to each other, thus generating a string of somehow interacting species which likely represents the actual situation within any natural microbiome (Fig. 2).

FISH-CLSM confirmed the results of pyrosequencing for Bacteroidetes and Chloroflexi, partially confirmed for Gammaproteobacteria and Alphaproteobacteria, and completely confuted for Betaproteobacteria. In fact, FISH-CLSM showed Betaproteobacteria as, by far, the most abundant group. FISH-CLSM allowed us to unravel the nature of the occurrence pattern−based correlations: cell-to-cell contact between Fs−Spm and An−Spb were observed *in situ*, thus their correlations (Fig. 2) can be interpreted as real interactions. Conversely, the correlation between Fs−Ps is more likely to be the effect of habitat sharing. Betaproteobacteria showed many interactions with Gammaproteobacteria and other bacteria which were not detected by occurrence pattern based correlations.

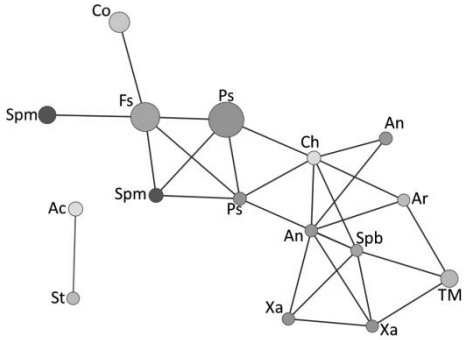

Figure 2. Network analysis showing correlations between abundant OTUs (>0.5% of the total microbiome). Nodes represent OTUs and edges represent strong positive (dark grey) or negative (light gray) correlations, respectively. Node size and labels indicate OTU abundance and identity, respectively. Ac: Acidobacteria−6, Ae: *Aeromicrobium*, An: Anaerolinae, Ar: *Arthrobacter*, Ch: Chloracidobacteria, Co: Comamonadaceae, Fs: *Flavobacterium succinicans*, Ps: Pseudomonadaceae, St: *Streptomyces*, TM: TM7-3, Spb: Sphingobacteria, Spm: *Sphingomonas*, Xa: Xanthomonadaceae.

Conclusions

FISH-CLSM is a useful tool to complement deep sequencing approach. Because it is based on direct visualization *in situ*, it adds a greater quality to the deep sequencing results, by validating or contradicting them. It can also discern between real interactions and habitat sharing among environmental bacteria, so increasing our capacity to assess ecological interactions between microbes within their natural habitats.

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O6.3

MYCORRHIZAL SYMBIOSES AFFECT THE PRODUCTION OF HEALTH-PROMOTING METABOLITES IN HOST PLANTS

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Introduction

Many plant secondary metabolites, known as phytochemicals, have revealed a variety of health-promoting functions, particularly related to aging, free radical scavenging, immune system stimulation, lipid metabolism control and anti-cancer properties. Phytochemicals producing health benefits, such as polyphenols and sulfur-containing compounds, are contained in variable amounts in many raw edible fruits and vegetables (Liu, 2003) and are affected by plant genotype, environmental conditions, soil quality, agronomic management and the establishment of beneficial symbioses with soil microbes (Giovannetti et al., 2013).

Arbuscular mycorrhizal (AM) fungi (AMF) are obligate biotrophs which colonise the roots of their host plants obtaining sugars, without causing any damage, in exchange of mineral nutrients, absorbed and translocated through a fine network of extraradical hyphae extending from the roots to the surrounding soil. They establish mycorrhizal symbioses with most crop species, promoting plant growth and health, and reducing the need of chemical fertilizers and pesticides (Smith and Read, 2008). Moreover, biochemical changes induced by AMF in their host plants increase vegetable and fruit contents in secondary metabolites with health-promoting properties and the activity of plant antioxidant enzymes.

Globe artichoke, *Cynara cardunculus* var. *scolymus* (L.) Fiori, show high contents of phytochemicals, including polyphenols and inulin, and is considered a functional food. Artichoke leaves are utilized by the pharmaceutical industry for their choleretic, hypocholesterolemic, and antioxidant bioactivities, as a result of high contents in chlorogenic acid, cynarine, luteolin, whereas flower heads are among the richest sources of dietary phenolic antioxidants (Fintelmann, 1996). Tomato (*Solanum lycopersicum* L.) is extensively cultivated worldwide, and its fruits have assumed the status of "functional foods" as a result of epidemiological evidence of reduced risks of certain types of cancers and cardiovascular diseases. Tomatoes are a reservoir of diverse antioxidant molecules, such as ascorbic acid, vitamin E, flavonoids, carotenoids, among which lycopene, characterised

by a strong antioxidant activity and by the ability to modulate hormones, immune systems and other metabolic pathways (Canene-Adams et al., 2005).
In our work AMF-inoculated plants of tomato and globe artichoke were monitored for their content in antioxidant compounds and phenolics of fruits and flower heads, respectively, with the aim of assessing whether mycorrhizal symbioses affected the production of the health-promoting metabolites in such plant species.

Materials and Methods
Globe artichoke plants were nursery-inoculated with the AM fungal species *Glomus intraradices*, *Funneliformis mosseae* or with a 1:1 mixture of them. After field transplant, antioxidant activity, expressed as antiradical power (ARP), total phenolic content (TPC), flower heads production and mycorrhizal colonisation were monitored for two years. Molecular identification of root AM fungal symbionts was carried out during the second year in the field. Tomato plants in symbiosis with *G. intraradices* and uninoculated controls were grown in the greenhouse. Fruits were harvested at the full-red stage and analysed for biochemical parameters (lycopene, gluthatione and total phenolics contents and total antioxidant activity), genotoxic activity (Ames test and micronucleus assay) and oestrogenic/anti-oestrogenic activities (yeast oestrogen screen assay).

Results and discussion
In the field, globe artichoke plants previously inoculated with the AMF mixture showed earlier flowering and larger main flower head fresh weights, compared with controls, not only in the first (+92.8%), but also in second year (+70.6%). Molecular studies, aimed at assessing the persistence of inoculated AMF in the field, showed that ITS rDNA sequences clustering with those of *F. mosseae* and *G. intraradices* were retrieved only from inoculated plant roots. Inoculated plants showed higher phenolics content in flower heads, compared with controls, with the highest values detected in plants inoculated with the AMF mixture. Antioxidant activity of flower heads followed the same pattern of phenolics accumulation: ARP increases were higher in plants inoculated with the AMF mixture, compared with controls (+52% and +32% in the first and second year in the field, respectively) (Fig. 1).
AMF inoculation of tomato plants positively affected the growth and mineral nutrient content of fruits, with enhanced concentrations of some mineral elements (Ca, K, P, and Zn) with respect to controls. Interestingly, fruit P and Zn contents were 60 % and 28 % higher than those of controls, suggesting an important role of the mycorrhizal symbiosis in improving the nutritional value of tomatoes. Lycopene content of tomato fruits produced by mycorrhizal plants was 18.5% higher than that of controls (Fig. 1). Such modifications of plant secondary metabolism were not linked with the production of putative unsafe

compounds, such as mutagenic ones, since the two genotoxicity tests used, Ames Salmonella/microsome mutagenicity assay and the human lymphocyte MN test, excluded the presence of phytochemicals with DNA-damaging activity. Moreover, tomato fruit extracts - both hydrophilic and lipophilic fractions - originating from mycorrhizal plants strongly inhibited 17-β-estradiol-human oestrogen receptor binding, showing significantly higher anti-oestrogenic power (+30-40%), compared with controls.

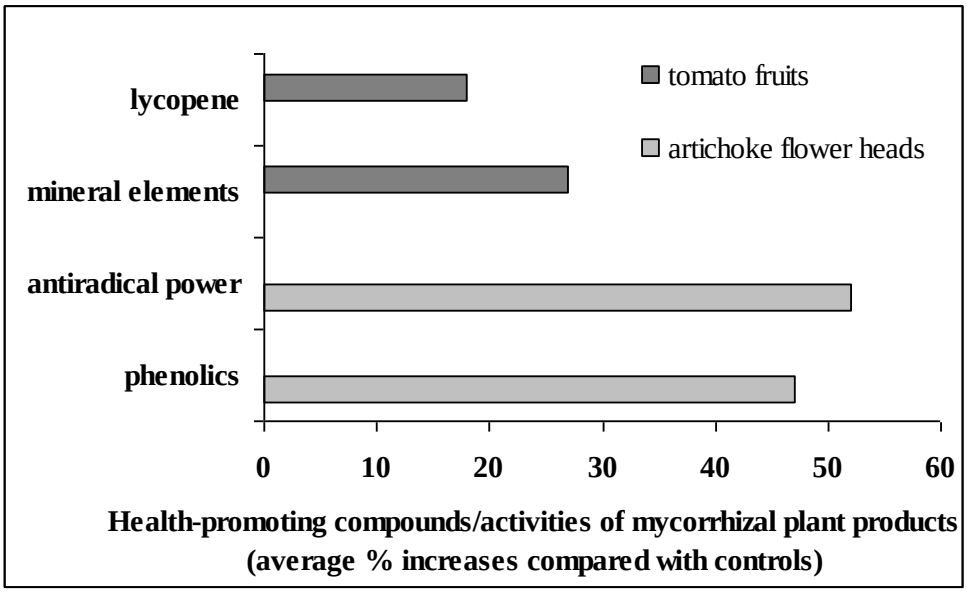

Figure 1. Percentage increases, with respect to controls, in health-promoting compounds/activities detected in food products of mycorrhizal plants.

Concluding remarks

Many studies reported that the consumption of fruits and vegetables may play a fundamental role in promoting human health, decreasing the risk of mortality from cancer and cardiovascular diseases.

AM symbionts determine higher plant growth rates, increase resistance to biotic and abiotic stresses and reduce the need of chemical fertilizers and pesticides, allowing a safe production of high-quality plant-derived food. Such beneficial fungi also enhance the biosynthesis of many different compounds with nutraceutical value in leaves, roots, and fruits of plants used for human nutrition

Our findings, indicating that shifts in plant secondary metabolic pathways as a result of mycorrhizal symbioses may lead to higher nutraceutical value of vegetable products, as in artichoke flower heads and tomatoes, suggest that plant inoculation with selected species of AMF may represent a suitable

biotechnological tool to be implemented in agri-food chains, with the aim of improving plant nutritional status and stress tolerance, while reducing the use of unsafe chemicals and producing healthy food.

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O6.4

TACKLING THE SPECIFICITY OF THE MARINE SPONGE MICROBIOME: A BIOGEOGRAPHICAL APPROACH

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Introduction

Recent evidence suggests geographical isolation plays a crucial role in driving microbial evolution and community structure in nature (Hanson et al, 2012). In this context, the study of microbial symbionts in animal hosts of restricted mobility is of particular interest given their proposed contribution to host fitness and survival. Recent research on microbial symbionts of benthic marine invertebrates such as sponges (*Porifera*) has unveiled high abundance and diversity of distinct prokaryotes, mainly within the domain *Bacteria*. These are believed to increase the fitness of their hosts (Taylor et al., 2007; Webster and Taylor, 2011) by providing them with readily-available nutrients, protection from disease, chemical defence and removal of metabolic by-products (Taylor et al., 2007; Webster and Taylor, 2011).

Studies using next generation sequencing (NGS) technologies have revealed tremendous bacterial diversity in varied sponge hosts (Lee et al., 2010; Webster et al., 2010). However, the ability of a given sponge host to maintain a core microbiota across biogeographical gradients has been poorly approached, in spite of being integral to our understanding of the marine sponge holobiont, its resilience and degree of specificity. Here, we make use of NGS technologies to determine the extent to which the shape of the sponge-associated microbiome is driven by the host organism and its biogeographical background, and to define the complementary role of seawater and marine sediments as "seedbanks" of microorganisms which make up its composition. To this end, specimens of the sponge genera *Ircinia*, *Sarcotragus* (Dictyoceratida, Irciniidae) and *Spongia* (Dictyoceratida, Spongiidae) were sampled at the Algarve coast (continental Portugal), the Madeira Island, and the Azores archipelago and subjected to 454 pyrosequencing profiling of bacterial 16S rRNA genes.

Methodology

Two species of sponge per locality were sampled during the summer of 2011: *Sarcotragus* and *Ircinia* spp.

from the Algarve and Madeira coasts, and *Spongia* sp. and *Ircinia* sp. from the Azores coast. Surrounding seawater and sediment samples were also collected. All samples were taken in triplicates.

Metagenomic DNA from all samples was extracted and the V4 hypervariable region of the 16S rRNA gene was amplified for in-depth analysis of bacterial community composition and diversity by 454 pyrosequencing on a Genome Sequencer GS FLX Titanium platform (Roche Diagnostics).

The obtained raw data was pre-processed using AmpliconNoise (Quince et al., 2011) for noise filtering, chimera removal and sequence sorting. Sequences among 150 and 260 bp were analyzed with the Quantitative Insights Into Microbial Ecology (QIIME) software package (Caporaso et al., 2010). Taxonomy assignment of representative sequences was performed within QIIME using the BLAST taxonomy assigner method with the Greengenes 13_5 database as reference sequences. The bacterial diversity and richness in the samples was estimated using the Shannon-Wiener diversity index and the Chao1 richness estimator, respectively. Multivariate analysis of community composition at the OTU level (97% sequence cut-off) was performed using the beta-diversity unweighted Unique Fraction metric (UniFrac, (Lozupone and Knight, 2005).

Results and Discussion

After AmpliconNoise filtering, 232251 chimera- and noise-free sequences were obtained, representing 5601 different OTUs.

Sponge microbiomes presented sharply contrasting composition of bacterial phyla in comparison with those present in seawater and sediments. Within sponges, *Ircinia* spp. possessed a more variable microbiota than *Sarcotragus* spp. and *Spongia* sp. The predominant taxonomic groups in *Sarcotragus* and *Spongia* were *Acidobacteria* and *Actinobacteria* (c. 20% relative abundance), followed by *Proteobacteria*, *Poribacteria*, PAUC34f, *Bacteroidetes* and *Chloroflexi* (c. 10% relative abundance each group). The aforementioned abundances remained similar in these sponges regardless of the sampling locality. Conversely, phylum-level abundances shifted across localities in *Ircinia* spp. In these hosts, the *Chloroflexi* displayed dominance at the Madeira site (31%) but was found to be a minority taxon at the Algarve (7%) and Azores sites (3%), where *Proteobacteria*, *Acidobacteria* and *Actinobacteria* were dominant groups at varying degrees. Surprisingly, bacterial communities from *Sarcotragus* spp. and the phylogenetically closer *Ircinia* spp. were less similar to one another than *Spongia* sp. and *Sarcotragus* spp. communities.

Regarding the biogeographical patterns exhibited by the studied communities at the approximate species (i.e., OTU) level, only the seawater samples were clearly dependent of the sampling location. Sediments and sponges clustered otherwise together

independently of the sampling site they were collected (Figure 1).

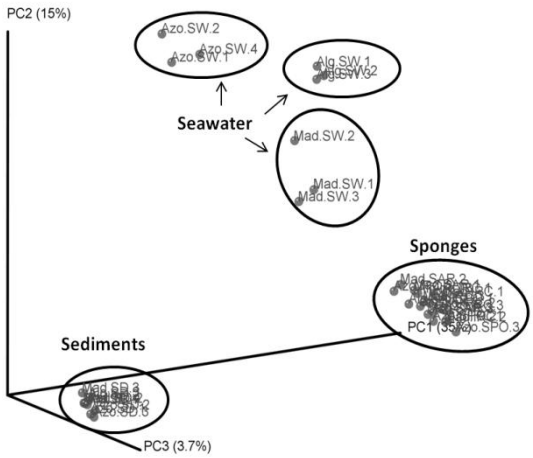

Figure 1. Beta diversity analyses based on weighted UniFrac distance matrices. The graphic evidences seawater sample grouping by locality. In contrast, sediment and sponge samples do not present or present a much less pronounced biogeographical pattern, respectively.

Based on the UniFrac metric, five clearly different groups were found: three different seawater clusters, each representing one sampling site, one sediment and one sponge cluster, encompassing all sediment and sponge samples, respectively, retrieved from all sample localities. In the case of the sponge samples, two differentiated sub-groups were found: *Ircinia* spp. and *Sarcotragus-Spongia* spp., reinforcing the results obtained from microbiome composition at the phylum level. *Ircinia* spp. were the most variable host regarding symbiont community composition. Yet they maintained a common pool of symbionts representing 23% (93 in 411) of the total *Ircinia*-associated OTUs detected across all examined sample sites (FIGURE 2). The number of shared symbionts in these hosts rose to 32 – 38% when only specimens from two sample localities were compared.

Our results suggest that the composition of the sponge associated microbiome is shaped by an intricate and cooperative interaction between the host organism and its environmental background. Further, compelling evidence for the recognition of marine sediments as the pivotal environmental source of highly specialized and abundant sponge-associated *Acidobacteria* was found.

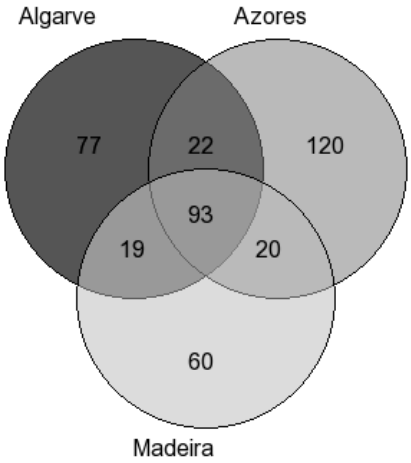

Figure 2. Venn diagrams of shared OTUs of *Ircinias* spp across localities.

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MD2013 - Torino (Italy), 23-25 October 2013

SPECIAL SESSION PROJECTS

SS1O.1

SOIL AGGREGATES: PROTECTIVE HOT SPOTS FOR MICROBES UNDER EXTREME ENVIRONMENTAL CONDITIONS

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Introduction

Among environmental impacts, *forest fires* represent a serious risk with negative effects on physical, chemical and biological soil properties. Soil aggregates the basic structural unit of soil, provide special microhabitats and are assumed to protect soil organic matter, including microbiota against harsh environmental conditions.

As the burning phenomenon is characterized by oxidation process and high temperature, we have simulated these conditions on isolated aggregates of different size by using plasma instrument and oven drying, respectively, to separately evaluate their effects on the autochthonous microflora in terms of soil microbial biomass and microbial community composition. Next to the protection of microbial cells (intracellular DNA) inside macro- meso- and micro-aggregates, focus was given on the protection of organic molecules free in the soil environment such as extracellular DNA.

Material and methods

Three different sizes of aggregates (0.5-1 mm, 500-100 µm and <100 µm) were calibrated by dry sieving from the 2 mm sieved fraction of a forest soil (Kristiansen et al., 2006). To simulate a *burning event*, each fraction of aggregates was subjected to 1) Low Temperature Ashing (LTA) by oxygen plasma treatment (D'Acqui et al., 1999) or to 2) high temperature by oven drying at 250°C, for 0, 5, 20 and 48 hours.

Aggregate protection-effects on soil microbiota were evaluated in terms of quality (molecular weight) and quantity (PicoGreen fluorometry) of the sequentially extracted extracellular (eDNA) and intracellular (iDNA) fraction of the soil metagenome (Ascher et al., 2009). The amounts of double stranded (*ds*) iDNA were used as index of microbial biomass (µg *ds* DNA g^{-1} soil). The microbial community structure (bacteria, archaea

and fungi) was assessed by comparative fingerprinting (PCR-DGGE) of eDNA and iDNA (Ascher et al., 2009), in order to increase the detection capacity of the performed molecular approach.

Results and discussion

The applied *fine-tuning DNA approach* (eDNA *vs*. iDNA), proofed to be a sensitive tool to assess the protective effect of aggregates not only on microbial cells (iDNA) but also on organic molecules free in the soil environment such as eDNA, this latter with evolutionary implication (genetic exchange by natural transformation).

Clear quantitative (TABLE 1) and qualitative differences were observed as a function of the treatment (oxidation, temperature), time of treatment, and pore sizes.

Microbiota was affected by the simulated *burning event* with the thermic stress being much more invasive than the oxidative one. Macro- meso- and micro-aggregates were found to exert different protective behaviours for microbial cells (iDNA) and organic molecules (eDNA) as was reflected also in the observed shifts in the community structure of all three analysed microbial domains.

Table 1. Yields of extracted DNA as a function of type and time of treatment, and aggregate size.

			OXIDATION		OVEN (250°C)	
type of aggregate	**time of treatment (h)**	**DNA**	***ds*DNA µg g^{-1} dry soil**	**SD**	***ds*DNA µg g^{-1} dry soil**	**SD**
0.5-1 mm	0	eDNA	1.38	0.30	1.38	0.30
	5		0.77	0.17	0.00	0.00
	20		0.19	0.01	u.d.l.	-
	48		0.01	0.00	-	-
500-100 µm	0		1.83	0.20	1.83	0.20
	5		0.65	0.07	0.01	0.00
	20		0.18	0.02	u.d.l.	-
	48		0.29	0.02	-	-
<100 µm	0		2.20	0.14	2.20	
	5		2.06	0.00	0.00	0.00
	20		0.03	0.00	u.d.l.	-
	48		0.14	0.00	-	-
0.5-1 mm	0	iDNA	1.87	0.82	1.87	
	5		1.00	0.16	0.00	0.00
	20		0.30	0.04	u.d.l.	-
	48		0.00	0.00	-	-
500-100 µm	0		2.51	1.12	2.51	1.12
	5		0.47	0.31	0.01	0.00
	20		0.27	0.00	u.d.l.	-
	48		0.11	0.00	-	-
<100 µm	0		1.30	0.17	1.30	0.17
	5		0.35	0.00	0.00	0.00
	20		0.07	0.00	u.d.l.	-
	48		0.56	0.00	-	-

u.d.l. = under detection limit

Conclusions

Our findings suggest the ecological relevance of soil aggregates in protecting organic molecules, such as DNA, against extreme environmental conditions, such as thermic and oxidative stress representative of forest fire.

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SS1O.2

MICROORGANISMS IN FOODS AND IN HUMANS: STUDY OF THE MICROBIOTA AND THE RELATED METABOLOME AS AFFECTED BY OMNIVORE, VEGETARIAN OR VEGAN DIETS

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Introduction

Human beings are reconsidered as "super-organisms" in co-evolution with their own indigenous microbial community (Ley et al., 2008). The composition of the oral and intestinal microbiota is influenced by genetic factors (Ktsoyan et al., 2008), age (NIH HMP Working Group, 2009) and diet (Larsen et al., 2010; Zimmer et al., 2012). Diet is the main reservoir of microbes and, especially, is the nutrient source for the host, and related oral and intestinal microbiota. The Diet4MicroGut project started in February 2013 and aims at studying how the omnivore, vegetarian and vegan diets may affect the oral intake of microorganims, and the composition of the oral and fecal microbiota.

Planning of the research activities

About 50 omnivore, vegetarian and vegan volunteers, for a total of 150 subjects have been recruited. Volunteers have filled diaries, describing their dietary habits, and have collected biological samples (saliva, feces and urine). Based on the different dietary habits, the presumptive intake of microorganims will be estimated using culture-dependent methods. Typing and antibiotic resistance of some food related microbial communities or foods will be also investigated. The metabolome

characterization will complete the overview on foods.

The oral and fecal microbiota will be studied. The viable cell number of several microbial groups have been estimated in fecal samples. Preliminarily, the microbial diversity of saliva and fecal ecosystems will be analyzed through PCR-DGGE. Selected numbers of biological samples will be further subjected to next generation sequencing, aiming at determining representative individuals/samples of the three types of diet.

Only for feces, representative samples (e.g., 4/5 for each diet) will be subjected to meta-omics analyses based on meta-genomic, meta-transcriptomic and meta-proteomic approaches. An integrated and iterative workflow will be also developed, by assembling an in house synthetic meta-genome. In agreement with an holistic approach, the metabolome analysis of saliva, feces and urine will complete the characterization of biological samples. All data available from the different research units will merge into a common database, whose structure will be developed during the project.

The Diet4MicroGut is a funded project by the Italian Ministry of Education, University and Research in the frame of the Research Projects of National Interest (PRIN) 2010-2011. It includes 10 Italian research groups. Update on the results obtained within the project will be regularly post on the project homepage (www.diet4microgut.it).

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SS1O.3

THE MIRRI PROJECT: THE ROLE OF COLLECTIONS OF MICROORGANISMS IN THE DEVELOPMENT OF ITALIAN BIOTECHNOLOGY

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Placed on the European Strategy Forum on Infrastructure (ESFRI) roadmap in 2010, MIRRI started its preparatory phase in November 2012. The aim of MIRRI, which actually involves 16 European partners, is the establishment of a pan-European distributed research infrastructure that will provide microorganisms and services facilitating access to high quality material, and associated data for research, development and application. It will connect resource holders with researchers and industries to deliver the resources and services more effectively and efficiently in order to meet the needs of innovation in biotechnology in accordance with the international legislation. During this preparatory phase a number of strategies for the implementation of MIRRI will be developed. These will, among others, include definition of a governance structure, operational practices as well as modes for sustainable funding of partner resource centres (for a detailed description of our work please visit the website www.mirri.org). As the success of these strategies depend on a full and open dialogue with different stakeholders we actively seek communication with users from the academic and bio-industry environments to give a feedback on their needs and expectations concerning the future MIRRI infrastructure.

For many decades European bio-industry has been one of our biggest stakeholder groups and as such they should profit most from a resource collection infrastructure with improved services and training and access to a much wider range of resources and associated data. To accelerate the mutual feedback between the stakeholder groups; the marginal and limited dialogue must be replaced by intensified and broadened communication channels. It is envisaged that removing existing obstacles such as yet under-utilisation of natural microbial resources and the diminishing expertise in their isolation and characterization, will be of benefit for the development of the European bio-economy. Therefore, MIRRI wants to take the opportunity to address leading figures of the European bioindustry taking the chance to present our vision.

MIRRI addresses the five fundamental challenges that Europe faces regarding

the use of microbial resources and their impact on the bio-economy:

1) Fundamental uncertainty in knowledge about where the latent value in microbial resources lies. MIRRI aims to pool existing information on microbial systems, sort them and make them more accessible to users. The idea is to create a user- and quality-driven virtual centre, resulting in a One-Stop-Shop for microbial raw material, expertise and legal advice.

2) There are significant gaps in the available resource base as well as gaps in the data and in taxonomic expertise that underpins resources. MIRRI will focus on the expertise and capacities of participating microbial resource centres (MRCs) to provide the specific resources to facilitate discovery of solutions to the grand challenges of climate change, improved agriculture and animal husbandry for food security, bioremediation, alternative energy sources and accelerated discovery in healthcare. To fight the loss of expertise well-structured training in different fields of modern microbiology will be offered.

3) Microbial diversity services need to be trusted and sustained. MIRRI will provide the services, including training to access new microbial diversity to support the bio-economy, based on a quality management system that increases the reliability of available organisms and associated data.

4) Places of origin need to be able to capture value and benefit from their microbial resources.

MIRRI will create a legal operational framework for access to microbial resources in full compliance with the Nagoya Protocol. This provides legal certainty in using microbial resources for the bio-economy.

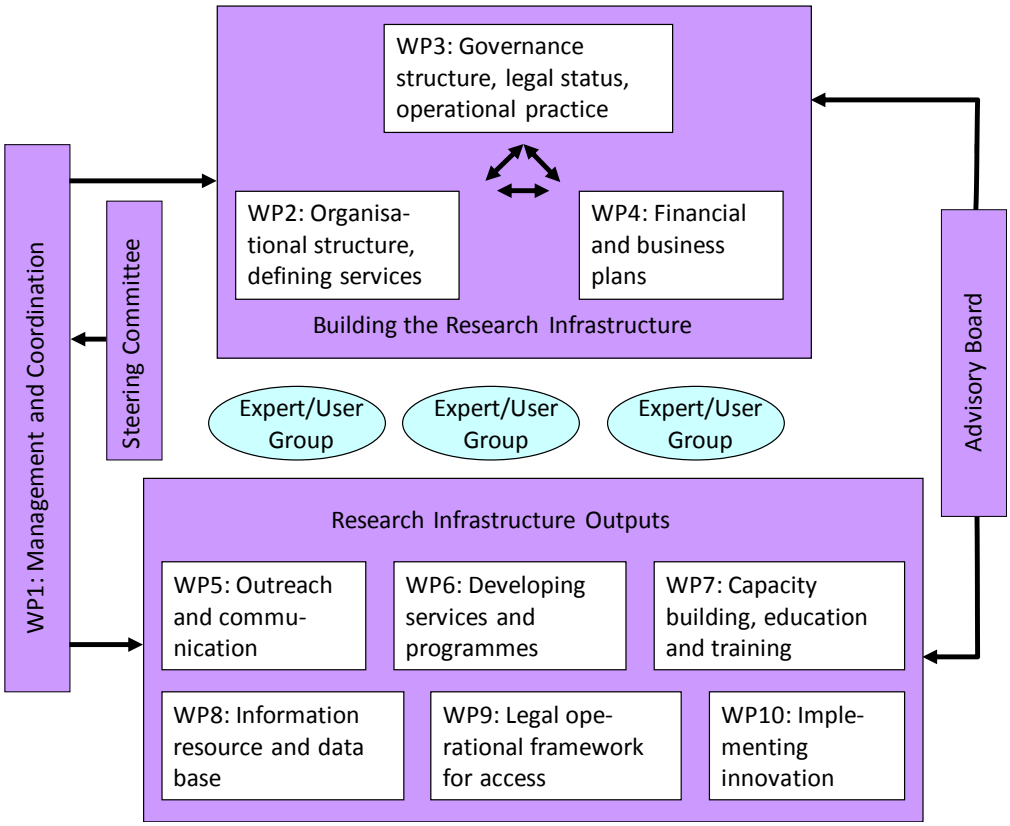
5) The quality and reproducibility of microbial science need significant improvement.

MIRRI supports the mandatory deposition of key resources included in the scientific literature and publicly funded research to safeguard valuable biological material for improving the credibility of science. MIRRI's aim is to establish an intensive communication with the European bio-industry, thereby identifying their needs and expectations from this pan-European infrastructure delivering microbial resources and expertise to support research and development. It is important to stress that the not-for-profit infrastructure MIRRI acts as service provider for the user in full support of the R&D strategies of bio-industry. By fulfilling our users' needs, MIRRI's goal lies in the improvement of discovery and innovation chain, leading to an increased competitiveness of the European bioeconomy.

The Preparatory Phase has been divided in 10 workpackages and the main activities will be:

- coordinate efforts of existing structures to design and build one pan-European infrastructure;
- define governance structure for Implementation Phase (incl. business plan and financial plan);
- define details of the future MIRRI infrastructure (e.g. minimum standards for MIRRI centres, quality control, access and supply of reliable material, services and expertise, data interoperability, …);

- consultation of decision makers concerning microbial components of the Nagoya protocol;
- organise funding and secure financial sustainability;
- organise consultation and cooperation with stakeholders;
- develop educational packages to ensure adequate training of scientists and BRC staff;
- establish linkages to non-European countries.



As regards Italy, the MIRRI project will have stimulating effects in many different ways. It will give the great opportunity to develop an Italian network of culture collections able to provide crucial services for both Academia and Industrial partners. From a practical point of view, if Italy wants to join MIRRI, the following tasks have to be developed:

- to list all collections of microorganisms present in Italy and check the quality of the material and of the relative dataset;
- staff training on technical and legal aspects for the establishment of common operational procedures within a legal operational framework;

- ensuring funding management of the collections;.
- development of National website for easy access to material and information.

Moreover, the creation of this network will have impact at several levels on society and economy: via an improved delivery of materials and information into the bio-based industry, it will generate employment opportunities as the discovery process is accelerated and new discoveries lead to new products and improved business.

In the Implementation phase MIRRI will be open for new members, provided that they will match membership criteria to be outlined within the next two years.

SS1O.4

GENETIC AND PHYSIOLOGICAL BASIS OF AEROBIC METABOLISM IN *LACTOBACILLUS RHAMNOSUS* AND *LACTOBACILLUS PARACASEI*: BASIC AND APPLIED ASPECTS

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Introduction

Lactobacillus casei, *L. paracasei* and *L. rhamnosus* are three closely related species (Felis and Dellaglio, 2007) involved in different food and health-related applications. As other lactic acid bacteria (LAB), these species respond to harmful conditions by activating general or specific stress response mechanisms, which can improve cell survival and strain performances. The growth behaviour and type of metabolism affect the robustness to stresses and recently, several studies have demonstrated that the aerobic/respiratory promoting conditions (oxygen, hemin and/or menaquinone in the substrate) induce in some LAB species helpful traits for industrial applications (Pedersen et al., 2012). To date, with exception of *L. plantarum*, reports on the aerobic/respiratory pathway in the *Lactobacillus* genus are still rare (Brooijmans et al., 2009) and data on defense mechanisms of *L. casei* group are limited to a small number of strains and stress conditions, generally investigated during anaerobic growth.

The Project "Genetic and physiological basis of aerobic metabolism in *Lactobacillus rhamnosus* and *Lactobacillus paracasei*: basic and applied aspects", funded under the framework of FIRB 2010, is focused on the studying, understanding and exploitation of stress response and aerobic/respiratory metabolism in wild and mutant strains of *L. casei* group. To achieve the overall aim of the Project a complex approach based on physiological, transcriptomic, proteomic, metabolomic and applicative studies will be carried out in order to obtain both pure (diversity of stress response, relations between strain ecology and survival, mechanisms of aerobic/respiratory metabolism) and applied (strains with respiratory phenotype and enhanced technological and nutritional properties) scientific knowledge in this field. A summary of the results of the first year is given here.

Material and methods

184 strains belonging to the *L. casei* group (identified by SDS-PAGE, DGGE-PCR, specific-PCR, multiplex-PCR, High Resolution Melting Analysis, and genotypically characterized by RAPD-PCR, Rep-

PCR, Sau-PCR) were screened for their ability to cope with several growth and stress conditions (different T°C, pH, NaCl, ethanol, and bile salt concentrations, antibiotics, ROS generating compounds; Ianniello et al., poster, this meeting; Reale et al., poster, this meeting). The shift towards aerobic or respiratory lifestyle (presence of oxygen and cofactors for the activation of electron transport chain, ETC) was also considered (Ianniello et al., poster, this meeting).

A more detailed study on the effect of anaerobiosis (AN; static cultivation), aerobiosis (AE; shaking cultivation, air) and respiration (RS; shaking cultivation, air, supplementation with hemin and menaquinone) was successively carried out on 60 selected strains. A new rapid approach based on the reduction of resazurin (change from blue oxidised form to colourless reduced form) was optimized and used to evaluate the oxygen uptake by AN, AE and RS cells in aerated buffer. The oxygen consumption by a polarographic electrode and the activities of oxygen-related enzymes POX (pyruvate oxidase), NOX (NADH oxidase) and NPR (NADH peroxidase) were also measured to confirm the adequacy of resazurin assay (Ricciardi et al., poster, this meeting). A comparative *in silico* analysis of the principal genes involved in the oxidative stress responses as well as in the aerobic and respiratory metabolism was performed.

Results and discussion

A wide genotypic and phenotypic diversity was found within the *L. casei* group. The molecular characterization confirmed the difficulty in distinguishing between *L. casei* and *L. paracasei*, while the physiological and technological screening detected strains (mainly *L. rhamnosus* and *L. casei* isolated from human faeces and wine) with interesting stress response pattern, able to tolerate the harshest conditions (pH 1.5-2.5, NaCl 6%, 1.5% bile salt, 12-15% ethanol, growth at 48-49°C and presence of antibiotics, 2 mM H_2O_2, 0.2 mM menadione, 50 mM pyrogallol). Unexpectedly, 4 *L. casei* strains exhibited a catalase-like activity (even if the available genomes do not include sequences annotated as catalase) and their evolutionary pathway is certainly worth investigation.

The presence of oxygen and ETC cofactors significantly improved the growth of *L. casei* group: several strains exhibited the common traits of aerobic and respiratory (concurrent increased biomass and pH values, capability to consume oxygen) pathway, while a smaller number of isolates grew better under anaerobic conditions. A good correlation in oxygen uptake was found between resazurin assay and polarographic measurement, and the new optimized method proved to be fast and effective for testing a large number of strains. As expected, the activity of POX, NOX and NPR were higher in the presence of oxygen, hemin and menaquinone supplementation.

Database searches revealed in *L. casei*, *L. paracasei* and *L. rhamnosus*, the presence of genes having homology with *pox5*, *nox5* and *npr2* of *L. plantarum* WCFS1 (minimum % of

identity from 40 to 50%) and the complete cytochrome gene-set (*cydAB* cytochrome D ubiquinol oxidase subunits I and II, and the *cydCD* cytochrome D ABC transporter ATP-binding and permease protein; from 50 to 60% of identity with *L. plantarum* WCFS1). The (mena)quinones biosynthesis complex *menFDXBE* present in *Lactococcus lactis*, is absent in all species of *L. casei* group. However, a methylase (metyltransferase) for ubiquinone/menaquinone biosynthesis was found.

Conclusions

These studies allowed the identification and selection of strains with promising robustness to harsh conditions that deserve further investigation. The respiratory pathway may confer physiological and metabolic advantages also in the *L. casei* group and the exploitation of oxygen-tolerant phenotypes could be useful for the development of starter and probiotic cultures. More generally, our results suggest a new possible and flexible classification (oxygen-sensitive anaerobes, oxygen-tolerant anaerobes, defective aerobes) of LAB strains considering their growth, metabolic and oxidative stress behaviors under aerobic conditions.

Then, during the second and third years of the Project, the effect of oxygen and respiratory pathway on the growth, biomass production, stress tolerance, technological fitness, synthesis of antioxidant enzymes, changes in gene expression, proteomic and metabolic profile of few selected wild and mutant strains will be evaluated.

Acknowledgements

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SS1O.5

BIOCLEAN, NEW BIOTECHNOLOGICAL APPROACHES FOR BIODEGRADING AND PROMOTING THE ENVIRONMENTAL BIOTRANSFORMATION OF SYNTHETIC POLYMERIC MATERIALS

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Introduction

The worldwide production of plastics materials obtained from fossil resources is about 230 mil ton/y. EU contributed for ¼ to such a production but it produces 75% of the most prominent petroleum-based plastics, i.e., polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC) and polystyrene (PS). In EU, the post-consumer waste is ~25,1 mil ton/y: ~14,9 mil ton is recovered (recycled or incinerated with energy recovery) and ~10,3 mil ton is disposed of (in landfills) (Plastics-The fact 2012; www.plasticseurope.org) where partially undergoes photo oxidation/degradation, producing small fragments, which, together with plasticizers/plastics additives, enter the marine environment and thus the food chain, where exert toxic effects (Tanaka et al. 2013; de Stephanis et al. 2013). Strategies addressed to increase recycling, incineration and the safe landfill disposal of such materials have been put in practice in several EU Countries. However, recycling requires prior tailored and expensive sorting, and the recycled products normally have inferior long-term properties, with limited market applications. Incineration produces emissions and ash wastes. Thus, large volumes of plastic wastes ended up in landfills in all major EU Countries. Biobased and biodegradable plastics can represent interesting alternatives to synthetic plastics in specific applications but the sector is still in its infancy (<0.3% of total plastics produced in Europe). Thus, innovative biotechnological eco-efficient solutions for degrading/detoxifying fossil plastics currently sent to/accumulated in landfills, intensifying the biodegradation of those entering composting/anaerobic digestion facilities and occurring in aquatic environments are needed. To date, only few data are available on the biodegradable synthetic polymers (i.e.,poly(vinyl alcohol), aliphatic polyesters, polycaprolactone, and polyamides, etc.) while a little is known about biodegradability of petroleum-deriving polymers/plastics (Shah et al. 2008; Mor et al. 2008). Moreover, despite the evidence that some physical and chemical pre-treatments improve

plastics biodegradability, no attempts to develop an integrated physical-chemical and biological polymers/plastics degradation process have been made.

Aims and strategy

BIOCLEAN, the collaborative project (FP7-KBBE; http://www.biocleanproject.eu/) involving 18 partners from 9 different EU Countries and 1 from China; representative of 6 Universities, 4 Research Institutes, 7 SMEs, a multimunicipality from Greece and PlasticsEurope, aims to develop innovative, eco-efficient pilot-scale and/or field validated biotechnological solutions for degrading (and possibly valorizing) plastic wastes in terrestrial (landfills, composting facilities) and aquatic environments.

The rationale of the project is that microbioma being for long in strict contact with aged waste plastics could be considered an important resource for selecting microorganisms with plastic degradative capabilities. Moreover combination of a physical/chemical pretreatment step to the microbial activity could be an important strategy for enhancing plastics biodegradation.

BIOCLEAN strategy consists in using waste plastics from various terrestrial (anaerobic digesters; landfills, composting facilities) and marine (sediment and water from Aegean and Norwegian seas) environments for the enrichment and selection of novel, robust naturally-occurring plastic-degrading mixed and pure cultures. Moreover, microbial strains from private and public culture collections will be screened for their degradative capability. These will be exploited, in parallel to degraders enriched from waste plastics, in hybrid physical/chemical-biotechnological processes for the biodegradation/detoxification or the valorization (i.e., transformation into useful chemicals) of PE, PP, PVC and PS plastics. The most active strains/consortia will be applied in bio-augmentation/biostimulation strategies in composting and waste treating facilities and in marine environments.

The project is structured in ten WPs consisting of: (i) Management and coordination; (ii) Isolation and selection of robust polymer degrading microorganisms and enzymes; (iii) Characterization of the most promising polymer/plastic degrading microorganisms and enzymes, identification of final products and of their possible valorization routes; (iv) Development of sustainable pre-treatments for improving plastic biodegradability; (v) Development of pilot-scale biotechnological processes for the bioremediation or valorisation of plastics; (vi) Development of bioaugmentation strategies for marine water and composting facilities; (vii) Demonstration of the effectiveness of bioaugmentation protocols in Aegean seawater and in a full-scale composting facility; (viii) Environmental and economic evaluation of developed processes and strategies; (ix) Development of policy tools in support of the Marine Strategy Framework Directive with respect to plastics; and (x) Dissemination, exploitation and knowledge transfer.

Expected outcomes

The project foresees: (i) the establishment of a collection of well characterized robust bacteria, fungi and enzymes able to degrade/transform PE, PP, PS and PVC polymers and plastics; (ii) the characterization of new pathways for the biotechnological valorisation (via tailored fragmentation towards useful products) of PE, PP, PS and PVC polymers and plastics; (iii) the development of new, pilot scale-validated eco-efficient chemical/physical-assisted bioprocesses for the degradation/detoxification (hopefully also the valorization) of wasted PE, PP, PS and PVC polymers/plastics; (iv) the development of new pilot- and field-scale validated biotechnological strategies for intensifying the plastic waste bioremediation in composting/anaerobic waste treatment facilities and in marine environments; and (v) development of site-specific measures for mitigating plastic pollution and improving the environmental status of Aegean Sea.

These new/innovative processes and solutions would remarkably contribute to the eco-efficient safe disposal of plastic wastes sent to/accumulated in landfills and the biodegradation/mineralization of those that enter terrestrial waste treating facilities and aquatic environments.

Conclusions

BIOCLEAN would result in innovative eco-efficient processes and strategies able to improve the environmental status of the EU aquatic environments with regards to marine litter as well as in novel and shared monitoring tools and mitigation measures necessary for the EU Member States for better addressing Marine Strategy Framework Directive requirements regarding the achievement and maintaining Good Environmental Status in the marine environment.

The preliminary results obtained during the first year project period will be presented and discussed.

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SS1O.6

EFFECT OF PBS-BASED PACKAGING ON THE SHELF-LIFE AND SPOILAGE PROFILES OF SOFT CHEESES

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Introduction

The intense use and accumulation of non-degradable plastics, which leads to the growing problem of waste disposal, and the lowering in the availability of fossil resources have strongly increased the interest and efforts in using renewable resources to develop biodegradable-based packaging materials. A number of bio-based materials and their innovative applications in food-related packaging have therefore gained much attention over the past several years. In particular the main bio-sourced polymer currently used in food packaging is polylactic. On the other hand, polybutylene succinate (PBS) can be considered a promising polymer from renewable resources for packaging industry for the coming years due to its good characteristics and the prediction of a large development of succinic acid production by fermentation of gluco-based resources. The potentialities of PBS in this area are explored within the EU project SUCCIPACK (Grant agreement 289196) which aims to develop sustainable, active, and intelligent food packaging materials based on green PBS that can be flexibly used by packaging and food industries. Aspects that are taken into consideration include the optimization of the synthesis and compounding of polymer and copolymer grades for industrial plastic transformation processes to obtain films, trays and pouches. Also tailored packaging functionalities are obtained through surface treatments to control gas barrier properties and introduce antimicrobials. The performance of the novel packaging materials are assessed for different food products including soft cheeses, raw meat, fish products, ready-to-eat vegetables and ready meals for vegetarian.

The objective of this preliminary work was to test the effects of PBS-based packaging materials on the shelf-life of an Italian soft cheese, i.e. ricotta, by evaluating the development of the spoilage-associated microbial

populations and the volatile metabolites release during storage.

Material and methods

Samples of ricotta (60g) were packaged in ordinary atmosphere in PBS-based bowls (Ø 80 mm) which were sealed with PET or PBS by-layer (MLC4) films. Also PBS bowls packaged into MLC4 bags were prepared. All the samples were stored at 4 °C for 20 days. Determinations of microbial counts, volatile organic compounds (by electronic nose and solid-phase micro-extraction gaschromatography/mass-spectrometry - SPME/GC-MS - analysis), pH, Aw, color and sensory properties were monitored over storage and compared to control products packaged with the polypropylene (PP) material.

Results and discussion

Monitoring of the pH, Aw and colour (L* and a* parameters) showed that data were comparable among the various samples, and differences were detected mainly in relation to the sampling time. On the other hand significant differences related to the packaging system were observed in the evolution over storage of the contaminating microbial populations. Total viable counts increased from 3 to 6 Log CFU/g in samples packaged in PBS sealed with PET showing a dynamics similar to the control (PP) ones. The use of MLC4 for sealing the PBS bowls resulted in a higher growth rate, and cell loads exceeding 7 Log CFU/g were achieved in 7 days. By contrast, their growth was significantly delayed up to 3 days when samples packaged into PBS bowls inside MLC4 bags were used. Similarly to the total viable counts, the load of *Pseudomonas* spp. was significantly lower in MLC4 bags than in all the other conditions, and counts higher than 7 log units were observed only after 13 days of storage. On the other hand, lactobacilli, lactococci and yeasts were unaffected by the packaging conditions and attained final populations of about 7, 6 and 3 Log CFU/g, respectively. The shelf-life, calculated as the time necessary to attain the critical threshold level of 7 Log CFU/g, was influenced by the packaging conditions, ranging from 7 days in PBS+MLC4 to 11 days in PP and PBS+PET, and 14 days in MLC4 bags.

The diversity in the evolution of the spoilage microbiota clearly gave rise to different trends in the production of volatile compounds detected in the headspace of cheese samples. The main volatile metabolites detected by SPME/GC-MS analysis included lcohols, aldehydes, ketones, esters and carboxylic acids. Quantitative data of the volatiles evidenced that ricotta cheese packed in MLC4 bags was characterized by the lowest amounts of alcohols, fatty acids and ketones. Moreover, Principal Component Analysis of the instrumental data showed that samples were distributed in the scores plot according to the storage time and packaging system.

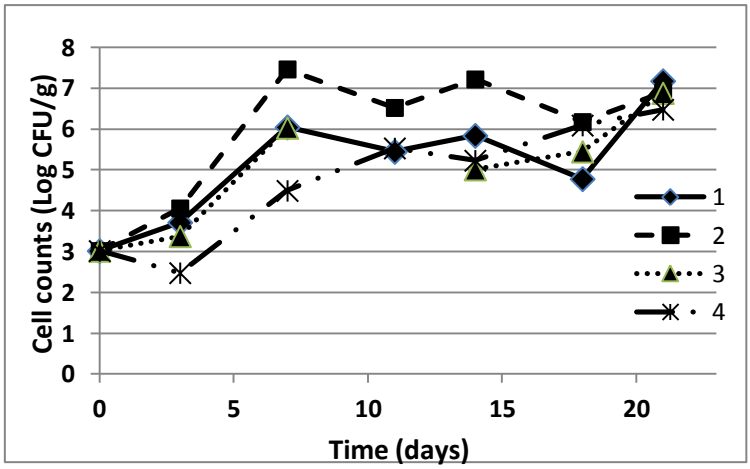

Figure 1. Evolution over storage of the total viable counts in ricotta cheese.
1: PP bowls; 2: PBS bowls sealed with PET; 3: PBS bowls sealed with PBS by-layer (MLC4) film; 4: PBS bowls packaged into MLC4 bags.

Conclusions

The comparison of all the data collected showed that the PBS-based packaging systems tested have good potential to be used by food industry to maintain or extend food shelf-life. In fact the packaging material and conditions used strongly affected the microbiota and its evolution during storage of ricotta cheese. In particular the packaging with PBS bowls inside MLC4 bags was the most effective in retarding the growth of total viable counts and *Pseudomonas* spp. and reducing the release of spoilage-associated volatile molecules with a potential impact on cheese sensory attributes.

MD2013 - Torino (Italy), 23-25 October 2013

SPECIAL SESSION YOUNG RESEARCHERS

SS2O.1

CHARACTERIZATION OF POTENTIAL PROBIOTIC BACTERIA FROM TABLE OLIVES FERMENTATIONS: TOWARDS A NEW FUNCTIONAL FOOD

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Introduction

In the past, microorganisms isolated from the gastro intestinal tract (GIT) were considered as the main potential probiotic bacteria, but recently the scientific community focused its attention to the fermented foods, recognizing them as valid and heterogeneous source of probiotic microorganisms. If the dairy products were broadly exploited both as source and carrier of lactic acid bacteria (LAB) and/or bifidobacteria potentially probiotics, few researches were carried out on fermented vegetable products. In this context, the table olives are the most important fermented vegetables in the international food trade and could be considered a promising probiotic food given that, as compared to dairy products, they do not pose problems for people intolerant to milk and milk products and those needing low-cholesterol diets. Moreover an edible portion of about 100 g of olives allows the ingestion of more than 10^9 live cells of selected *Lactobacillus* (*Lb.*) *paracasei* or *Lb. plantarum* strains corresponding to the daily dose recommended to obtain beneficial effects.

Therefore, the aim of this research was the identification of potential probiotic LAB strains isolated from table olives and their subsequent introduction in the same matrix of origin, in order to develop a new functional food.

Materials and methods

In the current study a broad collection of LAB strains native from table olives fermentations (Cocolin et al., 2013) was screened, paying attention to their tolerance to the hostile environment of stomach and intestine together with other suitable phenotypic features as the high autoaggregation, hydrophobicity and production of antimicrobial compounds (Bautista-Gallego et al., 2013). By using this approach we reduced the number of strains focusing the attentions to the most promising probiotics. Safety of these strains was assessed considering both their potential resistance to common antibiotics and the production

of biogenic amine (Argyri et al., 2013). Ascertained their safety, the potential cytotoxicity as well as adhesion capability were assessed in 2D and 3D intestinal model made with H4 human epithelial cells (Cencič and Langerholc, 2010). The characterization of the probiotic strains continued investigating their ability to inhibit the *Listeria* (*L.*) *monocytogenes* infection in 2D model and their effect on the epithelial barrier integrity in 3D mode (Koo et al., 2012). In parallel, to highlight the technological potentialities of the selected strains we evaluated their adhesiveness on the table olives surface. All data were subjected to ANOVA and Duncan's test.

Results

Hierarchical Cluster analysis was performed using as variables: the overall digestion survival (ODS) index after a simulated gastrointestinal passage, the autoaggregation and the hydrophobicity of the strains. Dendrogram resulting from the analysis highlighted a suitable probiotic phenotype for 17 strains belonging to the species *Lb. plantarum*, *Lb. pentosus* and *Leuconostoc mesenteroides*. High ODS index were shown by the *Lb. plantarum* strains O2T60C, S11T3E and O1T90E, compared to the probiotic *Lb. casei Shirota*. All strains were recognized as not harmful since neither production of BA nor cytotoxic effect versus human cells was detected. Moreover, none of these selected strains showed resistance to broad spectrum antibiotics.

Concerning the characterization of these 17 strains with 2D and 3D human intestinal models, we observed a high adhesion ratio for the strain O2T60C (over 9 %) when tested in the 3D functional model, which mimics closely the real intestinal conditions. Two *Lb. plantarum* strains (S1T10A and S11T3E) enhanced significantly (P<0,05) compared to the untreated control, the trans-epithelial electrical resistance and therefore the integrity of the polarized epithelium. Moreover, S11T3E showed the ability to inhibit the *L. monocytogenes* invasion in 2D epithelial model and in lesser amount in the 3D functional model. Regarding the potential reintroduction of the bacteria in the final product, the strains *Lb. plantarum* S11T3E and O2T60C showed the greater adhesiveness on the table olives surface.

Discussion

The use of table olives as probiotic source was already explored in several studies (Argyri et al., 2013; Bautista-Gallego et al., 2013), which evaluated by *in vitro* methods the probiotic characteristics of autochthonous LAB isolated from table olive fermentations. However, to our knowledge for the first time in this work the reintroduction of the best probiotics in the final product was kept in consideration as possible alternative way to develop easily a new functional food. However, the introduction of functional strains immediately at the beginning of the olives fermentations as starter culture can hardly guarantee an adequate amount of probiotics in the product until the end of the shelf life.

Conclusions

Lb. plantarum S11T3E could be considered the most promising candidate for the development of a new functional food, the probiotic table olives.

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SS2O.2

BACTERIAL DIVERSITY ASSOCIATED TO DIFFERENT DEVELOPMENTAL STAGES OF THE HONEYBEE, *APIS MELLIFERA*

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Introduction

Pollination is a crucial ecosystem service and pollinators exert a key role for orchard, horticultural and forage production (Klein et al. 2007). Among animal pollinators, the honeybee *Apis mellifera* represents the most important one.

Since few decades honeybees and bumblebees are tackling serious mass declines, due to stresses of diverse nature, such as abiotic stresses (e.g. the use of pesticides, the weather change and the lack of forage) and biotic ones (e.g. parasites, microsporidia, fungal and bacterial diseases). This population decline is seriously concerning the farmers and the scientific community, since the lack of the ecosystem services, exerted by the insect pollinators, influences negatively the agriculture sustainability. The scientific community is directing great efforts on the investigation of the interactions established among stressors, honeybees and microbial symbionts. Indeed, microbial partners of insects are widely recognized as an essential component for the host survival. They establish with insects strict relationships that influence different aspects of the host life, such as nutrition, development, reproduction, immune system and evolution (Dale and Moran, 2006; Crotti et al., 2012). For example, *Drosophila* microbiome sustains the gut homeostasis, prevents the host from pathogen colonization and influences the host homeostatic developmental programs (Ryu et al., 2008, Shin et al., 2011; Storelli et al., 2011).

With the aim to unveil the interactions established among honeybees, stressors and microbial symbionts, the knowledge of the structure and composition of the honeybee microbiome is mandatory. Investigations on honeybee microbiome has been performed on honeybees collected in different Countries, such as South Africa, USA, Australia, and some European Countries i.e. Sweden,

Germany and Austria (Hamdi et al., 2011). To our knowledge, the microbiome of Italian honeybees has never been investigated. Thus, the aim of this work is to characterize the bacterial community associated with different honeybee developmental stages collected in North Italy. In particular, larvae, pupae and adults were included in the analysis.

Materials and methods

Larvae, pupae and adults have been collected during spring/summer in northwestern Italy (Grugliasco, Torino) and kept at -20°C in ethanol until use. DNA has been extracted from the whole surface-sterilised larva or pupa or from the dissected adult gut, as previously described (Gonella et al., 2011). DNA has been then quantified with a Nanodrop ND-1000 spectrophotometer and employed as template in Denaturing Gradient Gel Electrophoresis (DGGE)-PCR. DGGE has been performed as previously reported (Gonella et al., 2011). Denaturant gradient of 38% to 55% has been used. Gels ran for 15 h at 90 V in 1X TAE buffer at a constant temperature of 60°C in a D-Code electrophoresis system (Bio-Rad, Milan, Italy). The gels have been stained for 30 min in 1X TAE buffer containing SYBR Green (Molecular Probes, Life Technologies-Invitrogen, Milan, Italy). Gels visualization has been performed with GelDoc 2000 apparatus (Bio-Rad, Milan, Italy), using the Quantity One software package version 4.6.6 (BioRad, Milan, Italy).

DGGE bands have been excised from the gels, eluted in 50 µl of MilliQ water and re-amplified by PCR as described in Gonella et al., (2011). Re-amplified DNA fragments have been sequenced at Macrogen Inc. (Seoul, South Korea). Then sequences have been compared to the sequence database at the National Center for Biotechnology Information (NCBI) using BLAST software.

Results and discussion

The bacterial community associated to different honeybee developmental stages has been investigated by DGGE. Figure 1 shows the DGGE profiles obtained for 9 samples which included 3 larvae, 3 pupae and 3 adults, respectively.

The identity of the sequences related to the excised bands is reported in table 1. Generally, the samples of each group show a similar bacterial assemblage, which increases in bacterial diversity along the insect growth. In fact, the bacterial diversity associated to adults is greater in quantity and composition than the ones associated to larvae and pupae. Sequences related to *Lactobacillus* spp. dominated the bacterial community of honeybee larvae (bands 1, 3, 4, 5, 6, 7, 8, 9, 10 and 11), with only one further sequence of *Clostridium* sp. detected (band 12). Pupae showed the presence of sequences related to *Lactobacillus* sp. (bands 16 and 17), *Ralstonia pickettii* (band 15) and *Staphylococcus xylosus* (bands 13 and 14). Adults have been found to be inhabited by several members of different classes: Alphaproteobacteria, such as bacteria of the order Rhizobiales (bands 19, 21, 22, 25, 26, 30 and 31) and uncultured *Gluconacetobacter* (band 27),

Betaproteobacteria, such as *Bartonella tamiae* (band 32), and Bacilli, such as *Lactobacillus* sp. (bands 20, 23, 24, 28 and 29).

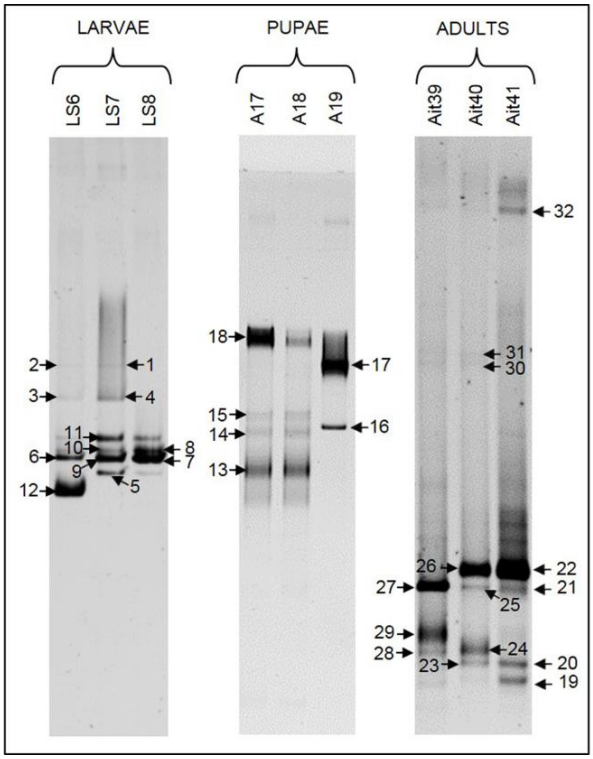

Figure 1. DGGE profiles of *A. mellifera* larvae, pupae and adults collected in North Italy. Numbers refer to the bands (marked with arrows) whose sequence identity is given in Table 1.

Conclusions

In the present study, we investigated the bacterial community associated to honeybee larvae, pupae and adults. Among the detected bacteria, *Lactobacillus* spp. were present in all the developmental stages of the insect. The bacterial communities described in the adult samples were consistent with the data reported in literature highlighting the hypothesis of a core bacterial assemblage conserved in adults from different geographical origin.

Table 1. Identification of microorganisms associated to according to DGGE profiles in Fig. 1.

Band	Origin[a]	Most related species	% nt ID[b] (GenBank Accension no.)	Classification
1-3-6-7-8-9-10-11-17	L-P	*Lactobacillus* sp. AcjLac9	97-100 (AB810030)	Bacilli, Lactobacillales
2-18	L-P	18S rRNA gene of *Apis mellifera* clone BKS_04	100 (KC413716)	-
4	L	*Lactobacillus* sp. AcjLac12	97 (AB810033)	Bacilli, Lactobacillales
5	L	*Lactobacillus* sp. Achmto2	99 (HM534754)	Bacilli, Lactobacillales
12	L	Uncultured *Clostridium* sp.	95 (FN394545)	Clostridia, Clostridiales
13-14	P	*Staphylococcus xylosus* strain 2SFHC	99-100 (KF233809)	Bacilli, Bacillales
15	P	*Ralstonia pickettii* strain Pa8	98 (KF111697)	Betaproteobacteria, Burkholderiales
16	P	*Lactobacillus* sp. Adhmto21	87 (HM534789)	Bacilli, Lactobacillales
19	A	Uncultured Rhizobiales sp. clone pAJ210	99 (AY370187)	Alphaproteobacteria, Rhizobiales
20-23	A	*Lactobacillus* sp. AmmhmR15	99 (HM534863)	Bacilli, Lactobacillales
21-22-25-26-30-31	A	Rhizobiales bacterium PEB0184	99-100 (JQ673261)	Alphaproteobacteria, Rhizobiales
24	A	*Lactobacillus* sp. AmmpolR3	100 (HM534867)	Bacilli, Lactobacillales
27	A	Uncultured *Gluconacetobacter* sp. clone pAJ205	99 (AY370188)	Alphaproteobacteria, Rhodospirillales
28	A	*Lactobacillus* sp.B A98	89 (JX896475)	Bacilli, Lactobacillales
29	A	Uncultured *Lactobacillus* sp. clone SHOG653	99 (HM113271)	Bacilli, Lactobacillales
32	A	*Bartonella tamiae* strain Th339	98 (EF672729)	Betaproteobacteria, Rhodocyclales

[a]L:larva; P:pupa; A:adult.

[b]nt ID: nucleotide identity

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SS2O.3

INTEGRATING SOIL CHARACTERISTICS, LAND MANAGEMENT AND SOIL MICROBIAL COMMUNITIES

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Introduction

Soil microorganisms are essential for numerous soil ecosystem functions, including nutrient cycling and organic matter decomposition. To truly understand the role that soil microbiology has in these different ecosystem functions, it is important to quantify a baseline range of microbial parameters across a spectrum of soils and land-use types. Biotic and abiotic factors, including pH, organic matter, soil carbon and nitrogen, moisture content, soil type along with land-use, management and above-ground vegetation have been identified as drivers of below-ground microbial community structure, diversity and abundance. This study aims to determine which parameters (including soil type and land use) shape soil microbial communities across Ireland. It covers 250 unique sample locations and soil depth profiles, and correlates soil chemical and physical data, determined for each sampled profile, to a series of microbial parameters. Presented here are results from analysis which focused on the microbial functional properties (nitrification and respiration) of 105 different soil surface horizon samples and their relation to intrinsic physicochemical parameters.

Materials and methods

Sampling sites around Ireland were chosen to provide a range of soil physical and chemical characteristics as well as 5 land-use types (improved and unimproved grassland, arable, forest and bog). Sampling was conducted over 2 years from March to September in 2012 and 2013. At each site, the horizon profile was exposed and pooled soil samples were collected from the surface horizon (0-10 cm) for chemical and biological analysis respectively. Soil for chemical analysis was dried and sieved to 2 mm. Soil for microbial analysis was stored at 4°C until arrival in the laboratory. Following sieving to 2 mm, soils were incubated at 20°C for one week prior to analysis. Physicochemical analyses were performed by the Irish Soil Information System (ISIS) team and included soil total carbon (C), total nitrogen (N), pH, particle size, cation exchange capacity (CEC), organic carbon (OC) and exchangeable bases Sodium (Na),

Potassium (K), Magnesium (Mg) and Calcium (Ca). Microbial biomass was measured using the fumigation extraction method (Vance et al., 1987). Ten grams dry weight equivalent for each soil was fumigated with chloroform for 24h. Soluble organic carbon and nitrogen was extracted from fumigated and unfumigated samples with 0.5 M K_2SO_4 (1:4 soil solution ratio) for 30 min on a side-to-side shaker. Extracts were filtered and carbon and nitrogen determined using a Shimadzu TOC-TN analyser. Microbial biomass carbon (Cmic) and nitrogen (Nmic) were calculated from the difference between fumigated and unfumigated samples using a conversion factor of 0.45. Substrate induced as well as basal respiration was measured by the MicroResp^{TM} method. This Community level physiological profiling (CLPP) method uses a microtiter plate design to asses catabolic activities of whole soil samples (Campbell et al., 2003). A range of 7 carbon substrates (Table 1) differing in complexity were applied at a concentration of 30 mg g^{-1} H_2O. Respiration rates were calculated (µg CO_2-C g^{-1} h^{-1}) from an independent calibration curve after 6 hour incubation at 25°C. Potential rates of nitrification were generated on a subset of 68 samples by using an augmented nitrification assay (Wheatley et al., 1997). At interval 0h, 24h and 48h nitrite was extracted with 1 M KCL followed by filtration. Nitrite concentrations were measured colorimetrically using the Griess test (Hood-Nowotny et al., 2010). For statistical analysis all data was normalised by log transformation. Physicochemical and microbial parameters were compared using the Pearson correlation and principal components analysis (PCA) (StatSoft Inc., 2010).

Table 1. Results of pairwise correlation analysis of microbial and physicochemical variables. Significance terms denote *** p<0.001; ** p<0.01; * p<0.05. (+) and (-) denote positive and negative correlations respectively.

	Microbial biomass		Soil physicochemical parameters								
	Cmic	Nmic	C	N	C:N	OC	pH	K	Mg	Ca	CEC
(a) Microresp (respiration rates)											
Carbon sources											
Water	***(+)	***(+)	***(+)	***(+)		***(+)		**(+)	**(+)	**(+)	**(+)
D-Galactose	***(+)	***(+)	***(+)	***(+)		***(+)		**(+)	**(+)	*(+)	*(+)
L-Malic acid	***(+)	***(+)	***(+)	***(+)		***(+)		**(+)	*(+)	*(+)	**(+)
gamma-Aminobutyric acid	***(+)	***(+)	***(+)	***(+)		***(+)		**(+)	**(+)	*(+)	**(+)
N-Acetylglucosamine	***(+)	***(+)	***(+)	***(+)		***(+)		***(+)	**(+)	**(+)	*(+)
D-Glucose	***(+)	***(+)	***(+)	***(+)		***(+)		**(+)	**(+)	*(+)	*(+)
alpha-Ketoglutaric acid	***(+)	***(+)		**(+)	***(-)		*(+)	**(+)	*(+)	**(+)	*(+)
Citric acid	***(+)	***(+)		**(+)	**(-)		*(+)	**(+)	*(+)	**(+)	**(+)
(b) Potential nitrification											
Nitrite concentration							***(+)		***(+)	***(+)	***(+)
(c) Microbial biomass											
Cmic		***(+)	***(+)	***(+)		***(+)		*(+)	**(+)		**(+)
Nmic	***(+)		***(+)	***(+)		***(+)		**(+)			

Results and discussion

Basal and substrate induced respiration increased with increased microbial biomass, C, OC and N. Alpha-ketoglutaric acid and citric acid were an exception to this general trend, with no significant correlation found to C and OC. For both a negative correlation with C:N and positive correlation with pH were observed. Increased amounts of all exchangeable bases as well as increased CEC correlated positively with respiration rates. Nitrite concentrations ranged from 0 to 87 µg g^{-1} d^{-1} and had a strong positive correlation to pH, CEC, Mg and Ca. PCA of respiration data did not display significant clustering by soil type or land use. However PCA %variances (PC1 80%, PC2 10%) were best explained by a combination of physicochemical variables (including Cmic, C, OC, pH and CEC). After classifying the soils into diagnostic organic matter groups, clustering in the PC1-PC2 plain was found to be highly significant.

Conclusion

These results suggest that although some factors significantly affect microbial parameters, it is the combination of variables that best describes shifts in microbial respiration. Further work is required to investigate how the combination of physicochemical factors can be used to describe soil microbial functionality. Future work in this study will also include data relating to soil microbial diversity and abundances through the use of molecular methodologies.

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SS2O.4

POPULATION STRUCTURE AND GEOGRAPHICAL DISTRIBUTION OF AUTOCHTHONOUS *S. CEREVISIAE* STRAINS ISOLATED IN THREE DIFFERENT OENOLOGICAL AREAS IN THE NORTH-EAST OF ITALY

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Introduction

Autochthonous *Saccharomyces cerevisiae* strains isolated from natural environments associated with wine production areas are now commercialized as active dry yeast for their ability to efficiently ferment grape musts and to produce desirable metabolites. Therefore, exploring the biodiversity of yeast strains isolated in vineyard can give an important contribution to elucidate the existence of a correlation between ecological and/or geographical origin and to understand strains phenotypic differences (Schuller et al., 2005). Several molecular methods were developed to study yeasts at both species and subspecies level, but mitochondrial DNA restriction analysis (mtDNA-RFLP) together with microsatellite typing are the most diffuse methods for *S. cerevisiae* genetic and phylogenetic characterization. Recently, in more than a few studies authors have chosen microsatellite markers, instead of mtDNA-RFLP analysis, to identify yeasts isolated from different ecological niches, as the formers are most appropriate in large-scale studies for the determination of yeasts genetic proximity and biogeographical distribution (Legras et al., 2007).
In this work the two different molecular methods were applied to a large-scale characterization of strains isolated in three Appellation of Origin areas located in the North-East of Italy: DOCG Prosecco of Conegliano-Valdobbiadene, DOC Piave and DOCG Lison-Pramaggiore).

Materials and methods

Yeasts were isolated after fermentation of single grape bunches collected in the three Appellation of Origin areas in Veneto region. Colonies with a *Saccharomyces*- like morphology were identified by a multiplex PCR (Nardi et

al., 2006) and a subsequent ITS-RFLP (Esteve –Zarzoso et al., 1999) for species determination. Afterwards mitochondrial DNA restriction analysis (mtDNA-RFLP) was performed to cluster the isolates at strain level (Querol and Ramon, 1996). Restriction profiles obtained were compared by the BioNumerics (Applied Maths) software. When the same mtDNA-RFLP profile was found in more than one fermented sample, an isolate from each sample was taken into account for the subsequent microsatellite typing that was performed by means of 18 microsatellites loci.

Results and discussion

A total of 219 autochthonous yeasts strains were collected during the 2004–2010 pre-harvest period in the three Appellation of Origin areas in Veneto region. The level of biodiversity obtained from fermentations of single grape bunches were different in relation to the three areas sampled as shown in table 1.

Table 1. Sampling and identification of *S.cerevisiae* strains.

Areas	Grape bunches sampled	Grape bunches with *Saccharomyces*	*S. cerevisiae* isolates	mtDNA-RFLP profiles
DOCG Prosecco	353	30	295	37
DOC Piave	78	54	254	129
DOCG Lison	203	18	197	17

In order to better understand yeast evolution, 34 commercial wine strains, including 26 strains whose genome sequence is available, and 8 selected on different substrates (beer, bread, laboratory, oak, sake, ragi and clinical) were analyzed. The mtDNA-RFLP analysis point out the presence of 183 different profiles on the total of 219 autochthonous yeast isolates tested. Moreover 3 strains from DOCG Lison and DOCG Prosecco were found in samples collected from different vineyards, while each mtDNA profile from DOC Piave area was obtained from single sample. The mtDNA analysis showed that there is not a clear cluster separation between strains of technological relevance and those isolated from different environments.

The microsatellites typing revealed 206 different genotypes out of the total 219 autochthonous strains isolated from vineyard. The Neighbour-joining consensus tree showed a cluster of strains isolated from environments different to wine. A second cluster containing eleven strains isolated from DOCG Lison-Pramaggiore, three from DOC Piave, two from DOCG Prosecco area and one USA oak strain was identified. Thus, these autochthonous yeasts revealed to be poorly related both to the other isolates collected from

vineyards and to the commercial ones that were equally spread in all the other clusters.

Conclusions

Our result point out the different biodiversity level present in the three winemaking regions. Strain geographical origin is not reflected by mitochondrial DNA analysis, but it is clearly revealed by microsatellites investigation that confirms to be a powerful tool in large-scale phylogenetic studies.

Results achieved on commercial wine strains were similar with both methods, indicating that mtDNA-RFLP analysis has a high discriminatory power if it is used to identify strains with distant phylogenetic origin, but it is lower when considering yeasts closely related.

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POSTERS

P1.1

Adaptation studies of *Scytonema millei* inhibiting in the biofilms on stone monuments of Santiniketan, India to elevated temperature and desiccation

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Phenotypic alteration and biochemical changes in the cells of cyanobacteria in biofilms colonizing stone monuments at Santiniketan, India in response to elevated temperature coupled with high solar insolation and desiccation was studied. One dominant cyanobacterium, *Scytonema millei* occurring on Buddha statue exposed to direct sunlight throughout the day was chosen as the experimental material (Keshari and Adhikary, 2013). Changes in the TTC reduction activity, pigment composition, macromolecular contents and superoxide dismutase (SOD) activity upon exposure to different temperatures (25°C, 35°C, 45°C and 55°C) and their recovery in culture in seven days after heat treatment was analyzed. Almost no adverse effect on the morphological feature of the cyanobacterium was observed when exposed to the temperature up to 45°C, however, exposure to 55°C in wet state for 1 hour was lethal for the cells. To the contrary in dried state the organism could tolerate and continued its metabolic activity up to exposure of 55°C, though the macromolecular as well as pigment contents showed a quantitative decrease in comparison to control with the exception that the cellular as well as extracellular carbohydrate content of the organism was increased significantly upon heat treatment. Extracellular polysaccharides secretion (EPS) by cyanobacteria in the biofilms after receiving monsoon coupled with high temperature thereafter showed its potentiality to cope with adverse conditions (Adhikary 1998; Gloaguen et al. 1995) and survived in dried state in the biofilms on stone monuments for prolonged periods even during the hot summer months of the tropics (Rossi et al. 2012).

Keywords: biofilm; cyanobacteria; adaptation to high temperature; desiccation; wet heat and dry heat

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P1.2

Formation of yeasts-lactic acid bacteria biofilms during the industrial elaboration of Spanish green table olives

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Recently, it has being evaluated the use of table olives as a carrier of functional/probiotic microorganisms to the human body. For this purpose, a first and essential step is the study of the capacity of microorganism to adhere olive epidermis and the subsequent formation of a complex biofilm. In this work, the ability of a Lactobacillus pentosus strain (LAB2) with potential probiotic activity to colonize olive epidermis during elaboration of Spanish green table olives at industrial scale was studied. The olives used in this work were of the Manzanilla variety, picked by hand at the green maturation stage during 2012/2013 season. Fermentation tanks with approximately 9,700 kg of olives were covered with 5,500 L of a 11% NaCl brine. Fermentations were started in October and the tanks were inoculated with the LAB2 strain at approximately 6 $\log_{10}$ CFU/mL. The process was followed during three months by the evolution of the main physico-chemical and microbiological parameters. For determination of microorganisms adhered to olive surface, biofilm disintegration was achieved by means of an enzymatic and mechanic procedure developed in our laboratory. Enterobacteriaceae were never detected on the epidermis of the fruits during all the time that fermentation tanks were monitored. On the contrary, LAB forming biofilm on olive skin reached population levels around 7 $\log_{10}$ CFU/g for all the studied tanks after the first month of fermentation. Then, the population showed the typical decline with time that can be explained by the depletion of nutrients and the effect of low pH due to the production of lactic acid. The molecular characterization (REP-PCR with primer GTG5) of the LAB population in the biofilm at the 30th day of fermentation revealed that the inoculated strain was always found on the fruit surface of the inoculated tanks. Thereby, isolates randomly obtained from the inoculated tanks shared a similarity level of 76% with the inoculated LAB2 strain, which was above the repeatability of the technique. On the contrary, the generated dendogram showed that the isolates obtained from the spontaneous tanks were grouped separately. The growth of yeasts on olive surface was very chaotic reaching a maximum population level around 5 $\log_{10}$ CFU/g. This population was molecularly identified by partial sequencing of the 26S gene with primers NL1 and NL4. The biodiversity of yeast species found in the spontaneous and inoculated

tanks was very low and it was reduced to *Candida tropicalis*, *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* species, which formed part of the mixed biofilms. Although the association between LAB and yeasts in brine was well known, there is scarce information about their relationship on olive epidermis when they form biofilms. Moreover, the possibility of controlling the LAB population during industrial fermentations with the use of beneficial and functional microorganisms is a complete novelty generated during this work. As result, the research has opened new and completely unexplored research lines that have expanded the frontiers of the olive fermentation and biofilms further away the current basis of the table olive investigation.

Keywords: Mixed biofilms; table olives; fermentation; yeasts; lactic acid bacteria

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P1.3

Bacterial biodiversity in an agricultural water residual lake

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Waste water ecosystems offer vast and complex habitats for diverse microbial communities. In this study we investigate the current status of Al-asfer lake microbial biodiversity and its ability to develop biofilm. Al-asfer lake is located close to Al-Ahsa city in the eastern province of Saudi Arabia and is composed primarily of agricultural drainage water. It is almost 25 km long hosting different kinds of trees and migratory birds.
The microbial life in Al-asfer lake was explored by cultural and non-cultural technique. DGGE- PCR Amplification and 16S rRNA sequencing were used with cultures isolates. DNA-DNA hybridization was used with DNA microarrays to identify microbial species that could not be grown or cultured in lab. The ability of isolates to develop biofilm was also determined by two methods. The isolates were cultivated in 6-well plates containing 6ml TSB and were then grown in dip flow reactor.
Several chemical parameters were determined in water and sediment samples include pH, Turbidity (NTU), Total Hardness, TDS, Conductivity, TOC, TC, and IC. Also, some elements were determined in the samples including Ba, Cr, Cu, Fe, Mn, Zn, As, Cd, Pb, Se, Al, and Hg. The analysis also, indicated the presence of several kinds of pesticides. The microbiology and molecular analysis performed isolated and identified 183 bacterial species belong to three genus. Bacillus was the most dominant genus followed by Vibrio and Citrobacter. The bacillus sp isolated

was able to develop biofilm. This biofilm was more significant in present of water from al-asfer lake which might be explained by its resistance to the toxicity of pesticides in the water al-asfer lake, therefore it became the dominated isolate.

Keywords: biodiversity; biofilm; water and sediment; bacterial species; pesticides

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P1.4

Role of *luxS* in biofilm formation and biocides resistance in *Listeria*

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Biocides are chemical compounds used in the food hygiene strategy to control pathogenic and spoilage micro-organisms. In food systems bacteria can be attracted to solid surface to form biofilm structures that can protect the cells from adverse environmental conditions and antimicrobial agents as biocides and antibiotics. This study was focused on the bactericidal action of two biocides, benzalkonium chloride and peracetic acid, on different *Listeria* sp. strains. Bacterial susceptibility to antimicrobials routinely used in food processing environments was evaluated both on liquid culture and on dynamically grown biofilms on different materials. A quantitative assessment of bacterial adhesion was carried out and the parameters that could affect significatively the biofilm formation were investigated: interspecific variability, nutrients, temperature, surface material. The capability of *Listeria* sp. to adhere and construct biofilm structures on the different materials considered was observed by Scanning Electronic Microscopy. The effect of antimicrobials to counteract the biofilm formation was also evaluated by this technique.
The role of quorum sensing gene *luxS* in biofilm development process was investigated in *L. innocua*. *luxS*–null mutant strain of *L. innocua* was obtained through plasmid insertion and evaluated for its capacity to form biofilm. The disruption of *luxS* gene led to a strong reduction of adhered cells and, consequently of biofilm thickness, confirming the effective role of *luxS* in the adhesion on abiotic surfaces and in reducing significantly the resistance of biofilms to biocides.

Keywords: *Listeria*; *luxS*; biofilm; biocides

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P1.5

Biofilm formation by *Candida boidinii* and *Lactobacillus pentosus* on the olive epidermis of Gordal fruits processed according to Spanish-style

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In this survey, we have evaluated the ability of *Candida boidinii* TOMCY42 and Lactobacillus pentosus IG43 to colonize olive epidermis of Gordal fruits processed according to Spanish-style. Fruits were treated with a NaOH solution (1.7%, 9 h, 25ºC) and then washed (16h) to remove excess of alkali. Prior to inoculation, olives were sterilized by immersion during 15 min at 37ºC in a sodium hypochlorite solution (50mg/L active chlorine) and washed twice with sterilized water to remove excess of chlorine. Thereby, all kind of microorganisms were removed from olive surface. Inoculation was carried out at laboratory scale in fermentation vessels of 1L capacity (600 fruits+475 mL of 10% NaCl brine), in single and mixed cultures at approximately 5 $\log_{10}$ CFU/mL. The process was followed during three months by the evolution of the main physico-chemical and microbiological parameters in both cover brines and olive surface. For determination of microorganisms adhered to olive epidermis, biofilm disintegration was achieved by means of an enzymatic procedure developed in our laboratory using lipase, β-galactosidasa and α-glucosidasa enzymes. Both species, *L. pentosus* and *C. boidinii*, were able to form biofilm on olive skin in the single inoculations, reaching population levels up to 8.55 $\log_{10}$ CFU/cm^2 (7 d) and 9.63 $\log_{10}$ CFU/cm^2 (30 d) for the bacteria and yeast species, respectively. In the mixed inoculation, the population levels reached by both microorganisms were very similar without significant differences among treatments. At the end of the experiment (3 months), 7.15 $\log_{10}$ CFU/cm^2 (for LAB) and 8.23 $\log_{10}$ CFU/cm^2 (for yeasts) were obtained on olive surface yet. In situ observation of Gordal epidermis slices by scanning electron microscopy revealed a strong aggregation and adhesion of the bacteria and yeast to olive surface, by the formation of a complex biofilm with an exopolysaccharide matrix which embedded the microorganisms. These kinds of

studies are very useful for the evaluation of table olives as carrier of beneficial microorganisms to the human body.

Keywords: table olives; biofilms; fermentation; yeasts; lactic acid bacteria

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P1.6

The hard fight against bacterial biofilms in industrial environments

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Bacterial biofilms represent a considerable problem in the major part of industrial plants which use water as a process fluid (cooling towers, seawater/freshwater cooling water systems, paper mills, steelworks, etc.) or as an ingredient (food&beverage production, pharmaceutical, etc.). These microorganisms can increase energy consumption (e.g. covering thermal exchangers, thus reducing their efficiency), structures deterioration (e.g. accelerating metal corrosion) and biological risk. Indeed, even when not made by pathogens, biofilm represents the ideal environment for the survival and growth of such potentially harmful bacteria. In order to limit this kind of biological growth inside industrial water lines, the most widely applied solution is represented by the dosage of chemical compounds ("biocides" or "sanitation agents") in the water. Usually such treatments are applied on a regular time-basis (daily/weekly/…) or when problems like visible deterioration of products or drop in process performance happens, without any check on the real need of the treatment and on its effectiveness. Since those chemical compounds are finally discharged into sewers, rivers and seas, an optimization of sanitation treatments is strongly required, in order to reduce their environmental impact and, at the same time, the overall cost of biofilm-related issues. An innovative biofilm monitoring system (Pavanello et al., 2011), able to detect bacterial growth on surfaces since its first phases, on line and in real-time, has been tested and validated in different kinds of industrial environments including, among the others, cooling towers, paper mills, food production plants and mineral water bottling. With the support of such technology, a new approach towards the problem of biofilm in industrial water lines has been adopted, allowing to make a wise use of chemical biocides, preventing/reducing potential microbiological contaminations, cutting management costs and verifying cleaning

effectiveness.

Keywords: Biofilm; CIP; sanitation; biocides

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P1.7

The use of bacterial community structure to integrate classical water spring monitoring

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Water springs in high elevation environments are one of the most endangered habitats because of their vulnerability to environmental changes. Because of this reason they should be taken in particular consideration for conservational issues. Bacteria have shown to be major actors in ecosystem metabolism but at the moment little studies have been carried out in bacterial communities in water springs. We tried to combine traditional chemical and physical approaches with the analysis of bacterial community structures. In this way we intend to assess their quality as tracking of water origin and the relationships between bacterial diversity and the main water parameters measured in water spring monitoring. Quantitative Amplified Ribosomal Intergenic Sequence Analysis (qARISA) was done on DNA extracted from sediments collected in three water spring in the upper Matsch Valley (South Tyrol, Italy; 2300 m a.s.l.) four times during the summer. The fingerprinting matrix was analyzed through canonical correspondence analysis (CCA). Shannon (H`) diversity indexes obtained from the fingerprinting matrix were analyzed for correlation with water 2H and 18O isotopic ratios, electric conductivity (EC) and water temperature. The latter two features yielded the highest correlation with bacterial diversity ($r=0.75$ and $r=0.58$). Through a multiple regression analysis, bacterial diversity appears to be indeed causally related to EC and water temperature (adj-$R2=0.5$, $p<0.05$). Our results suggest that EC can be influenced by both temperature and bacterial diversity and bacterial community structure can be used as tracking tool for water chemistry and residence time (correlated to EC) in oligotrophic freshwater environments but not for water origin.

Keywords: Bacterial community structure; water spring; fingerprinting; alpine

environment

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P1.8

Novel starter strains for beer production from sourdough and wine environment

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Saccharomyces cerevisiae is a domesticated microorganism that possesses genetic, genomic and metabolic peculiarities that depend on its technological use. Brewer's, baker's and wine strains of *S. cerevisiae* have been selected for hundreds of years on the basis of specific attributes such as flavor production, sugar attenuation, and others, thus leading to the achievement of different strains within the same species. In this respect, the deliberate inoculation of strains on food matrices other than those of their domestication, could allow the transfer of new and specific sensory properties to wine, beer and bread. Thus, the aim of this work was to assess the possibility of using baker's and wine strains of *S. cerevisiae* as starters for beer production. Baker's strains isolated from artisanal sourdough were tested for their ability to ferment wort. Those strains showed an efficient uptake and utilization of trehalose, that share common transporters with maltose, the most important disaccharide in wort. From wine environment, a biofilm forming strain was selected in order to produce a biofilm on beer, to reduce alcohol production and to characterize beer aroma.

Keywords: *Saccharomyces cerevisiae*; sourdough; wine, beer; biofilm

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P1.9

Antimicrobial activity of paints added with titanium and doping agents: a preliminary study

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The antimicrobial photocatalytic activity of titanium dioxide (TiO_2) is gaining more attention regarding its utilization for the sanification of the surfaces. The action mechanism of TiO_2 is related to the degradation of cytoplasmic membranes due to the formation of reactive oxygen and improves in presence doping agents.
TiO_2 may be added to paints, which are used for the sanitation of different surfaces. For example, they are employed in hospitals in order to limit the development of bacteria responsible for diseases in hospitalized patients. Furthermore, many diseases that attack animals in cattle breeding develop thanks to the contamination of pathogenic bacteria that produce biofilms on the walls where animals live. Therefore the development of new paints enriched in titanium, with a suitable formulation for the application on stable's walls may decrease the incidence of common bacterial diseases in breeding systems.
This study represents the preliminary stage of a project " PHOTOTiO_2 (Sviluppo di nanostrutture TiO_2 per prodotti vernicianti ad attività fotocatalitica per la riduzione delle cariche batteriche)" that has as final goal to create new formulations of paints added with TiO2 for a future use for the sanitation of the stables walls.
We tested the antimicrobial activity of several paint formulations on both Gram+ and Gram- stains in experiments conducted at laboratory scale. *Lactobacillus fermentum* and *Salmonella tiphimurium* were inoculated on surfaces treated with different formulations of antimicrobial paints and, after incubation, the viable bacterial counts were determined. Moreover, inoculated paints were analyzed by scanning electron microscopy to evaluate possible damages induced by active molecules on cell walls of the tested microorganisms.
Our preliminary results highlight different bactericidal efficiency related to paint formulations and to bacteria cell wall characteristics. These aspects must be taken into account in order to optimize the antimicrobial effect.

Keywords: antimicrobial; paints; TiO_2; stable wall

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P1.10

Quantification and characterization of biofilm formation in table olive fermentation vessels

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Lactic acid bacteria and yeasts are the responsible microorganisms for the fermentation of a wide range of foods including table olives. Plastic vessels as abiotic surfaces might lead in the development of different microbial associations. The objective of the present study was the quantification of biofilm formed in plastic vessels at the end of table olive fermentation and the further characterization of the biota assembling this community by molecular fingerprinting. Brine and olives were removed from the vessels (vessel a and vessel b, 15lt capacity each, duplicates of spontaneous spanish-style table olive fermentation) and subjected to sampling at three different spots (high spot, middle spot on the wall and bottom) following two cleaning treatments, namely washing with hot tap water (treatment A) and washing with hot tap water, soap and commercial bleach (treatment B). Population (expressed as log CFU/10cm^2) of total viable counts, lactic acid bacteria and yeasts were enumerated by the standard method of agar plating. on Tryptic Soy Agar (TSA), de-Man-Rogosa-Sharpe Agar (MRS) and Rose Bendal Chloramphenicol Agar (RBC), respectively. Bulk cells (whole colonies) from agar plates from each media and for all conditions were isolated for further characterization by PCR-DGGE. Regardless of the cleaning treatment no significant differences were observed between the different sampling spots in the vessel. The initial microbial population (before cleaning treatments-controls) ranged between 4.0-5.5 log CFU/10cm^2 for LAB and 5.0-5.5 log CFU/10cm^2 for yeasts. Cleaning treatments exhibited the highest effect on LAB which were recovered at around 2.5 log CFU/10cm^2 after washing with hot tap water and 1 log CFU/10cm^2 after washing with hot tap water, soap and bleach, whereas yeasts were recovered at around 3.0 log CFU/10cm^2 even after washing with soap and bleach. High diversity of yeast biota was observed between the different treatments and sampling spots as indicated by PCR-DGGE. The obtained profiles were compared with profiles of yeast strains previously isolated from black table olive fermentation (used as reference strains). *Wickerhamomyces anomalus* was found to be the most abundant species followed by *Debaryomyces*

hansenii and *Pichia guilliermondii* while other species like *Pichia membraniefaciens* were also detected. Among lactic acid bacteria, in most of the cases *L. plantarum* group was present which was further characterized by multiplex PCR as *Lactobacillus pentosus*.
Acknowledgement. This work was financially supported by the European Union (FP7) under GA 243471 project PROBIOLIVES (www.probiolives.eu).

Keywords: biofilm; table olives; lactic acid bacteria; yeasts

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P1.11

Mono- and dual- species biofilm formation on the surface of black olives under different sterile brine solutions

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Table olives are generally regarded as microbiologically safe since they are consumed fermented. A mixed species community of lactic acid bacteria and yeasts which colonize the surface of the fruits are responsible for the fermentation process thus inhibiting the growth and survival of other undesirable or hazardous microorganisms and enhancing the sensory characteristics of the final product. The lactic acid bacterium *Lactobacillus pentosus* B281 in both monoculture and co-culture with the yeast *Pichia membranifaciens* m3a were studied for their ability to attach and colonize the surface of black olive drupes under different sterile brine solutions. *Lactobacillus pentosus* was selected due to its *in vitro* probiotic potential (Argyri et al., 2013) and the yeast due to its dominance amongst yeast species at the end of black table olive fermentation (Doulgeraki et al., 2013). A low salt concentration, 6% (w/v) NaCl, was used as initial concentration in the brine. The brine was supplemented with 0.5% (w/v) glucose or 0.2% (v/v) lactic acid, or the combination of glucose and lactic acid at the same concentrations and all brine solutions were sterilized at 120^{o}C for 15min. Black oxidized olives, thermally processed by the manufacturer, were submerged into 20 ml sterile brine solution and inoculated with 5 log CFU/ml *Lactobacillus pentosus* or with 5 log CFU/ml *Lactobacillus pentosus* and *Pichia membranifaciens*. Each brining treatment was studied in duplicate and stored at 20oC for 30 days. At regular time intervals olive

drupes of each brining treatment were sampled for quantification of biofilm cells and the pH of the brine was measured. At the end of storage, olive samples were also observed under scanning electron microscopy (SEM) for extracellular polymeric matrix production. In the case of mono-species biofilm formation, the initially attached population (day 1) of *L. pentosus* varied between 4.14 - 5.29 log CFU/g depending on the brining treatment while by the end of storage the microorganism was recovered in high numbers (above 6.5 log CFU/g) under all brining treatments. In the case of dual-species biofilm formation, the recovered population of *P. membranifaciens* at day 1 ranged between 2.96 - 4.43 log CFU/g depending on the brining treatment and progressively increased during storage reaching around 5 log CFU/g. *L. pentosus* exhibited as well a progressive increase during storage and it was in general recovered in higher levels that the yeast by 1.5 to 2 log units. Biofilm formation on the olive skin was confirmed by SEM. Aggregates of the lactic acid bacterium and the yeast joined together with extracellular matrix were observed, mostly located in the stomatal openings and on the epidermis. Characterization of the extracellular matrix and the role of the biofilm structure on olive drupes remains unknown. Further research in this context would help to better understand how microorganisms adhere and interact with one another in biofilm communities thus facilitating the development of controlled table olive fermentation.
Acknowledgement. This work was financially supported by the European Union (FP7) under GA 243471 project PROBIOLIVES (www.probiolives.eu).

Keywords: biofilm; table olives; lactic acid bacteria; yeasts

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P1.12

Mixed-species biofilm on abiotic surfaces

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Bacterial and fungal cells are able to attach to a surface, produce extracellular

polymeric substances, create a cell-to-cell bridge and irreversibly colonize a surface, producing a structure called biofilm. Biofilms are complex systems which can be considered as protective niches, in fact they allows the survivor and growth of micro-organisms when the environments is inhospitable. Biofilms are known as a significant hazard in both food sector and clinical environment. Thus, the development of biofilm on food industry equipment, premises and medical devices surfaces such as catheters, valves and prosthesis is a safety issue. Biofilms are important in food production and nosocomial environment because their formation contributes to bacterial survival since are difficult to remove and the bacteria in biofilm are highly resistant to treatment with antimicrobial agents. The microbial colonization depends on the nature of attachment surface, on the characteristics of the cell and on several environmental factors. Since few information is available on mixed-species biofilms and on the dynamic of biofilm formation when bacteria and fungi grown in combination, we have started a study involves two fungal species, *Trichosporon faecale* and *Candida albicans* and two bacterial species, *Enterococcus faecium* and *Staphylococcus aureus*, in order to evaluate their ability to form biofilm. Colonization were observed on two different substrates, stainless steel and polyethylene, largely used in food processing and clinical setting. These species were as example of opportunistic or pathogenic micro-organisms. The study was performed both on simple cultures and on the co-cultures. A total of 11 conditions were tested (4 simple cultures, 6 pair-associations and one association includes all the species taken into consideration). In order to have a standardized surface usable for all the experiments, tests were carry out in static conditions using 1 cm^2 stainless steel washers and 1.5 cm^2 polyethylene washers. The assessment of biofilm formation and development were performed by plate count and scanning electron microscopy (SEM).
Results vary significantly with the different experimental conditions, suggesting that the ability of forming biofilm is closely related to interaction among the different species and the environment.

Keywords: Biofilm; bacteria; fungi; stainless steel; polyethylene

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P1.13

Tracking of biofilm development and composition in a cooling lubricant system

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Cooling lubricants are used in a large variety of metalworking processes like milling or turning. Cooling lubricants consist of different amounts of oil, emulsifiers, detergents and other long chain hydrocarbon compounds diluted in water. As these compounds are suitable to be used as energy source for microorganisms, the lubricants are subject to microbial contamination. The lubricants are degraded by microorganisms, thereby losing their functionality. Of further importance is that the microorganisms found, can be potential pathogens like *Mycobacterium immunogenum*. As a countermeasure against the contamination biocides are added to the lubricant. But still lubricants are contaminated after a certain time. One of the main sources for contaminations of the liquid is most probably a biofilm in the whole lubrication system of a machine. The aim of this work was to analyze the temporal change of the biofilm in the cooling lubricant tank of a milling machine after filling it with freshly prepared cooling lubricant. The biofilm was grown on polyurethane foil in the tank of the lubrication system over a period of six weeks during spring time. The microorganisms were isolated and identified with classical microbiology and 16S rRNA sequencing. The isolation of the microorganism out of the biofilm was achieved by direct plating and a mechanical process. Additionally two further contamination sources were investigated, these being the tap water used for diluting the lubricant and airborne microorganisms in the machining hall. The results show that the diversity of the microflora in the biofilm is low, the most predominant microorganisms being *Micrococcus luteus* and *Staphylococcus warneri*. *Micrococcus luteus* could also be isolated from the used tap water and was the predominant airborne microorganism in the machining hall. The bacterial load in the lubricants was not detectable most of the time, only on three sampling days the bacterial load was between 10^2 – 10^5 CFU/ml with *Staphylococcus spp* being the predominant species. This shows that the biocides in the lubricant are effective in controlling planktonic bacteria, but not the development of a biofilm in

the system. Therefore to ensure full functionality of the lubricant it is important to adapt the biological monitoring of cooling lubricants and cleaning strategies to the degradation of biofilms.

Keywords: Cooling lubricants; contamination; mixed biofilms

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P1.14

Exploring the anti-biofilm activity of zosteric acid via high-throughput screening of a small molecules scaffold-based library

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The understanding of the interaction of microbes with surfaces is the basis for managing emerging threats, ranging from recalcitrant infectious diseases to food safety. While these may be viewed as assorted items, they actually have a common denominator: the biofilm lifestyle. Nowadays, the worldwide safety is seriously jeopardized by the emergence and spread of microorganisms in form of biofilm that are resistant to traditional biocides (Flemming, 2011). In addition, the antimicrobial arena is experiencing a shortage of lead compounds progressing into both clinical and industrial trials (Lam, 2007). An innovative approach is the use of biocide-free antibiofilm agents with novel targets, unique modes of action and properties that are different from those of the currently used antimicrobials. Using bio-inspired molecules at sub-lethal concentrations is an elegant way to interfere with specific key-steps that orchestrate biofilm formation, disarming the microorganism without affecting its existence, sidestepping drug resistance and extending the efficacy of the current arsenal of antimicrobial agents. At present, the main bottleneck to the spread and use of this technology is the incorporation of the antibiofilm molecule into a system able to resist to biofilm over a working timescale. The ideal approach would create a permanently non-leaching, long-lasting bio-hybrid material by covalent functionalization of its surface with the anti-biofilm compound. In our previous research, we demonstrated that zosteric acid (ZA), the secondary metabolite produced by the seagrass Zostera marina, might be suitable for implementation as a preventive or integrative approach

against biofilm (Villa et al., 2011, Villa et al., 2013). The goal of this work was to understand the structural characteristics responsible for the anti-biofilm activity of ZA in order to identify the binding site of the molecule for immobilizing ZA on an abiotic surface allowing its bioactive moiety to exert the anti-biofilm action (http://www.anfomat.unimi.it/). To this end, a small synthetic compounds library of around 50 molecules, related to the scaffold of ZA, was synthesized. These compounds were subjected to high-throughput biological screening to evaluate both their toxicity and anti-biofilm performance against *Escherichia coli* and *Candida albicans*, used as model systems of bacterial and fungal biofilm respectively. The dataset was made by more than 5000 data obtained from biofilm assay assessed quantitatively using fluorochrome-labelled cells in black microplate wells.

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Keywords: anti-biofilm compounds; zosteric acid; sub-lethal concentrations; covalent functionalization

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P2.1

Use of specifically selected rhizospheric microorganisms as a way to restore soil quality and biological fertility and to improve crop yields under drought stress

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Desertification and soil biodiversity loss are at present considered among the main risks in policies for prevention and reclamation in sustainable development programs throughout the world. Water deficiency is since longtime a problem in arid countries, but due to climate changes it is also becoming a serious problem in temperate countries, where a competition for water between agricultural and other forms of human needs is happening. Main cropping systems adopted in these countries pay scarcely attention to soil biodiversity; as a consequence a general quantitative and qualitative loss of soil biological fertility is widespread. Several beneficial rhizospheric microorganisms, such as *Arbuscular mycorrhizal* fungi (AMF) and Plant Growth Promoting Rhizobacteria (PGPR) can improve the tolerance of plants to water deficiency; then their addition to soil is a possible tool to increase crops yields under water deficiency and to improve soil biodiversity resilience. In this work several drought-tolerant AMF and PGPR strains were selected from a sahelian rhizospheric soil and mixed in a consortium. A field trial was carried out with maize, where the consortium was inoculated in the rhizosphere; control plants were non-inoculated. Two different cropping systems were checked (minimum tillage and conventional); for each cropping system a further comparison was carried out between non-watered and watered plants. Plant physiological response (gas exchange parameters, water use efficiency, chlorophyll index, morphometric analysis) and yields were analysed. Rhizospheric cultivable microbial populations and roots AMF colonization were determined and tensiometric analysis of soil was carried out. The results shown that physiological behaviour and yields of plants inoculated with the rhizospheric microbial consortium were different from the control plants. In particular, stomatal conductance was higher in inoculated plants; this means a better capability to maintain photosynthetic activity also under drought stress, and as a consequence to reach higher productivity levels. The higher yield increases were observed mainly in non-watered trials and in conventional cropping system, and for green biomass rather than for ears. The lower effect of the inoculum observed in minimum-tillage

trials confirms that this cropping system is suitable to respect and maintain soil biological fertility. Microbiological and tensiometric analysis of soil showed an improvement of soil structure and biological fertility due to the microbial consortium inoculation. In conclusion it can be deducted that specific selection for drought tolerance and management of rhizospheric microbial strains can be a way to improve the crop performances and the soil biological and physical quality in arid climates but also in temperate countries where a risk of desertification exists.

Keywords: soil; biodiversity; drought; desertification; PGPR

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P2.2

Microbial bioremediation of tar produced by steam gasification

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Biomasses are a valuable choice to conventional fossil fuels for energy production through gasification. This thermo-chemical process unfortunately produces several toxic condensable compounds (tar), mainly composed by polycyclic and monocyclic aromatic hydrocarbons (PAH and BTEX). The tar may represent till 10% of the original woody biomass used in the process. Up to now the problem of the disposal of tar from biomass gasification is not solved. Bioremediation of tar with a mixed microbial consortium, despite its complexity, seems a highly promising and innovative approach for the handling of these complex family of byproducts.The aim of this study was to assess the degradation dynamics of a laboratory-scale microcosm where different microbial consortia have been used for the treatment of tar obtained during steam gasification of wood. Tar (BTEX 26.35 mg/L; PAH 8460 mg/L) was incubated with a complex microbial consortia

selected from pinewoods and polluted soils. Triplicate microcosms were setup and incubated at room temperature for 30 days. Aliquots were taken at different time intervals to assess the structure and diversity of the biodegrading bacterial communities by Automated Ribosomal Intergenic Spacer Analysis (ARISA) and 16S and 18S rRNA gene pyrosequencing, respectively. Chemical analyses were carried out by HPLC and/or GC/MS before and after microbial growth. Bacterial growth could be observed after two days. Tar color changed visibly after six days. Both the bacterial growth and the change in color were not detected in the negative controls. At day 30 the inoculated microcosms were completely odorless, whereas the negative controls emitted the characteristic tar scent as intensively as at day 0. Chemical analysis confirmed the occurred degradation of the volatile fraction. ARISA reliability of distinct replicated microcosms was very high (97%). ARISA and pyrosequencing showed a complex bacterial/fungal consortium. Pyrosequencing gave 214,578 bacterial 16S rRNA OTUs and 53,719 fungal 18S rRNA OTUs. The analysis showed the presence of 59 bacterial and 22 fungal genera who participate in tar degradation: after 30 days, while 24 bacteria genera were actively present, only 4 fungal genera were detected (*Galactomyces geotrichum* mostly). Bacterial species grew accordingly to a "cross-feeding" behavior, involving the use of metabolic outputs of one species as an energy source for another one. Further results on chemical analysis, on the dynamics of the biological process of bioremediation and of the behavior of the most representative bacterial isolates will be shown.

Keywords: biodegradation; microbial consortia; microbial resource management; tar; gasification

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P2.3

The diversity of arbuscular mycorrhizal fungi isolated from the roots of *Senecio inaequidens* growing in a heavy metal polluted ash dump downtown Venice.

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Sacca San Biagio is an ash disposal island (4.5 ha) in the central lagoon of Venice (Italy), that hosted a municipal solid waste furnace operating from 1973 to 1984 and producing bottom ashes that were disposed all over the island. Such ashes contain high levels of heavy metals (mainly Cu, Pb and Zn) and are considered ecotoxic, genotoxic and immunotoxic. In the last 29 years the island has been naturally colonised by plants and soil biota, representing a unique site for the study of plant-microbe associations in heavy metal polluted environments. Arbuscular mycorrhizal (AM) fungi (AMF) are important beneficial root symbionts living in association with most plant species in all terrestrial ecosystems worldwide (Smith and Read, 2008; Turrini and Giovannetti, 2012), including polluted soils (Gaur and Adholeya, 2004). In a previous work AMF were found in 67% of Sacca San Biagio plant species. Although no spores were retrieved from rhizosphere soil, molecular methods allowed the characterisation of AMF occurring in the roots of three plant species (Bedini et al., 2010a, b). In this work, in order to induce the production of spores and to isolate AMF strains able to live in such a harsh environment, trap cultures of *Senecio inaequidens* from Sacca San Biagio were set up. Four main spore morphotypes were successfully retrieved and morphologically identified. Molecular characterisation was carried out on two *Rhizophagus* morphotyes, which were identified as *R. intraradices* and *R. irregularis* using primer pairs for the small subunit (SSU) gene and the internal transcribed spacer (ITS) region of the ribosomal DNA. Molecular analyses of DNA extracted from *S. inaequidens* roots showed the occurrence of five sequence types: two types matched with those of *R. intraradices* and *R. irregularis* retrieved from spores, other two types did not show any correspondence with described AMF species, one sequence type was homologous to *Claroideoglomus etunicatum* sequences. The two *Rhizophagus* morphotyes identified were isolated and propagated in pure culture. The availability of AMF isolates, capable of living in harsh environments, such as Sacca San Biagio heavy metal polluted ashes, represents a fundamental step for their utilization as inoculants in successful revegetation management for the reclamation and remediation of contaminated soils.

Keywords: Arbuscular mycorrhizal fungi; heavy metals; SSU gene; ITS region; bioremediation

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P2.4

Are the bio-cleaning technologies safe for Cultural Heritage?

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The main aim of this work is to verify and confirm the safety of the methodologies based on the use of biological techniques for the cleaning of the cultural heritage (CH). The principal technologies to study are the so called bio-cleaning which use microorganisms for restoration. The confirmation of the freedom from danger of these methodologies application will permit the recovery of Cultural Heritage that present serious alterations without damaging our heritage due to its lack of toxicity. The basic idea came from the observation that only few microorganisms have negative role in the natural processes, whilst the majority of them are responsible of "virtuous" processes, such as biogeochemical cycling, wastewater treatment, etc. In the field of CH conservation, the use of microorganisms is an interesting alternative to traditional solvents or other aggressive methods; in these alternative methods microorganisms are cleaning agents of artworks, archaeological pieces and architectural historical monuments. Up until now few scientific teams have work on this methodologies efficiently applying them for the bio-recover of alterations of different materials related to CH (Zanardini E., et al., 2003; Ranalli et al., 2005; Lustrato G., et al., 2012; Bosch et al., 2013). Diverse research teams are here combining their experiences working together on this proposed project in order to verify if these technologies are really softer and safer for artworks, restorers and environment. In fact the frequently asked questions to be answered here are: are these technologies really safe for CH? Is the potential high gain bigger than the potential high risk? In order to answer all those worries this study is focused on strategies for controlling and monitoring eventually new microbial interactions on bio-cleaned artworks. We present a monitoring plan including on site advanced technologies based on non-invasive tools to understand the potential risks on bio-cleaned tangible heritage (Rampazzi et al., 2011; Raimondi V. et al. 2013). Focus may be paid on the risks of re-colonization of bio-cleaned fresco surface by new microbial communities on the Camposanto Monumentale of Pisa, Italy (Ranalli et al., 2005) and on the Santos Juanes church of Valencia, Spain

(Bosch et al., 2013).

Keywords: Cultural Heritage; Safety; Bio-cleaning technologies; Monitoring

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P2.5

Combining fungal and bacterial biotechnological potentials for the functional and eco-friendly bioremediation of polluted industrial wastewaters

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Industrial wastewaters represent one of the most complex environmental issue, due to the growing worldwide industrialization. They have a heterogeneous composition that may also vary during time: salts, surfactants and a great variety of aromatic compounds, including dyes, hydrocarbons, etc. can be found even at high concentration, posing serious concerns about their discharge in the environment.
Due to the complexity of the water-to-treat, several techniques have to be considered. Any method has clearly its specific target of action and only by combining multiple processes, their remediation can be successful.
A biological approach has many advantages, being competitive to the already in use chemico-physical ones. Indeed, the use of high amount of potentially harmful chemicals is avoided and the operative conditions do not require elaborate and energetically costly technologies: bioremediation processes often result environmental and economical sustainable.
Bacterial treatment has been long studied for almost one century, representing nowadays the central oxidation process in many wastewater treatment plants (WWTPs). However, it shows several problems, limiting its efficacy towards some components of industrial effluents, i.e. dyes. In alternative, fungi can be considered as promising biocatalysts, thanks to their capability to mediate extensive degradation reactions towards several aromatic compounds, by means of their

extracellular oxidative enzymes.
According to this general context, the main goal of this project was to suggest and investigate a new solution for wastewaters treatment. More in detail, textile effluents and landfill leachates were taken into consideration: they were highly coloured and toxic, as assessed by several ecotoxicological tests. The limits of activated sludge immediately appeared: bacteria were able to extensively reduce COD, but almost no effect was observed towards colour and toxicity.
The first issue was the selection of powerful fungal strains, able to conserve their oxidative capability in the harsh chemico-physical conditions of not-sterile industrial wastewaters. The screening allowed to identify strong and versatile strains able to survive and remain active in those extreme environments. The main fungi strength was to transform coloured compounds in colourless ones. Furthermore, the process optimization was conducted aspiring to a future industrial application: different mycelium immobilization techniques and reactor technologies were taken into consideration, to focus the attention on industrially feasible processes development.
Interestingly, bacteria and fungi showed a different but not overlapping activity, justifying the hypothesis to set a combined biological treatment, able to strengthen the potential of both organisms. The optimized fungal process was coupled with activated sludge and the sequential treatment resulted very effective. As regards textile effluents, fungi and bacteria alone achieved bioremediation yields not even close to the combined process ones: when they worked in a synergic way, colour, COD and toxicity were all extensively reduced, complying the threshold limits of the Italian law.
At the moment, few parameters have still to be optimized to allow the application of the studied biological methodology at higher working volumes. Anyway, the acquired results were so promising that a WWTP is actually working to set up a pilot reactor for the scale-up of the biological treatment of textile effluents.

Keywords: activated sludge; bioremediation; fungi; industrial wastewaters.

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P2.6

Production of bioethanol from effluents of the dairy industry by *Kluyveromyces marxianus*

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Whey and scotta are the main effluents of dairies coming from cheese and ricotta processing respectively. Whey contains minerals, lipids, lactose and proteins; scotta contains mainly lactose. Nowadays scotta is just considered a waste, while whey can be reused by several ways, such as protein extraction or animal feeding; in spite of this, due to very high volumes of whey produced in the world, it still poses serious environmental problems for its disposal. Alternative destinations of these effluents, such as biotechnological transformations, can be a way to reach both goals of improving the added value of the agroindustrial processes and reducing their environmental impact. In this work we investigated the best way to produce bioethanol from lactose of whey and scotta and to optimize the fermentation yields. *Kluyveromyces marxianus* var. *marxianus* was chosen as lactose-fermenting yeast. Batch, aerobic and anaerobic, fermentations and semicontinuous fermentations in dispersed phase and in packed bed reactor were carried out of row whey, scotta and mix 1:1 whey:scotta at a laboratory scale. Different temperatures (28–40°C) were also tested to check whether the thermotolerance of the chosen yeast could be useful to improve the ethanol yield.
Both whey and scotta showed to be suitable for bioethanol production. Anaerobic fermentation of whey can easily be managed in several ways, always reaching good results, while scotta, that has not sufficient features as growth medium due to lack of nutrients necessary to yeast cells for anaerobic metabolism, can give rise to good fermentation performances only in semicontinuous fermentations. In general, the best performances were reached at low temperatures (28°C), but high temperatures allow good ethanol yields in short times in whey fermentations: in particular, batch anaerobic fermentation carried out at 40°C with high biomass inoculum showed the best performances.
The production of bioethanol from scotta can be considered avery good opportunity, as nowadays this effluent is considered just a waste without other possible destinations. Aerobic fermentations of whey and scotta are not suitable for bioethanol production; however, a different goal could be the production of microbial biomasses, contemporarily allowing the waste purification, then the reduction of their environmental impact and disposal costs. Finally, using whey and scotta as growth media for biotechnological transformations is an important chance which can allow us to improve the added value of agroindustrial processes,

contemporarily reducing the effluents disposal costs.

Keywords: bioethanol; *Kluyveromyces marxianus*; whey; scotta; fermentation

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P2.7

Diversity and evolution of 1,2-DCA Reductive Dehalogenases in polluted marine and freshwater environments

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Halogenated organic compounds are extensively used in a wide range of industrial applications and have caused, as a consequence, serious environmental contaminations. Among them, 1,2-dichloroethane (1,2-DCA) is one of the most important quantitatively, being used as an intermediate for polyvinyl chloride production. Microorganisms capable of deriving energy for their growth from the reduction of halogenated compounds are widespread in natural ecosystems. Organohalide-respiring bacteria belong to several phyla, including *Proteobacteria*, *Firmicutes* and *Chloroflexi*. The metabolic process known as reductive dehalogenation has been demonstrated to be a key microbial metabolism for groundwater bioremediation. Since dehalogenase homologous genes have been retrieved both in contaminated groundwater and seawater sites, aim of this work was to study and compare the phylogenetic and functional diversity of the reductive dechlorinating microbiota of freshwater and marine environments with different contamination backgrounds. Microcosms, inoculated with water and sediments from contaminated aquifers and coastal sites, were set up and spiked with known amounts of 1,2-DCA as the only electron acceptor and different

sources of electron donors. A series of subsequent culture transfers, where an aliquot of the microbial consortia were inoculated in new microcosms containing fresh media, 1,2-DCA and the proper electron donor, were established with the aim of selecting the best adapted bacterial dehalogenating consortium. Bacterial phylogenetic diversity was monitored by *16S rRNA* gene-based PCR-Denaturing Gradient Gel Electrophoresis (DGGE) and barcoded pyrosequencing, while the functional diversity was described by DGGE fingerprinting on dehalogenase encoding genes. The results indicated that both groundwater and seawater contaminated samples have dehalogenating potentials for remediation, showing complete degradation of 1,2-DCA in anaerobic microcosms and the enrichment of different dehalogenase homologous encoding genes characterized by sequence domains specifically associated to 1,2-DCA metabolism. Despite a conserved 1,2-DCA reductive dechlorinating potential, the different environments and contamination context lead to the selection of specific communities with functional and phylogenetic diversity adapted to the peculiar geo-chemical settings.

Keywords: reductive dehalogenation; 1,2-Dichloroethane; bioremediation; pyrosequencing; DGGE

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P2.8

Purification and characterization of invertase from *Aspergillus* spp. grown on potato and carrot peels

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Food processing industry produces an enormous amount of carbohydrate wastes, which pose increasing disposal costs and environmental challenges, approximately 45% of total organic industrial pollution generates from this sector. Beside their pollution and hazardous aspects, these organic wastes are rich in biodegradable materials, and have potential to be used as suitable substrates for biotechnological productions. Extensive studies have been done by using synthetic medium for preparation of enzymes while a little attention has been paid on their production from less expensive sources. The present study was planned to produce invertase

from indigenous wastes, carrot and potato peels, as substrates for five different *Aspergillus* species through solid state fermentation (SSF). Higher activities of invertase were observed by *Aspergillus niger* on carrot peels and *A. terreus* on potato peels at 30°C over 72h of incubation. Invertase (β-D (fructofuranosidase, EC 3.2.1.26) is one of the important commercial enzymes used in food industry. The process parameters influencing the production of invertase by A.niger and A.terreus in SSF were optimized at 90% moisture content, 72 and 60 h of incubation period for *Aspergillus niger* and *Aspergillus terreus* respectively and 2.5% Inoculum's size. Enzyme purification was carried out, and 512 IU of crude Invertase from *Aspergillus niger* was partially purified to maximum specific activity (11.5 IU/mg protein) after 40-80% ammonium sulphate precipitation. Similarly, in *Aspergillus terreus* enzyme purification was carried out, and 354.8 IU of crude Invertase was partially purified to maximum specific activity (9.46 IU/mg protein) after 40-80% ammonium sulphate precipitation. During characterization, it was observed that invertase from *Aspergillus niger* was stable from pH 5.5 to 6.5 with maximum activity at pH 5.5. The temperature range for enzyme stability was between 20 to 50°C. When the effect of different substrates on invertase activity was assessed, it was found that sucrose showed highest activity both at higher and lower concentrations. As metal ions cobalt and sodium showed highest activity while mercury significantly inhibited invertase activity. While Invertase from *A.terreus* was stable from pH 4.0 to 6.0 with maximum activity at pH 6.0. The temperature range for enzyme stability was from 30 to 60°C. Similar results were observed in case of substrate specificity, metal ions inhibition.

Keywords: invertase enzyme; solid state fermentation; agro-wastes

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P2.9

Effect of environmental stresses on plant grow promoting characteristics of the arsenite oxidizer (*Pseudomonas*) sp. strain N2

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Heavy metals resistant bacteria with plant growth-promoting (PGP) characteristics can be used to facilitate the proliferation of plants under environmentally stressful conditions, such as drought, salinity and heavy metals contamination. Moreover, arsenic-transforming PGP bacteria, influencing arsenic mobilization in soil, can

enhance arsenic uptake by plant.
The aims of this study were: (i) to characterize an arsenic-resistant (*Pseudomonas*) sp. strain N2 for resistance to osmotic stresses, production of stress-related phytohormones, PGP traits and arsenic transformation, and (ii) to evaluate the effect of arsenic on these characteristics.
The capability to respond to osmotic stresses (-1.5 MPa) was evaluated by measuring the bacterial growth in LB medium separately added of: 175 mmol/L of Na_3AsO_4, 400 mmol/L of NaCl, or 26% (w/v) PEG6000 (Sosa et al., 2005). Production of stress-related phytohormones (indole acetic acid IAA, and jasmonic acid, JA) was determined in yeast mannitol medium (YEM) in the absence and in the presence of arsenate (50 mmol/L), arsenite (3 mmol/L), and PEG6000 (13.7% w/v), as stress agents. Tested PGP characteristics were: the ability to grow on 1-aminocyclopropane-1-carboxylic acid (ACC) as sole nitrogen source, the production of siderophores and proteolitic, chitinase and phosphate solubilising activities. Phosphate solubilisation activity was evaluated in YEM with $Ca_3(PO_4)_2$ (5 g/L) in the absence or in the presence of arsenite or arsenate. Arsenic transforming capabilities were evaluated during 72 h growth in Tris Mineral Medium (Mergey et al., 1985) supplemented with 0.6% (w/v) gluconate (TMMG) spiked with 3 mmol/L of arsenite or arsenate.
Strain N2 was able to tolerate -1.5 MPa: osmotic stress generated by the presence of arsenic was tolerated better than those generated by NaCl and PEG6000. The strain was able to produce 68 pmol/ml of IAA and 0.18 pmol/ml of JA and to solubilise 100 mg/L phosphate. While the production of IAA was enhanced by the presence of arsenic forms it was not affected by osmotic stress. On the contrary, phosphate solubilization was impaired by arsenic. Strain N2 possessed all the tested PGP characteristics and it completely oxidized arsenite to arsenate, while it did not reduce arsenate.
Our results suggest that strain N2 possesses PGP characteristics not affected by the presence of arsenic and of osmotic stresses. Moreover, the ability to solubilize phosphate may contribute to improve plants' phosphorous nutrition. (*Pseudomonas*) sp. strain N2 might be a candidate inoculum useful to amplify plant resistance in stress conditions.
Acknowledgments: Funding for this work was provided by: Fondazione CARIPLO (project 2010-2221) and PRIN (project 2010JBNLJ7_004).

Keywords: PGP; osmotic stress; phosphate solubilization; arsenic resistance; arsenite oxidation

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P2.10

Aerobic remediation of BTEX and MTBE-contaminated groundwater through lab-scale biobarriers

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Biobarriers are an innovative technology for the in-situ treatment of contaminated groundwater. In these systems, groundwater flows through the barrier core, which contains a filling material with adhered biomass. Thus, organic contaminants can be removed by biological processes. For optimal in-situ performances a careful setup is required, with particular attention to the configuration and the location of the barrier and the residence time of the contaminated water within the reactive zone. This work was aimed at identifying the best working parameters in a lab-scale biobarrier for the aerobic treatment of groundwater contaminated with the gasoline-derived compounds BTEX and MTBE. Pumice was chosen as the most suitable filling material on the basis of its hydraulic, chemical-physical and biomass sorption properties. Several microbial strains were isolated from hydrocarbon-contaminated soil and groundwater by enrichment cultures using BTEX and MTBE as sole carbon sources. The isolated strains were combined to prepare different inocula, which were tested in batch systems for their suitability as inoculum for the lab-scale system. The best results were obtained with the inoculum composed by *Rhodococcus* sp. CE461, *Methylibium petroleiphilum* PM1 and *Rhodococcus* sp. CT451, which degraded all BTEX in 7 days and 19% of MTBE in 25 days in batch experiments. Lab-scale tests were carried out in a column filled with unsaturated pumice, either inoculated or not. The removal efficiency of hydrocarbons from the input water was between 42% and 72% for MTBE, and between 78% and 97% for toluene. However, no significant differences were observed between inoculated and no inoculum experiments. Samples of pumice for molecular analyses were also taken along the column. Both DGGE and T-RFLP analyses on 16S rRNA gene confirmed the persistence of the inoculated strains all along the column until the end of the experiment. On the contrary, in the column without inoculum, the same analyses revealed that an indigenous microbial community different from that hosted by the pumice developed during the experiment. Particularly, the genera *Hydrogenophaga* and *Thauera*, known for their MTBE and BTEX degrading abilities, were present in the final part of the column. Q-PCR analyses were also performed on toluene

monooxygenase and on xylene monooxygenase genes. The results indicated that the copy abundance of both genes increased over time in both experiments, and it was generally higher in the inoculated column, although it did not lead to a higher hydrocarbon degradation. Overall, the results suggested that autochthonous microorganisms were able to effectively degrade the hydrocarbons. Therefore, a bioaugmentation with allochthonous inocula would not be necessary to improve the remediation performance.

Keywords: gasoline; permeable reactive barriers; in-situ bioremediation.

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P2.11

Bacterial and archaeal community in a flooding uranium mine, Königstein (Germany)

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The former uranium mine Königstein in Germany is currently in the process of remediation and represents an underground acid mine drainage (AMD) environment. Due to technical leaching with sulphuric acid, the mine water is characterized by low pH, high concentrations of toxic heavy metals and uranium (up to 3×10-4 M) (Arnold et al. 2011). Biofilms in the Königstein mine grew underground in the mine galleries in a depth of 250 m (50 above sea level) either as stalactite-like slime communities (snotites) or as acid streamers in the drainage channels (Zirnstein et al. 2012). Since 2010 the underground mine is no longer accessible because of flooding. Biomass of the mine water community was retrieved by three different in-situ systems: vacuum filtration of the mine water, flow cell with slides and reactor with PE carrier. The diversity of the planktonic microorganisms and of the biofilms of the Königstein samples of two consecutive years were characterized by catalysed reporter deposition fluorescence in-situ hybridization (CARD-FISH) and a bar-coded pyrosequencing approach.
The identified microbial communities showed low diversity. 24,630 archaeal sequences and 5,706 bacterial sequences could be classified into 5 classes

(archaea) and 13 phyla (bacteria) including candidate divisions. CARD-FISH analysis showed that Bacteria were more abundant than Archaea. The dominating phylum of bacteria was *Proteobacteria*, especially *Alphaproteobacteria* and *Gammaproteobacteria*. Furthermore, sequences of *Actinobacteria*, *Nitrospira*, *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, *Acidobacteria*, *Chlorobi*, *Chloroflexi*, *Planctomyces*, OD1, TM7 and *Gemmatimonadetes* were found. Most of the archaeal sequences belong to the class Thermoplasmata, especially *Ferroplasma sp.*.

The obtained results for the microbial community in the flooded mine site is completely different in comparison to the microbial community observed in the underground environment before flooding. Before flooding the mine galleries were dominated by biofilms composed predominantly of Betaproteobacteria affiliated with *Ferrovum myxofaciens*, also designated "*Ferribacter polymyxa*".

Knowing more about the acidophiles in former uranium mines helps to explain how microorganisms live in such extreme environments and how they affect the water chemistry and how to use them for biomining and bioleaching technologies.

Keywords: microbial community; biodiversity; bacteria; archaea; pyrosequencing.

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P2.12

Microbial communities in flooded underground uranium mines of East Germany

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After the German reunification the Wismut GmbH, formerly the 3rd largest U producer of the world, started to remediate the legacies of their U mining activities. As a part of the remediation strategy, the pit body was flooded which induced reductive processes. Although flooding of the mines Pöhla and Schlema-Alberoda was already finished about ten years ago, the mine water still contains elevated concentrations of toxic contaminants such as U, As and Ra. Thus, expensive and

long-lasting monitoring and waste water treatment is required. Since microorganisms can influence the toxicity of metals directly or indirectly, one alternative approach is to use them for bioremediation (Anderson and Lovley, 1997). To remediate U contaminated sites recent studies mainly focused on the applicaton of dissimilatory Fe(III) and sulfate-reducing microorganisms which are capable to reduce U(VI) to U(IV) thus resulting in a decreased U mobility (Lovley and Philips, 1992; Lovley et al., 1993). To investigate whether indigenous microorganisms in the mine water of Pöhla, Schlema-Alberoda and an older uranium mine site, Zobes, could have an influence on contaminant toxicity, it was first necessary to analyze the microbial community. Mine water samples were either filtrated (10 l) or collected from an *in situ* flow cell. For the Zobes site natural biofilms grown on activated carbon were also collected. *Bacteria* as well as *Archaea* were analyzed by state-of-the-art pyrosequencing of the 16S rRNA gene. The analysis of the bacterial diversity of the Pöhla mine water resulted in 1196 sequences which represent members of 11 phyla. For the Schlema-Alberoda mine water 1915 sequences were analyzed which were divided into 9 phyla. In comparison to the mine water of Pöhla and Schlema-Alberoda the bacterial composition of the Zobes mine water is very similar. The dominant bacterial phylum in all samples is the *Proteobacteria*, especially sulfur-oxidizing bacteria of the *Epsilonproteobacteria* such as *Sulfuricurvum* were frequently found. Interestingly, the analysis of biofilm samples of Zobes revealed a different bacterial community compared to the respective mine water. *Geobacter*, a known Fe(III)-reducing and U(VI)-reducing bacterium, was found to be the dominating genus in the bacterial biofilm community. For the investigation of the archaeal diversity of the mine water a dataset of 33658 (Pöhla), 19184 (Schlema-Alberoda) and 11401 (Zobes) sequences was analyzed. In all samples the archaeal sequences mainly represent the three following classes: *Methanobacteria*, *Thermoprotei* and *Methanomicrobia*. In this study Fe(III)-reducing bacteria capable of reducing U (VI) could be found in biofilms of the Zobes mine water probably influencing U mobility *in situ*. Future experiments will focus on the analysis of biofilm samples from the Pöhla and Schlema-Alberoda site and will include the determination of metabolically active microorganisms.

Keywords: microbial community; diversity; uranium

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P2.13

Synergistic effects of cadmium and iron on siderophore production in *Anabaena oryzae*

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Siderophore production in cyanobacteria is very much dependent on iron content. *Anabaena oryzae*, a nitrogen fixing *cyanobacterium*, commonly found in rice fields contributes in the nitrogen economy for rice cultivation. In the current scenario cadmium gains a global attention because of its high water solubility, relative mobility, and phytotoxicity even in minute amounts. The high water solubility of cadmium might have a high impact on rice and associated microorganisms. In the present investigation, effect of cadmium on behavioural properties of *Anabaena oryzae* and siderophore production under iron supplemented and depleted conditions were observed during the late phase of growth. Through experiments it was found that individually, 50 µM of iron ($FeCl_3$) and 1.0 µM of cadmium ($CdCl_2$) were the optimum concentrations for the growth and siderophore production in *Anabaena oryzae*. In addition to this, the interactive effect of 1.0 µM cadmium and 50 µM FeCl3 on Siderophore production in *Anabaena oryzae* have also been investigated and it was found that siderophore production was reduced markedly with enhance level of electrolyte leakage and antioxidative enzymes such as SOD and catalase. In both the cases, induction of hydroxymate type siderophore was observed. Further, whole cell protein pattern in cadmium (1.0 µM) Fe^{+3} (50 µM) and Cd^{+2} in combination with Fe^{+3} was also evaluated in order to observe appearance and non appearance of different size of bands at exponential phase. Results suggest that 1.0 µM of Cd^{+2} and 50 µM Fe^{+2} individually have stimulatory effect possibly because regulation of Cd^{+2} creates oxidative stress to the cell reflecting high anti-oxidative responses of *Anabaena oryzae* for survival. Upto1.0 µM Cadmium can be acquisitized by the siderophores produced by *Anabaena oryzae* from the soil. Thus, it can be concluded that under iron sufficient condition (Fe^{+2}) the siderophore produced removes cadmium from the soil through complex formation. Under iron deficiency (Fe^{+3}) siderophore is needed to chelate the iron from environment to maintain its normal biological processes and defence mechanisms needed to reduce Cd^{+2} impact. Thus, *Anabaena oryzae* not only plays a significant role in nitrogen fixation but also helps in metal ion regulation to maintain soil health.

Keywords: cadmium; siderophore; phytotoxicity; relative mobility.

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P2.14

Biochar stability in soil microcosms

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Energy security has become a matter of big concern in the past decades. Given the rising prices and diminishing reservoirs of oil, not to mention the environmental impact of the use of fossil fuels for energy purposes, the switch to sustainable renewable sources has become a improcrastinabile need. Energy production from biomass is a ready-to-market technology, it represents about 70% of EU renewables, and its use as a fuel permits to have a carbon-neutral cycle. Syngas production through pyrolysis and gasification is regarded as the most environmentally friendly and one of the most efficient technologies available to exploit biomass energy potential. However, pyrolytic breakdown of biomass also produces different by-products in quite big amounts, and studies have been undertaken to find out applications.
Biochar is one of them, and it is being studied all around the world for its soil-ameliorating and soil fertility-enhancing characteristics. Biochar stability within soil is a matter of big interest, mainly because its carbon sink-activity depends on its recalcitrance. Presence of biodegrading microbial consortia able to degrade biochar aromatic structure is therefore a good indicator for its long-term effects on soil and environmental health.
To assess whether microbial consortia in soil were able or anyway adapted in the course of time to degrade biochar aromatic structure while exploiting it as a carbon source, quantification and comparison of the total living bacterial community and of the degrading one were carried out at 6 different time points: by incorporation,

and after 1, 2, 5, 12 and 24 weeks. In order to account only for active bacteria, RNA was extracted from the different samples and analysed. The total bacterial abundance was quantified through Quantitative Real-Time PCR of the highly conserved 16S rRNA region. A further Quantitative Real-Time PCR of the polycyclic aromatic hydrocarbon-ring hydroxylating dioxygenase (PAH-RHDα) gene gave us as a result the amount of aromatic-degrading bacteria in the samples. A growth in the total population was noticed in most of the samples, independently from biochar addition to soil, but no relevant presence of PAH-degrading bacteria was noticed both in soil only and in biochar amended pots. The fact that no PAH-degraders were detected even if the whole bacterial community raised, supports the hypothesis that biochar is a stable form of carbon, playing an important role in the atmospheric carbon-sequestration.

Keywords: biochar; biodegradation; RNA; qPCR; dioxygenase

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P2.15

A microcosm study of chlorinated solvents microbial degradation: a possibility to remediate a contaminated area in Central Italy

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Perchloroethene (PCE), Trichloroethene (TCE) and other chlorinated solvents are widespread groundwater pollutants. They are among the most common pollutants in industrial sites due to their extensive application in chemicals production, metal degreasing and dry cleaning. These compounds are environmentally persistent and carcinogenic.
Chlorinated solvents, once released into the environment, are subject to both chemical-physical and, in particular, microbial degradation. They often behave as electron acceptors because of their substituents' electronegativity, and are then reduced. In particular the anaerobic reductive dehalogenation occurs mostly in compounds with a high number of halogen substituents (such as Tetrachloroethene and Trichloroethene), which are not degraded by aerobic microorganisms.
Many cases of serious contamination of groundwater - that might compromise their use in industry, agriculture and private - are present in Italy. Among these, the

critical case of a Central Italy valley located in the province of Teramo, characterized by a significant contamination by chlorinated solvents.
Microcosms were prepared by using soil and groundwater samples from this contaminated site, by adding suitable elector donors and carbon source (lactate or butyrate) and a mineral medium containing resazurin (redox indicator), a metal solution and calcium, magnesium and potassium ions (Tandoi et al., 1994). In addition, some microcosms were additioned with an anaerobic sludge from a wastewater treatment system. Microcosms were set-up in duplicate in 250 mL serum bottles and sealed with Teflon-faced butyl rubber stoppers and aluminum crimp caps (Fennell et al., 2001). Headspace was flushed with a 70% N_2- 30% CO_2 gas mixture, to ensure anaerobic conditions (Aulenta et al., 2007). Microcosms were incubated statically in the dark at room temperature.
The amended microcosms only were positive for dechlorination of chlorinated compounds. In the abiotic controls and in the non amended microcosms, dechlorination has not played and PCE was not degraded. This indicates that is no possible a chemical and abiotic degradation of organochlorine and is not even feasable a natural attenuation; this indicates also the presence of an active native dechlorinating population in the subsurface, but possibly insufficient. Microcosms bioaugmented with the anaerobic sludge dechlorinate PCE to DCE, vinyl chloride and also ethene. DGGE analysis shows a complex microbial diversity with high dynamic and functional organization and with different real and potential dechlorinating species. These microcosm studies indicate that in the contaminated site is possible an Enhanced anaerobic dechlorination of PCE and TCE, through an appropriate addition of electron donors and/or through bioaugmentation with dechlorinating cultures, even originating from an anaerobic sludge.

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P2.16

Effect of development of *Sporosarcina pasteurii* on sand consistency

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In this study we provide the results obtained after inoculation in controlled

experimental conditions Sporosarcina pasteurii in samples of different grain sands. The methodology used was as follows: 25 g of each of the samples of sand were placed in sterile petri dishes of 9 cm diameter. To each plate was added 5 mL of 3M urea, 5 mL of 3M calcium chloride and 10 mL of a suspension of Sporosarcina pasteurii prepared in BHI and a concentration of about 1-2x10^8 CFU/mL. Incubaronen plates were aerobically at 37°C and over several weeks. Control plates were maintained sands to which was added no cultivation Sporosarcina pasteurii. At intervals of seven days, we proceeded to the observation of the plates. Three weeks after the start of the investigation, it was found that in samples of sand size exceeding 5 mm in diameter the activity of bacteria was remarkably promotes the cohesion of the sand particles, transmutation themselves deposit material on the plate in a compact and robust. In controls this phenomenon is not observed. Thus we can say that the addition of crops to Sporosarcina pasteurii sand samples under the conditions laid determines its transformation into a compacted material for possible application in technology of building materials

Keywords: Sporosarcina pasteurii; sands; bioremediation.

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P2.17

Fingerprinting characterization of prokaryotic communities in sediments from three tourist ports in the Mediterranean area

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Port areas pose major concerns due to the presence of hydrocarbon contamination, associated with the heavy boat traffic and related facilities and to consequently harmful effects on the marine ecosystems and human health. Hydrocarbon degraders and particularly obligate hydrocarbonoclastic bacteria carry out a global activity in biological removal of petroleum hydrocarbons in marine habitats. Moreover, distinct bacterial populations specifically degrade structural different classes of hydrocarbons. Despite their abundance, diversity and significant role in marine ecosystems, the response of archaeal communities to hydrocarbon pollution has been less extensively examined. In oxic environments, Archaea seems to have a limited role in hydrocarbon degradation showing a high sensitivity to oil exposure. A contribution in PAH degradation of methanogenic community has been recently suggested in anoxic sediments.
In this context MAPMED project is aimed to improve the environmental sustainability of tourist coastal areas in the Mediterranean Sea Basin with regard to monitoring and reduction of marine hydrocarbon pollution. Aim of our work was the characterization of prokaryotic communities in 3 tourist ports (Cagliari, IT; El Kantaoui, TN; Heraklion GR), with particular regard to the indigenous hydrocarbon degrading populations. Two sampling campaigns were carried out during winter and late spring, before the touristic season, and a third one in September, after the touristic season. In each port, samples of superficial (SS) and anoxic sediments (AS) were collected at different stations. Each port was characterized using a combination of culture-dependent (MPN of hydrocarbon degraders) and independent approaches (T-RFLP of 16S rRNA gene). The MPN technique was employed in order to enumerate microbial communities able to grow on yeast extract, as non-hydrocarburic organic compounds, diesel, as hydrocarbon mixture representative of contaminations resulting from marine transport, phenanthrene, as model compound of polycyclic aromatic hydrocarbons, and crude oil. The highest viable titles of heterotrophs were obtained in samples from Heraklion for both matrices. The highest abundance of hydrocarbon degraders was found in superficial sediments from Cagliari and Heraklion and in

anoxic sediments from El Kantaoui. T-RFLP analysis was used to assess spatial and temporal changes in prokaryotic communities. Shannon index, as a synthetic measure of biodiversity, showed lowmedium values for Bacteria and Archaea in both matrices. Correspondence analysis, Sørensen's distances analysis, and clustering of T-RFLP profiles showed, for both Bacteria and Archaea communities and in both matrices, ordination of samples based mainly on sampling campaign. In order to fully characterize microbial communities of the study sites, metagenomic NGS sequencing, using Illumina MiSeq, will be performed. Our approach devises the use of different primers for the amplification of Bacterial V4 and Archaeal V3 hypervariable region of 16S rRNA gene (that will be tagged with different indexes) and their analysis in the same MiSeq run. To do so, we are currently performing PCR conditions optimization.

Keywords: port sediments; hydrocarbon contamination; bacteria; archaea; microbial community analysis

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P2.18

Flavolipid biosynthesis genes in *Flavobacterium* sp. strain MTN11

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Many microorganisms from various taxonomic groups and diverse habitats synthesize a wide variety of secondary metabolites of low molecular weight called biosurfactants. Generally, these biomolecules are not essential for the growth and development of microorganisms and occur only under environmental stress conditions or during some stages of the life cycle of microorganisms (Lee et al., 2008). Due to the biosurfactant intrinsic characteristics (emulsifying, dispersing and foaming) in addition to its low toxicity and ready biodegradability, its specific conditions of high temperature, salinity and pH extremes compared with synthetic surfactants, these molecules have a large range of applications within most notably are bioremediation of petroleum hydrocarbons in groundwater and soil, and the degradation of hazardous compounds, biological control, biofilms and antibiotic activity thus impacting on the health and economy of our society (Huang et al.,

2010). Structurally, biosurfactants are amphipathic molecules constituted by both a hydrophobic and a hydrophilic domains, this peculiarity confers biosurfactants reduce the interfacial tensions. The hydrophilic portion of these compounds has been used to classify the biosurfactants as glycolipids, lipopolysaccharides, lipopeptides, phospholipids, fatty acids, polymeric biosurfactants and the recently described flavolipids (Baek et al., 2004). The flavolipids are produced by Flavobacterium sp. strain MTN11 which is an aerobic, Gram-negative and non-fermenting soil bacteria. The polar portion of these molecules is similar to the structure of the siderophores aerobactin and artrobactin, while the non-polar moiety comprises two branched acyl chains (Bodour et al., 2004), however little is known regarding the genes involved in the biosynthesis of flavolipids therefore, the aim of this study is the identification and characterization of genes responsible for the biosynthesis of flavolipids. By random transposition assays, we identified three genes encoding proteins involves in the biosynthesis of these interesting molecules. Additionally, our working group has evaluated the biosurfactant production capacity of the generated non flavolipids-producing mutants.

Keywords: biosurfactants; flavolipid; genes; *Flavobacterium* sp.

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P2.19

A new selection-based approach of yeasts and bacteria for the bioremediation of olive oil mill wastewaters (OMW)

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Olive mill wastewaters (OMWs), by-products of the olive oil industry, are highly pollutant and harmful to the environment, due to their content in antimicrobial and

phytotoxic compounds. In the last 20 years, the treatment and disposal of OMW represent an emerging problem in the Mediterranean Basin. Several different approaches have been developed for OMW biological treatment.
In particular, different yeast species were proposed as biotechnological tool for OMW bioremediation. Several bacterial strains were also used to remove recalcitrant and toxic compounds from OMW by aerobic and anaerobic treatments. In this study, an innovative protocol was set up for isolation of aerobic microorganisms, yeast and bacteria, and step-by-step selection of strains able to detoxify OMWs. In the first step, microorganisms, deriving from different OMWs, OMW-contaminated soils samples and commercial products for waste-water treatment were sampled and the associated microbial populations were firstly isolated in non-selective media and then selected for their ability to grow in selective media, each added with different carbon sources, in order to stimulate the activation of specific metabolic pathways. In the second step the selected isolates were assayed for their ability to grow on OMW as unique carbon source. They were then selected in a third step for their capacity to grow in presence of different mono- and poly-phenol compounds generally present in OMW. The isolates obtained at the end of the described procedure were identified at molecular level: bacterial isolates belong to the genera *Acetobacter*, *Bacillus*, *Peanibacillus* and *Sphingomonas*, whereas all yeast isolates were identified as belonging to *Candida boidinii* species.
To perform the fourth step of selection, a new synthetic formulation of OMW was developed and applied. Identified bacterial and yeast isolates were inoculated in synthetic OMW and tested for their ability to reduce phenolics, chemical oxygen demand (COD) and antimicrobial compounds. This analysis allowed to identify the best performing bacterial and yeast isolates that represent promising candidates to be used in free or immobilized form, in single or combined inocula, for OMW bioremediation.

Keywords: bioremediation; waste waters; aerobic treatment

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P2.20

Biosysnthesis of Se (0) nanoparticles, using *Pantoea agglomerans* UC-32 strain, isolated from Atacama Desert, Chile

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Selenium (Se), as a functional material, is an important semiconductor and photoelectric element due to its special physical properties (Zhang et al., 2011). Therefore, Se is used in many applications ranging from photocells, photographic exposure meters and solar cells to semiconductor rectifiers. When amorphous, Selenium nanoparticles (SeNPs), possess unique photoelectric, semiconducting and X-ray-sensing properties (Dhanjal and Cameotra, 2010). The SeNPs also have Biologic activity and good adsorptive ability due to interaction between the nanoparticles and NH, C=O, COO–, and C–N functional groups of proteins (Zhang et al., 2004). Recently, there has been increasing interest in the synthesis of nanoparticles using biologic systems, leading to the development of various biomimetic approaches (Mohampuria et al., 2008). However, most methods used to synthesize SeNPs are characterized by elevated temperatures and high pressures and are hazardous to the environment (Zhang et al., 2011). The bio-reduction of selenite (Se (IV)) generates nanoparticles with sizes ranging between 30 and 300 nm (Thakkar et al., 2009). Biologic properties of Se nanoparticles, e.g., antioxidant activity, are dependent on the nanoparticle size; smaller particles have greater activity (Dhanjal and Cameotra, 2010). In this study, the bio-reduction of selenite by *Pantoea agglomerans* strain UC-32 under aerobic conditions and room temperature to produce bioactive Se nanoparticles smaller than 100 nm was demonstrated. Isolation and purification of the nanoparticles was performed by alkaline lysis. These purified nanoparticles were stabilized with L-cysteine (4 mM). The visualization and characterization of nanoparticles were performed by transmission electron microscopy, energy dispersive X-ray spectroscopy, and scanning electron microscopy. The antioxidant activity of nanoparticles was determined by production of reactive oxygen species using human umbilical veinendothelial cells. Transmission electron microscopy images showed the

accumulation of spherical selenium nanoparticles as intracellular and extracelular deposits. The size of Se nanoparticles varied with incubation time. Amorphous Se nanoparticles with size in the order of 100 nm were obtained before 24 h of incubation; but, at 24 h of incubation, the size of the majority of the nanoparticles was in the desirable order of 100 nm and they were not aggregated. Energy dispersive spectroscopy spectra indicated that nanoparticles were composed entirely of selenium. Antioxidant activity of stabilized selenium nanoparticles demonstrated high antioxidant activity when compared to selenite and selenium nanoparticles without stabilization. Stabilized biologically synthetized selenium (0) nanoparticles with size less than 100 nm have a potential application as a food additive with antioxidant properties relevant to human health.

Keywords: Se-nanoparticles; bio-reduction; selenite; Antioxidant activity

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P2.21

Characterization of a bacterial consortium capable of degrading roxarsone in aerobic condition

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Roxarsone (ROX) is an organoarsenical compound used as food additive in the poultry industry and it is mainly excreted unchanged in feces and urine (Garbarino et al., 2003). ROX has the potential risk to contaminate the environment, mainly by the use of poultry industry manure as fertilizer, releasing inorganic arsenic to the soil and water (Jackson et al., 2003; Stolz et al., 2007). The aim of this work was to isolate and characterize a bacterial consortium capable to degrade ROX under aerobic conditions. A bacterial consortium was cultured from a soil sample obtained from a field fertilized for years with poultry litter from a poultry industry using ROX. The consortium was enriched in a basal medium added with ROX. ROX degradation and growth kinetics were determined by incubation of the bacterial consortium in the presence of 0.5 mM ROX at room temperature and

under aerobiosis conditions. The bacterial consortium were characterized molecularly by PCR-DGGE and metabolically, using Biolog Ecoplates. The detection of inorganic arsenic was carried out through precipitation with silver nitrate ($AgNO_3$). The consortium was also analyzed by scanning electron microscopy. The results showed that the growth rate (k) of the bacterial consortium was 1.4 fold higher in presence of ROX. The bacterial consortium had the ability to transform, after 7 days of incubation, 81.04% of the ROX present. Molecular characterization revealed the presence of different bacterial groups, being *alphaproteobacteria* and *firmicutes* were the groups that showed the highest count in the both corsortiums. The metabolic profile of the consortium did not change in the presence of this organoarsenical compound, but it showed a greater ability to oxidize amines, suggesting that the reduction of the nitro group, producing a functional amine, should occur first, followed by the decrease of the stability of the aromatic ring resonance energy, the principal problem associated with aromatic compounds degradation.

Keywords: roxarsone; arsenic; biotransformation; soil; bacterial consortium

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P2.22

Detection and community dynamics of denitrifying bacteria in riparian buffer strips and agricultural soils

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Nitrate runoff from agro-ecosystems is a major environmental concern but soil

nitrogen management and nitrate reduction can be achieved through the exploitation of defined microbial activities within appropriate off-field set ups. Among these, purposely afforested buffer strips surrounding water streams can foster the reduction of nitrate loads through effective denitrification. In the present study we aimed at characterizing the variations of the denitrifyier community in relation to the hydrological regime and to compare them with those occurring in neighbouring agricultural soils. After a chemical soil characterization we assessed the denitrification potential and targeted the *nirK* nitrite reductase gene by DGGE analysis, band separation and sequencing. Factors affecting the phenomenon and the corresponding microbial guilds included organic carbon availability, soil depth and land use. In parallel RealTime PCR-based quantification protocols were optimized for the detection of *nirK* in soil bacterial communities. Standardized curves for the enumeration of gene copies per gram of soil were constructed using plasmid-cloned *nirK*. The dynamics of denitrifyiers fluctuation in the presence of known amounts of different organic fertilizer substrates was measured in potted soils over a two-months period. Results allowed to verify the quantitative PCR performance and to estimate the amount of soil-borne *nirK*-bearing genomes.

Keywords: buffer strips; denitrification; nitrite reductase; *nirK*

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P2.23

New insights into the relations between epiphytic bacteria in different vineyard microbial ecosystems

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Plants sustain a complex micro-ecosystem, which harbors a diverse array of

bacteria, able to colonize different plant organs and tissues, including roots, leaves, flower clusters, and fruits (Hallman et al., 1997; Berg et al., 2009). Despite its importance in plant health and crop quality (Compant et al., 2005), the diversity of epiphytic bacteria on grape berries and other plant parts, like leaves and bark, remains poorly described, as does the role of telluric bacteria in plant colonization. Few studies have reported the presence in soil of bacterial species associated with grape berries. For instance, some lactic and acetic acid bacteria species found in wine environments (Nisiotou et al., 2011) have also been detected in vineyard soil (Yanagida et al., 2008). However, no comparative studies of the structure of bacterial communities on grapevine parts and in soil had previously been conducted. In this study, we compare the bacterial community size and structure in vineyard soils, as well as on grapevine bark, leaves and berries. Analyses of culturable bacteria revealed differences in the size and structure of the populations in each ecosystem. The highest bacteria population counts and the greatest diversity of genera were found in soil samples, followed by bark, grapes and leaves. The identification of isolates revealed that some genera - *Pseudomonas*, *Curtobacterium*, and *Bacillus* - were present in all ecosystems, but in different amounts, while others were ecosystem-specific. About 50 % of the genera were common to soil and bark, but absent from leaves and grapes. The opposite was also observed: grape and leaf samples presented 50 % of genera in common that were absent from trunk and soil. The bacterial community structure analyzed by T-RFLP indicated similarities between the profiles of leaves and grapes, on the one hand, and bark and soil, on the other, reflecting the number of shared TRFs.
The interaction between bacterial populations in vineyard soil and the epiphytic bacteria present on the various parts of grapevines, suggests that part of the plant epiphytic population may have a telluric origin. In addition, the bacterial population of the vegetative (leaf) and reproductive (fruit) structures of the vine may also be affected by trunk bark. This first investigation is of particular importance, considering the role of bacteria in plant health and the fact that grape berries are the primary source of microbial communities that play a prominent role in the winemaking process and impact wine quality.

Keywords: epiphytic bacteria; grape berries; leaves; soil; bark

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P2.24

Characterization of the microbial diversity in the water fluxes of a wooded riparian strip set up for nitrogen removal

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This research is part of a project aimed at verifying the potential of a specifically assessed wooded riparian zone in removing excess of combined nitrogen from the Zero river flow for the reduction of nutrient input into Venice Lagoon. Seasonal fluctuations of microbial populations in the water entering and leaving the wooded riparian strip were determined for at least two years. Combined approaches involving cultivation, microscopic approaches and DNA bases techniques were adopted to characterize both culturable and total microbial community. Seven major bacterial lineages, namely Firmicutes, Gammaproteobacteria, Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Flavobacteria, and Sphingobacteria were present in water samples as revealed by 16S rDNA sequence analysis of the culturable fraction of the bacterial population. Gammaproteobacteria were the most dominant both in spring and fall, although distinct bacterial communities were clearly detectable for the two seasons.
However, while DGGE cluster analysis did not reveal significant differences between irrigation and drainage ditches, a significant alteration was detected by PCA based on 16S rDNA of the culturable fraction. Since the wooded riparian strip was vigorously working in terms of N removing by plant uptake and especially by microbial denitrification, as demonstrated by parallel studies performed on the same experimental site (Gumiero et al., 2011), it is reasonable to suppose that the culturable bacteria fraction is the one effectively carrying out the required task. In other words, the wooded riparian buffer zone specifically assessed for water remediation (nitrogen removal) is efficiently working as a result of the special conditions there produced to support the work of specific microbial populations. This is confirmed by the increase of metabolically active bacteria detected at the drainage ditches.
Taken together, the overall results provide key indications for the management of a phytoremediation site.

Keywords: riparian strip; water flux; nitrogen removal; bacterial diversity;

unculturable bacteria.

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P2.25

Biotransformation of roxarsone by bacterial community associated to agricultural soil

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Roxarsone is widely used as a feed additive in the production of broiler chickens. About 70% of broiler chickens are feed between 23 and 45 g roxarsone ton-1 for controlling coccidial intestinal parasite and sustain rapid growth. Most of the roxarsone is excreted untransformed in manure; however, several soil bacteria could degrade the roxarsone. Farmers commonly apply poultry litter as fertilizer. This practice, contributes considerable amounts arsenic to farm fields that can have polluting side effects. The current maximum contaminant level for As is 10 ugL-1, ingestion of As contaminated water above this level has been documented to cause cancer and many other health problems. The goal of this works was evaluate the biotransformation of roxarsone in different soil layers, in microcosm experiments. Four columns were implemented with agricultural soil and chicken manure supplement with Roxarsone (0,5mM) (CMROX); (A) sterile soil-CM-ROX sterile; (B) Soil-CMROX; (C) sterile soil-CM-ROX and (D) Soil-CMROX sterile. The columns were incubated at room temperature and dark, during 45 days. Water was periodically added. Bacterial community (aerobic (A) and anaerobic (B) layers) were characterized by means BioLog Ecoplate and PCR-DGGE. Biotransformation studies, of two layers, were performed by spectrophotometry. Toxicity was assessed for roxarsone and biotransformation of compounds generated by roxarsone by means Chromotest Toxi-Kit and HUVECs analyses. The results showed that in aerobic conditions, soil microbial communities of both layers, were able to transform roxarsone (100%). In anaerobic conditions, only layer B was able to transform roxarsone (52,32%). DGGE pattern showed differences in the bacterial communities composition of soil, in presence and absence of roxarsone. The metabolic profile of the microbial community, present in the layer A of all columns, showed significant differences in substrate utilization. Instead, the

community of layer B showed no significant difference between columns B and D. The increased carbon availability, mobilization of bacterial species present in the manure to the soil and the use of roxarsone as carbon source, may positively affect indices diversity and richness obtained by ecoplate Biolog. Moreover, it was observed that after 30 days, the richness it significantly reduces in several layers, which may be attributed to the presence of inorganic arsenic compounds, most toxic to microorganisms. Chromotest-Toxi results showed that roxarsone and column leaching control, showed low toxicity rates (7.5 and 11.4%, respectively) and leachate samples of the B, C and D columns, show a higher percentage of toxicity (36.9 to 74.2%). HUVECs assays showed that the percentage of cell viability decreased significantly compared to the control sample and exposed to 50 ul of B, C and D leachates. We can be concluded that the degradation of roxarsone and the leachate toxicity are directly related to bacterial community structure present in every soil layers.

Keywords: biotransformation; roxarsone; soil; bacterial-community

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P2.26

Different environmental applications of *Trichoderma* broth containing peptaibols

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Peptaibols are a group of small peptides characterized by short linear chain lengths (<20 residues), C-terminal alcohol residues, and high levels of non-standard amino acids, principally-aminoisobutyric acid (Aib), isovaline (Iva), and the imino acid hydroxyproline (Hyp). Naturally occurring peptaibols have been isolated from soil fungi mostly from the genus *Trichoderma* (teleomorph Hypocrea) that is a group of filamentous ubiquist fungi in soil that are capable of protecting crops from root-invading fungi, parasitizing mushrooms and other fungi, and in some instances being opportunistic human pathogens (Mukherjee et al., 2008; Verma et al., 2007). Thus, these fungi occupy important roles in agriculture, medicine as well as industry (Brotman et al., 2010; Schuster and Schmoll, 2010).
In this work was tested the activity of secondary metabolites produced from *Trichoderma harzianum* (strain T-22) and *Trichoderma asperellum* (strain B1),

tested against typic phytopathogen agents of crops (*Rhizoctonia solani*, *Pythium ultimum* and *Fusarium oxysporum*) and against fungi and bacteria colonies founded on the cultural heritage. In fact the bio-cleanig is a challenge and it is considered a promising alternative to synthetic pesticides. The antifungal and antimicrobial activities were performed by agar diffusion assay and the diameter of inhibition zones was measured and compared.
Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for screening, identification and elucidation of amino acid sequences of peptaibols. These results open interesting perspectives on the possibility of using derivatives of fungal for the formulation of new commercial products can protect the plants and the environment.

Keywords: *Trichoderma harzianum*; *Trichoderma asperellum*; peptaibols; biocontrol

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P2.27

Non-target effects of bioinoculants on rhizospheric microbial community structure of *Cajanus cajan*

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Studying the effect of application of bioinoculants on the indigenous microbial community is a pre-requisite for formulating an environmental friendly consortium. Therefore, a thorough risk assessment study should be performed before bringing any such biological agents to field application. We critically evaluated the non-target effects of bioinoculants in the rhizosphere of one of India's most important pulses, namely *Cajanus cajan*. We selected three bioinoculants, viz. *Bacillus* sp. (B), *Pseudomonas* sp. (P) and *Trichoderma* sp. (T),

for plant growth promotion of *C.cajan*. Both cultivation-dependent and -independent approaches were used to study their impact on microbial community structure in the plant's rhizosphere. Seeds treated with bioinoculants individually and in combinations were grown in pots under field conditions. Rhizospheric populations of phosphate solubilizing *Bacillus* sp., *Pseudomonas* sp., and fungal communities were enumerated on specific selective media. Fingerprinting of rhizospheric populations was done using automated ribosomal intergenic spacer analysis (ARISA). Specific rhizospheric communities of two of the most dominant bacterial groups, β-Proteobacteria and Actinobacteria, were quantified using qPCR. The best growth effect, in terms of dry mass (by 330%) and grain yield (by 238 %), was observed when *C. cajan* was inoculated with mixed consortium of the three bioinoculants (B+P+T). An increase in abundance of phosphate solubilizing *Bacillus* sp. (73%), *Pseudomonas* sp. (42%), and fungal population (53%) was observed at maturity stage of the plants, as compared to control plants. Actinomycetes and β-proteobacteria gene copies ranged from 4.1×10^{10} to 6.4×10^{12}, and 3.2×10^{7} to 7.0×10^{12} copies per gram dry soil, respectively. ARISA profiles showed distinct community profiles for various treatments, with samples of the same treatment at different sampling points clustering together. The study is the first to employ both cultivation-independent and dependent approaches to assess the non-target effects of traditional bioinoculants, as well as their non-conventional combinations, in rhizosphere of *Cajanus cajan*.

Keywords: *Pseudomonas*; *Bacillus*; *Trichoderma*; ARISA; qPCR

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P2.28

Deep desulfurization of hydrodesulfurized diesel and heavy crude oil by an isolated bacterium

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Sulfur emission due to fossil fuel combustion is a global problem. Biodesulfurization is an attractive alternative for the removal of sulfur from organosulfur compounds as it is environmentally safe. A number of microorganisms have been reported that can desulfurize dibenzothiophenes and benzothiophenes but only limited studies have been done on benzonaphthothiophene (BNT). BNT is a recalcitrant compound present in heavy crude oil and it persists in the heavy fractions of oil. We have isolated a novel

bacterium from petroleum contaminated soil that can desulfurize BNT. The bacterium was identified as Gordonia sp. by biochemical and molecular approaches. The biodesulfurization of BNT was found to follow 4S pathway, where BNT was first converted into BNT sulfone. The major metabolite was found to be a hydroxy b phenyl naphthalene. The reduction in sulfur content in heavy crude oil was also investigated and 76% reduction in the total sulfur content was observed. Similarly, when hydrodesulfurized diesel was used more than 90% reduction in the sulfur content was observed within four days. The present results suggest that the bacterium has a very high potential for deep desulfurization of diesel and other heavy oils.

Keywords: Benzonaphthothiophene; biodesulfurization; heavy crude oil

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P3.1

Understanding the adaptive growth and survival strategies of *Lactobacillus plantarum* during plant fermentation and storage

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Microbial growth and maintenance of high cell viability during the stationary phase, under challenging and hostile environmental conditions such as those characterizing plant-derived matrices, is one of the main aspects, which needs a deepest physiological explanation and has applicative repercussions. More than other food ecosystems, raw fruits and some vegetables possess intrinsic chemical and physical parameters, which make them as particularly hostile environments for microorganisms (Di Cagno et al., 2013). When environmental conditions are favorable, resources are primarily devoted to growth, whereas under nutrient limitation, most of the energy is invested on survival (Nyström, 2004). The metabolism is, therefore, redirected to alternative substrates and global responses are induced (Redon et al., 2005). This study aimed at investigating the growth and survival of several strains of *Lactobacillus plantarum* under hostile environmental conditions such as those characterizing vegetables and fruits. A panel of various metabolome approaches was used to describe the responses. Strains of *L. plantarum* were grown and stored in cherry (ChJ), pineapple (PJ), carrot (CJ) and tomato (TJ) juices, and in wheat flour hydrolysate (WFH) and whey milk (W), which mimicked the source of isolation. MRS broth was used as the control. Metabolome approaches were used to describe the responses. Data were elaborated through multidimensional statistical analyses. ChJ and PJ, which showed the most adverse chemical composition, negatively affected the kinetic of growth of *L. plantarum* strains. Viability during storage depended on media and source of isolation. Glucose and fructose were largely used during fermentation of CJ and TJ but the consumption was not found in ChJ and PJ. Malolactic fermentation was noticeable, especially during fermentation and storage of the most acidic juices (ChJ and PJ). A decrease of branched chain amino acids (BCCA) and His was found in almost all fermented juices. The synthesis of VOC depended on the strain behavior, which was related to the juice matrices. Pseudo-heatmap showed positive correlation between some aldehydes and alcohols. Diacetyl was the ketone found

at the highest level in all fermented juices. Pseudo-heatmap highlighted the correlation between several VFFA and Glu and γ-amino butyric acid, which were correlated to BCCA.

Keywords: Plant fermentation; metabolome approaches; metabolic responses

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P3.2

Functional genomic of *Lactobacillus rossiae* DSM 15814T

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Lactobacillus rossiae is an obligate heterofermentative lactic acid bacterium that is frequently isolated in sourdoughs, spelt flour, pineapple fruits and in the gastro-intestinal tract of animals and humans. Genotypic and phenotypic diversity of *L. rossiae* strains isolated from sourdough was described (Di Cagno et al., 2007). Some strains were selected for their antifungal activity (Valerio et al., 2009) and applied in sourdough biotechnology for glutamate production (Stromeck et al., 2011) and for wheat germ fermentation (Rizzello et al., 2010). Genomes of several lactobacilli species typically (e.g., *Lactobacillus brevis* and *Lactobacillus plantarum*) or solely (*Lactobacillus sanfranciscensis*) isolated from sourdoughs have been sequenced and annotated so far (Vogel et al., 2011). The genome sequence of the type strain of *L. rossiae* DSM 15814T (=CS1T=ATCC BAA-822T) will be useful to explore its biotechnological properties. A total of 30,544,098 whole-genome shotgun 100bp pair-end reads were generated by using Illumina sequencing technology. Library preparation was performed using TruSeq DNA-seq sample preparation protocol. Reads were assembled to a 2,9 Mb draft version (N50 of 150 kb) with CLC Genomics Workbench assembler. The annotation was done by merging the results obtained from the RAST (Rapid Annotation using Subsystem Technology) server and checked by BLAST analysis when needed. In addition, the scaffolds were searched against the KEGG, UniProt, and Cluster of Orthologous Groups (COG) databases to annotate the gene

descriptions. RAST genome annotation evidenced 2,722 predicted coding sequences (CDSs). Comparative genomic analysis indicates that the closest genome is that of *L. brevis* (Genome ID: 387344.13) and *L. plantarum* WCFS1 (Genome ID: 220668.1). There are many carbohydrate, amino acid and derivatives subsystem features, including genes involved in central carbohydrate, monosaccharide, and fermentation metabolism. There are also many protein and DNA metabolism subsystem features. The genome sequence provides new avenues to further explore gene-based functional and technological applications of *L. rossiae*. In addition, comparative genomics analysis and functional genomics analysis could also be carried out to trace the origin and evolution of *L. rossiae*.

Keywords: *Lactobacillus rossiae*; genome; fermented food and beverages

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P3.3

From raw ewes' milk to ripened Canestrato cheese: microbial community dynamics through high-throughput sequencing

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Canestrato Pugliese is a traditional cheese from the Apulia region, with a protected-designation-of-origin (PDO) status, manufactured from raw ewes' milk without starter cultures. The cheese derived its name and traditional shape from the rush basket "canestro", in which the curd is ripened (Di Cagno et al., 2003). This study was undertaken in order to gain insight into the microbiota that develops and evolves during the manufacture and ripening of Canestrato. Two batches of cheese were manufactured at industrial plant (Molino a Vento, Biccari, Foggia, Italy).

Canestrato cheese was ripened for 90 days at 11°C. Samples were taken after 0, 1, 3, 7, 15, 30, 45, 60, 75 and 90 days of ripening and analyzed in triplicate. The evolution of bacterial diversity from raw ewes' milk to ripened cheese was studied by culture-dependent approach and 16S rRNA gene pyrosequencing. The yeast biota was also investigated by 26S rRNA based PCR-DGGE followed by band sequencing. Both DNA and RNA extractions were carried out and pyrosequencing analyses were performed on cDNA. The evolution of the bacterial diversity (Shannon index of diversity, the substrate richness and the substrate Evenness) was also studied by the community level catabolic profiles by BIOLOG 96-well Eco-Microplates. Compositional analysis and proteolysis assessment were also determined. Pyrosequencing highlighted a large bacterial diversity (Alegría et al., 2012). Members of the most abundant bacterial genera in dairy products, e.g., *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, and *Enterococcus* were identified. However, other subdominant bacteria were identified. Insights on the source and dynamics of non starter lactic acid bacteria (NSLAB) during Canestrato cheese ripening were provided through high-throughput sequencing. NSLAB constitute the major factor that remains uncontrolled during cheese manufacture (Crow et al., 2001). The contribution of autochthonous NSLAB to the main biochemical events occurring during cheese ripening was highlighted.

Keywords: Canestrato cheese; pyrosequencing; microbial diversity; NSLAB

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P3.4

Antibiotic resistances: a competitive factor that allows *Enterococcus faecium* dominance in several environments

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The Antimicrobial resistance (AMR) is a serious threat to public health in Europe, leading to mounting healthcare costs, treatment failure, and deaths. The surveillance programmes indicate that there is a general Europe-wide increase of

antimicrobial resistance. Antibiotics are still extensively used in farm animals as therapeutic and the food chain is considered to be the most important vector for spread of resistance between man and animals.
In the case of *Enterococcus faecium*, a species which is a commensal of the gastro-intestinal tract of humans and other animals, is used as probiotics and in fermented foods and is responsible for hospital infections, the presence of antimicrobial resistance is considered a leading factor for human infections.
The wide use of antibiotics in the farm environments acts as selective pressure for gut associated bacteria and can influence the bacteria growing in fermented food of animal origin. Thus, the presence of one or more resistances to veterinary relevant antibiotics is a competitive factor to survive in the gastro-intestinal tract of treated animals and colonize the products thereof. In the framework of a study to detected antibiotic resistant *Enterococcus faecium* in ready to eat fermented foods, a multidrug resistant strain of *E. faecium* was isolated. The analysis of the antibiotic resistance profile of *E. faecium* UC7251 revealed that it is resistant to almost all antibiotic categories include β-lactamics, aminoglycosides, tetracycline and macrolides (erythromycin and clindamycin), when compared to the cut-off values defined by CLSI, EUCAST and EFSA. In order to deeper analyze this strain, a genomic approach was followed. The UC7251 genome sequence revealed the presence of several antibiotic resistance genes. We found two genes (*pbp5-R* and a *β-lactamase*) involved in ampicillin resistance; three genes (*aph3iiia*, *aad6* and *aadE*) encoding aminoglycosides resistance; *ermB* linked to macrolides resistance and two genes (*tetL* and *tetM*) for resistance to tetracycline. Further genome analyses underlined that *tetM* in carried by Tn916, a conjugative transposon. Mating experiments using as recipient strain *Enterococcus faecalis* OG1rf demonstrate the horizontal gene transfer of this mobile element in other strains of the same genus.
A phylogenetic analysis using MLST (Multi Locus Sequence Typing) on the *gdh*, *purK*, *pstS*, *atpA*, *gyd*, *adk*, *ddl* genes, and subsequent eBURST analysis grouped the multidrug resistant strain UC7251 with a clinical isolate, but not in the CC17, the clonal complex typical of the majority of clinical isolates.

Keywords: *Enterococcus faecium*; antibiotic resistance; genome sequence; food chain

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P3.5

Drawing the genotipic and metabolomic features of *Kluyveromyces marxianus* from Pecorino di Farindola cheese

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Pecorino di Farindola is a traditional variety of Italian cheese made from ewes' milk, and manufactured mostly in artisan plants according to local traditions, by using raw milk and pig rennet without the addition of natural or commercial starter cultures. Among its microbiota, yeasts range from 104 at the start of fermentation to 102 at the end of ripening. The majority of yeast isolates belong to the species *Kluyveromyces marxianus*. We studied 127 *K. marxianus* isolates from Pecorino di Farindola in comparison with the type strain and other isolates from Parmigiano Reggiano cheese and fermented milk. Growth kinetics in whey revealed that under anaerobic conditions the strains had different behaviours, although all of them resulted to be Crabtree negative strains. Differences were also observed for caseinolytic activity and metabolomic profiles. Genotypic typing was carried out on all the *K. marxianus* strains using RAPD-PCR fingerprinting that revealed a high genetic heterogeneity. Moreover, PFGE analysis showed a basic set of chromosomes by most of the *K. marxianus* strains ranging from five to seven chromosomes. The polymorphism observed for number and size of chromosomes was correlated with the strain origin. In particular, this genetic difference was associated to a biogeographical origin rather than to differences in the technological processes adopted. The degree of ploidy was evaluated by flow cytometric analysis. This research focused on the genetic and phenotypic intraspecies biodiversity of the dairy yeast *K. marxianus* provides useful data to increase the knowledge on the biology of this species and to understand its potential role in biotechnological applications.

Keywords: *Kluyveromyces marxianus*; biodiversity; dairy products

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P3.6

Bacterial invasion potential in water is determined by nutrient availability and the indigenous community

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In drinking water and the distribution systems, bacterial growth and biofilm formation has to be controlled for drinking water safety and taste, odour and piping problems. After a contamination with undesired bacteria, temperature, nutrient availability and predation will influence the survival of these invaders. The growth of invading bacteria in drinking water has always been a big concern and the influencing parameters are poorly understood. In this study, *Pseudomonas putida* – a model invader – was spiked in different water samples in order to examine the effect of different nutrient concentrations and the indigenous microbial community on the growth and survival of *P. putida*.
When spiking in drinking water, *P. putida* concentrations tripled within one day, but lowered soon again. When adding C as acetate, *P. putida* grew higher, but after two days, this concentration lowered again fast. C possibly promoted initial growth of *P. putida* but the survival time in drinking water was not elongated. Addition of only P and/or N indicated the importance of P for growth. After two days, the *P. putida* concentrations had risen very similar if either P or C was dosed. When adding combined C, N and P, *P. putida* grew for 5 days extensively and survived for a long period. Surprisingly, when a similar experiment was conducted in surface water, there was initial growth but after five days, no *P. putida* could be detected anymore. While nutrients were brought to the same level drinking water and surface water, the indigenous bacterial community stayed different. To examine this effect, indigenous bacterial communities of both samples were switched, resulting in drinking water harbouring surface water bacteria and vice versa. C, N and P were added and *P. putida* was spiked. In surface water with drinking water bacteria, an extended growth and survival was seen like before in the nutrient-enriched drinking water. In the drinking water with the surface water community, growth was more limited, although the survival time was also extensive. Previous authors already reported on antagonistic or protagonistic effects of indigenous bacteria on invaders. However, none could fully explain our results and a microbial community analysis with DGGE was performed. The higher richness in surface water could possibly lead to the higher invasion resistance, as stated by the diversity-invasibility hypothesis, however, the difference in richness was limited. Alternatively, a higher initial evenness can also result in a higher invasion resistance. This was in accordance with our results,

where surface water was shown to have a higher evenness than drinking water.
In conclusion, the antagonistic or protagonistic effect of indigenous water communities on invaders is hard to predict without testing the specific water. The invasion depends on the bacterial community structure and each water source has its own unique bacterial community. However, these experiments could confirm the combined importance of carbon and phosphorus. Extensive limitation of one of both can prevent the long-term growth or survival of an invader and therefore this can be a possible pathway for providing safe, biostable drinking water, not susceptible for invasion.

Keywords: Drinking water; invasion; pseudomonas

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P3.7

Dynamics of microbial ecology turning flours into mature sourdoughs for bread making

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Cereal fermentation using sourdough as natural leavening agent retained much attention in the last decade, largely because of the many advantages that sourdough offers over baker's yeast. Many studies have undoubtedly demonstrated the nutritional, sensory, texture and shelf-life advantages, which rely on the use of sourdough for making baked goods (De Vuyst et al., 2009). Mature sourdough is a necessary prerequisite as it confers distinctive characteristics, high sensory properties and shelf life to the resulting products (Gobbetti et al., 2005). The mature sourdough represents a very complex biological ecosystem where yeasts and, especially, lactic acid bacteria occur through consecutive refreshments. In this study, the evolution of bacterial diversity from flour to mature sourdough was studied by 16S rRNA gene pyrosequencing (Ercolini, 2013) and the yeast biota was investigated by 26S rRNA based PCR-DGGE followed by band sequencing. Rye and wheat (*Triticum durum* or *T. aestivum*) flours were used. Sourdoughs were daily propagated for eleven days, and samples were taken after 0 (dough), and 1, 2, 5 and 10 (sourdough) days of propagation. Both DNA and RNA extractions were carried out and the DGGE and pyrosequencing analyses were

performed on DNA and cDNA. Biochemical parameters were also determined during fermentation. DGGE analysis revealed that *Saccharomyces cerevisieae* was the dominant yeast in all the samples. Before fermentation, several bacterial phyla occurred in the dough, including *Proteobacteria*, *Cyanobacteria* and *Firmicutes*. Nevertheless, only *Firmicutes* dominated during late sourdough propagation as shown by both DNA and RNA analyses. The microbial species driving the fermentation were shown to be all occurring in the dough and none of them was detected only after a certain number of propagations. Before fermentation, *Weissella* sp. was already the dominant species of the rye dough, and it dominated during propagation. On the contrary, *Triticum* flours did not harbor an evident dominance of lactic acid bacteria, whereas *L. sakei* group, *Leuconostoc* sp. and *Weissella* sp. developed during propagation and contributed to the structure of the mature sourdough microbiota. Most of the dominant species were also clearly associated to the main metabolic activities taking place during propagation. In fact, statistical analyses showed correlation of the dominant lactic acid bacteria and decrease in pH, concentration of lactic and acetic acids and increase in free amino acids (FAA). Remarkably, some less abundant species such as *Pediococcus pentosaceus* and *Lactococcus lactis* also contributed to the fermentation as their occurrence was correlated with increase in FAA and acidification, respectively. The OTU network based on weighted UniFrac analysis provided a complete view of the fermentation dynamics: with increasing fermentation time the samples became more similar and the number of shared OTUs increased. The number of OTUs, which was detected before fermentation, decreases with refreshments. A better knowledge of the microbial diversity and dynamics of rye and wheat sourdoughs during propagation was provided with this study. Flours and doughs before fermentation are microbiologically complex ecosystems including different bacterial phyla. From this complexity, only very few lactic acid bacteria develop and drive the fermentation, together with other temporary sub-dominant species.

Keywords: Sourdough; food microbial ecology; microbial diversity; high-throughput sequencing

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P3.8

Artisan bakery or laboratory propagated sourdoughs: influence on the diversity of lactic acid bacterium and yeast microbiotas

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Traditional sourdoughs are characterized by continuous (daily) propagation (back-slopping) of the dough, using tap water and (non-sterile) flour. A microbial consortium mainly consisting of obligately and/or facultatively heterofermentative lactobacilli and yeasts usually dominates the mature sourdough (Gobbetti, 1998). Recently, a number of studies (Scheirlinck et al., 2009; Venturi et al., 2012; Vogelmann and Hertel, 2011; Vrancken et al., 2011) have considered the influence of environmental microbiota, its metabolic activity, bakery environment and specific technology parameters on the microbial diversity and stability of traditional sourdoughs. Nevertheless, some questions are still debated. When mature, is the sourdough microbiota always stable? Further, when all the other parameters are kept constant, is the production environment or the type of flour the major source of microorganisms? This study aimed at showing the influence of the environment of propagation on the diversity of the lactic acid bacterium and yeast microbiotas of seven sourdoughs, which were used for the manufacture of traditional Italian leavened baked goods. Sourdoughs were propagated at artisan bakery or at laboratory using the same batch of flour and applying the same technology parameters. Dominating lactic acid bacteria and yeasts were monitored over time (80 days) through culture-independent and -dependent methods. Multivariate statistical analyses were used to find correlation among the composition of the sourdough microbiota, the biochemical characteristics of sourdoughs, and the environment of propagation. The cell density of presumptive lactic acid bacteria and related biochemical features (e.g., pH, total titratable acidity, and concentration of organic acids) were not affected by the environment of propagation. On the contrary, the number of yeasts and the concentration of related metabolites (e.g., ethanol) markedly decreased from artisan bakery to laboratory propagation. During late laboratory propagation, Denaturing Gradient Gel Electrophoresis (DGGE) showed that the DNA band corresponding to *Saccharomyces cerevisiae* was not more detectable in several sourdoughs. Twelve species of lactic acid bacteria were variously identified through a culture-dependent approach. All sourdoughs harbored a certain number of species and strains, which were dominant throughout time and, in several cases, varied depending on the environment of propagation. As shown by statistical permutation

analysis, the lactic acid bacteria populations differed between sourdoughs propagated at artisan bakeries and laboratory levels. *Lactobacillus plantarum*, *Lactobacillus sakei* and *Weissella cibaria* only dominated in some sourdoughs back-slopped at artisan bakeries, and *Leuconostoc citreum* seemed to be more persistent under laboratory conditions. Strains of *Lactobacillus sanfranciscensis* were indifferently found in some sourdoughs. Together with the other stable species and strains, other lactic acid bacteria temporarily contaminated the sourdoughs and largely differed between artisan bakery and laboratory levels. A better knowledge of the parameters that lead to variations of the bacterial sourdough microbiota during the back-slopping should allow better control of industrial processes and standardization of high-quality baked goods. This study showed that the environment of propagation has an undoubted influence on the composition of sourdough yeast and lactic acid bacterium microbiotas.

Keywords: traditional sourdough; back-slopping; environment; lactic acid bacteria; yeasts

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P3.9

Determining the growth potential of foodborne pathogens as affected by indigenous microflora in the finished composts

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Introduction: Composting of animal wastes is not only recycling the nutrients for plant growth but also inactivating manure-borne human pathogens. However, the finished compost may still pose the microbiological risk due to the growth potential of a few pathogenic cells which either have survived the active composting or are cross-contaminated from the compost surface or environment

(Russ and Yanko, 1981; Kim et al, 2009). Although the composting operators or producers rely on the microbiological test results on fecal coliforms, *Escherichia coli*, *Salmonella*, *E. coli* O157:H7, and maturity or stability tests to determine if the compost can be released to end-users, there are no valid scientific data to link these tests with the potential for pathogen regrowth in the finished compost. Therefore, there is a need to fully understand the factors contributing to the growth of human pathogens in the finished compost.

Objectives: The objectives of this study were to analyze a variety of compost samples for their physicochemical and microbiological properties, and factors affecting the growth potential of foodborne pathogens in compost products.

Materials and Methods: The finished compost samples were collected from several states across the United States. The physico-chemical properties such as pH, aw, C:N ratio, organic matter, Solvita CO_2 and NH_3 test, and microbiological properties including mesophiles, thermophiles, actinomycetes, fecal coliforms, fungi, *E. coli* O157:H7 and *Salmonella* were tested. The growth potential of ca. 3 log CFU/g of 3-strain mixture of *E. coli* O157:H7 or Salmonella spp. was determined in these samples. The community-level physiological profiling (CLPP) of indigenous microflora of these compost samples were assayed with the Biolog EcoPlate™ method, and the data were analyzed via Principal Components Analysis (PCA) (Weber et al., 2007).

Results: A total of 31 compost samples were analyzed in this study. The populations of *E. coli* O157:H7 and *Salmonella* increased ca. 0.1 ~ 0.8 and 0.2 ~ 0.6 log CFU/g within 3 days at 22ºC in 8 compost samples, respectively. Those composts supporting pathogen growth had the pH of 8.7 ~ 9.2, moisture content of 39 ~ 43%, water activity of 0.98 ~ 0.99, low organic matter of 17.8 ~ 20.6% as compared with 27.2 ~ 58.7% in other samples, and low number of fungi (2.6 ~ 3.3 vs. 4.9 ~ 7.0 log CFU/g). Different compost samples were clustered into four groups based on the utilization of 31 carbon sources in the Biolog Ecoplates by compost microbial community. To further investigate if pathogen regrowth is translated in any changes in metabolic profiling, the principal component analysis was performed using the Biolog data from the samples supporting pathogen growth along with other two compost samples negative for pathogen growth. Interestingly, the compost samples that supported regrowth, esp. those plant-based composts were grouped together using PCA analysis.

Conclusion: Our results suggested that certain types of compost may have the potential for supporting pathogen growth due to nutrients, types and levels of indigenous microorganisms, although all these composts met the microbiological criteria and maturity of the finished compost.

Keywords: compost; indigenous microflora; *Escherichia coli* O157:H7; *Salmonella* spp.; growth potential

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P3.10

Selfish and cooperative strategies, two energetic behaviors driven by environmental constraints

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Natural habitats of some microorganisms may fluctuate erratically, whereas others, which are more predictable, offer the opportunity to prepare in advance for the next environmental change. In this context, microorganisms may have evolved the bioenergetics machinery to anticipate environmental fluctuations by adapting to their temporal order of appearance. Food matrixes represent an example of 'predictable' fluctuating environments, generated by anthropic activities, able to drive the speciation of several microorganisms. The nutrient richness, and specifically the abundance of mono- and disaccharides that characterize several food matrixes, such as milk and grape juice, have allowed the speciation of lactic acid bacteria and yeasts with a high fermentation capacity instead of the energetically favorable respiration metabolism. In these environmental contexts, fast sugar consumption, lactic acid or ethanol production, accumulation and tolerance, together with the ability to propagate in the absence of oxygen are some of the 'winning' traits, and have apparently evolved and became specialized to perfection in these fermenting microorganisms. Here we will focus the attention on the evolutionary forces that allow the selection between a selfish and a cooperative energetic behavior (Pfeiffer et al., 2001) in food-associated microorganisms. The analysis of the energetic behaviors will be carried out taking always in consideration the environmental context where microorganisms evolves and discussing the hypothesis of a metabolic adaptive prediction (Mitchell et al., 2009) towards environmental changes that happen during the microbial growth in a food matrix.

Keywords: bioenergetics; food adapted microorganisms; evolution; metabolic adaptive prediction

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P3.11

Characterization and identification of bacteria isolated from 'Hurma' olives grown in Karaburun peninsula

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Olive is an important agricultural product for Turkey and an indispensable component of the Mediterranean diet. Olive has a reputation to prevent certain kind of cardiovascular diseases and some kind of cancers owing to be rich in phenolic compounds especially oleuropein. In this respect, increasing interest to the Mediterranean diet is lead to be consumed natural food products such as olive and olive oil. Turkey is an important table olive producing country with diverse olive varieties, most of them are consumed in Turkey without exportation. Among these varieties, 'Hurma' olives grown mainly in Karaburun Peninsula are differ from other varieties that maturates on the tree losingly its bitterness caused by phenolic compounds especially oleuropein. Thus, they can be directly consumed when they are harvested and they do not require to undergone debittering process to make them edible by removing their bitterness components. It was stated in limited literature that the debittering phenomena during maturation period occurs by a fungus called Phoma olea that hydrolyses oleuropein (Panagou, 2006). Besides, in small number of studies stated that similar olive varieties were grown in Greece and Tunisia (Jemai et al., 2009; Panagou, 2006). To the best of our knowledge there is no any study about microbiological characterization of Hurma olives grown in Turkey.

In this study bacterial growth and colonization and change in olive bacterial microflora during the period of between the start of debittering to full ripeness (maturation period) which lasted about eight weeks between October-December were investigated in the crop seasons of 2011 and 2012. The media used for isolation and enumeration were Plate Count Agar (PCA) for total viable count, Lactobacilli MRS agar (MRS), Violet Red Bile Glucose Agar (VRBGA) for Enterobacteriaceae, Baird Parker Agar (BPA) supplemented with egg yolk tellurite emulsion for staphylococci and micrococci, Pseudomonas Agar base (PSA) supplemented with SR 0102 E. As a result of counting lactobacilli, pseudomonads, staphylococci and micrococci were not detected in Hurma olive samples during maturation period. Therefore bacterial cultures were isolated from PCA and

VRBGA. In 2011, bacterial microflora comprised of Gram negative rods (60.9%), Gram negative cocci (29.2%), Gram positive cocci (7.3%) and Gram positive rods (2.43%) while in 2012, the bacterial groups composed of Gram negative rods (78%), Gram positive rods (14%) and Gram negative cocci (29.2%). Most of the isolates have 16S rDNA sequences closely related to Enterobacteriaceae family.

Keywords: Hurma olive; bacteria; characterization; identification; 16S rDNA

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P3.12

Shelves microbiota involved in the Orange-Red coloration of Fontina cheese: investigation by 454 high-throughput pyrosequencing

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Fontina PDO is a semi-cooked cheese produced from full cream, raw cow's milk from a single milking, which is fermented with selected autochthonous *Streptococcus thermophilus*, *Lactococcus lactis* and *Lactobacillus delbrueckii* strains. A defect that could occur during cheese ripening is a spoil-coloration of the under-rind cheese section from light-yellow (LY) to orange-red (OR) color. This coloration could be generated from caretonoids and other pigments produced from ripening bacteria (Galaup et al. 2007) but also from bacteria present in the store environment like shelves. The aim of the present study was to characterize the microbial composition of cheeses and shelves by pyrosequencing of 16S rRNA gene amplicons; therefore, in the current study, bacterial communities of 16 Fontina cheeses after 3 months of ripening and respective storing shelves were analyzed with 454 barcoded pyrosequencing. Cheeses were sampled both under the rind and in the core section. Shelves are by red larch wood and were sampled onto the surface in contact with the respective cheese. After analyzing 570,247 bacterial pyrosequences, we found that almost all the cheese bacteria were members of the phylum *Firmicutes* both in OR and LY colored cheese (95% and 99%

respectively), with only a small portion belonging to *Proteobacteria* (3.6% and 0.7% respectively). The bacteria community isolated from shelves showed higher biodiversity: the bacteria phylum more represented were: *Actinobacteria* found both in shelves used for OR and LY colored cheeses (50.1% and 45.8% respectively), *Bacteroidetes* (31.7% and 31.2% respectively) *Proteobacteria* (11% and 16.6% respectively) and *Firmuctes* (6.2% and 5.5% respectively). Among the 319 identified bacterial genus, the ones belonging to *Streptococcaceae* were the dominant both in Core and Under-Rind cheese section (72.4% and 80.8% respectively) and this was expected considering that two strains of *Streptococcus thermophilus* and *Lactococcus lactis* are commonly used as starter in Fontina production. In shelves samples, Flavobacteriaceae and Corynebacteriaceae were dominant bacterial families but if Flavobacteriaceae were similar both in shelves used for OR and LY colored cheese (26.6% and 26.3% respectively), *Corynebacteriaceae* showed different percent of presence (28.1% and 19.9% respectively). Community comparison analysis of cheeses suggested that samples collected from OR and LY colored cheese exhibited a similar bacterial composition, cause of the starter that is in huge proportion if compared to other bacterial Families. The differences of bacterial community between OR and LY colored cheeses were more clearly represented by pyrosequencing analysis of shelves microflora. When compared by the cheese coloration (OR and LY), the shelves overall bacterial population showed statistical significant differences and in particular *Actinobacteria* and *Corynebacteriaceae* families were present in higher percent in shelves of OR than LY colored Fontina cheeses.

Keywords: Fontina cheese; bacteria; orange-red color; 454 pyrosequencing

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P3.13

Yeast community structure in grape juices from two major PDO wine producing regions in Greece

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Indigenous yeast community structure in two major PDO regions in Greece, Peza and Nemea, was surveyed. Strains were isolated from spontaneously fermented 'Vilana', 'Mandilaria' and 'Kotsifali' grape musts, cultivated in 16 vineyards in Peza, Crete, and 'Agiorgitiko' samples collected from 11 vineyards in Nemea, Peloponnese.

Species diversity, as assessed by restriction fragment length polymorphism combined with sequence analyses of the 5.8S-ITS rDNA region , revealed a relatively high level of species heterogeneity in the two regions. A total of 2219 isolates were assigned to the species *Candida incommunis, C. zemplinina, Issatchenkia orientalis, Hanseniaspora uvarum, H. guilliermondii, H. osmophila, Lachancea thermotolerans, Metschnikowia pulcherrima, Meyerozyma caribbica, Pichia anomala, Pichia* sp., *Saccharomyces cerevisiae* and *Torulaspora delbruecki*. *S. cerevisiae* was scarce on grapes, since it and was only encountered in two vineyards from Nemea (7.5% of total samples), at populations ranging from 2-4 log10cfu/ml. As the fermentation proceeded, *S. cerevisiae* became the dominant population in most cases and could be recovered from 91% of the samples at levels of 7-8 log10cfu/ml. Non-*Saccharomyces* yeasts were encountered on grapes at populations from 2.2 to 6.2 $\log_{10}$cfu/ml (average of 5.7 $\log_{10}$cfu/ml) and 2-7.8 $\log_{10}$cfu/ml (average of 5.2 $\log_{10}$cfu/ml) in samples from Nemea and Peza, respectively. In 15% of total samples, non-*Saccharomyces* yeasts were below the detection limit. At the final stages of fermentation, about 75% of the samples harbored wild yeast populations, at 2-7 $\log_{10}$cfu/ml. These populations may possess potential enological importance, as they could survive the elevated ethanol concentrations encounter at these late stages. *C. zemplinina, I. orientalis, L. thermotolerans, M. pulcherrima, H. uvarum* and *P. anomala* were the most abundant species within the samples from Peza, while other species, like *M. caribbica* and *Pichia* sp., were also present, but at low frequencies. The same species also dominated the Nemea samples, except from *M. pulcherrima* and *P. anomala* that were replaced by *H. guilliermondii*. *M. pulcherrima* along with *H. osmophila. T. delbrueckii* and *C. incommunis* were also recovered, albeit from fewer samples and at low frequencies. Importantly, *C. zemplinina*, *I. orientalis*, and *L. thermotolerans*, alone or in coexistence, were identified to predominate and complete fermentation in about 10 % of the Peza samples, while *L. thermotolerans* in about 3% of the Nemea samples. Among them, *L. thermotolerans* and *C. zemplinina* were able to yield considerable amounts of ethanol (at least 12 % vol). This is the first comprehensive study on the diversity of autochthonous yeasts in two of the most important viticultural areas in Greece.

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Keywords: alcoholic fermentation; yeast species diversity

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P3.14

Integrated approach based on 16S rDNA PCR-DGGE and hsp60 qPCR assay for monitoring dynamics of a *L. rhamnosus* probiotic strain in table olive fermentation

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Recently, vegetarian fermented foods have reached an increasing attention as working base alternative to milk-based foods for the development of probiotic-type functional foods. Table olives have been demonstrated an excellent food carrier suitable for delivering probiotics to humans (Lavermicocca et al., 2005; Abriouel et al., 2011). Management of fermentation for developing probiotic-carrier table olives is critical since the use of a different starter strain, in addition to probiotic one, could hamper probiotic's multiplication and survival by depletion of nutrients in brine. The use of probiotic strains as starter to pilot the fermentation can overcome this drawback [3]. Alternatively, probiotic strains could be added during fermentation process. Here, we developed an integrated approach based on 16S rDNA PCR-DGGE profiling and a *L. rhamnosus*-specific qPCR assay to detect the dominant species in Sicilian table olives and to monitor the implantation of the *L. rhamnosus* probiotic H25 strain, inoculated both in presence and in absence of a indigenous *L. plantarum* starter. The amount of H25 strain was compared to that of LGG strain.
Based on PCR-DGGE profiling, few microbial shifts throughout fermentation time were revealed in brine samples, while many different dominant bands among samples were achieved, indicating that both starter and probiotic inoculums could influence brine microbiota. In particular, DGGE results of brine samples revealed that samples without *L. plantarum* starter exhibited a high biodiversity with more pronounced dominant bands, including the dominance of the *L. rhamnosus* probiotic strain H25 until the end of fermentation. Brine samples inoculated with probiotic LGG strain showed the presence of few dominant bands and a weak presence of the probiotic strain up to the end of the process. DGGE results of olive samples revealed a marked microbial shifts during fermentation time and a higher biodiversity in samples without *L. plantarum* inoculum. Nevertheless, the presence of H25 and LGG probiotic strains was not detected.
In order to species specifically quantify *L. rhamnosus*, a SYBR Green qPCR assay was designed with a primer set targeting chaperonin hsp60 gene sequence. qPCR

assay was effective in detecting the *L. rhamnosus* target DNA. *L. rhamnosus* genome copy number shifted from 6.13 (±0.04) to 6.40 (±0.01) log_{10}/ml in brine sample with starter, after 60 days of inoculum with *L. rhamnosus* probiotic H25 and from 6.72 (±0.03) to 7.86 (±0.02) log_{10}/ml in brine samples without *L. plantarum* starter. Similarly, LGG genome copy number was from 6.54 (±0.03) to 7.53 (±0.02) log_{10}/ml after 60 days of inoculum in brine samples without starter. Evaluating olive samples, H25 *L. rhamnosus* genome copy number was registered at 2.91±0.04 and at 3.99±0.05 log_{10}/g after 60 days of inoculum in both samples with and without *L. plantarum* starter, respectively. These values were slightly higher than those found for LGG (3.07±0.03 log_{10}/g).
In conclusion, the integrated approach based 16S rRNA gene PCR-DGGE and hsp60 qPCR, was effective in monitoring implantation of a *L. rhamnosus* probiotic strain on microbial population inhabiting olives and brines. Preliminary results also suggest that olives could support the survival of *L. rhamnosus* probiotic H25 strain.

Keywords: qPCR; probiotic; table olive; *Lactobacillus rhamnosus*; PCR-DGGE

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P3.15

Gram-negative bacteria have an impact on bacterial diversity, biogenic amine content and sensory characteristics of an experimental smear soft cheese

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The impact of Gram-negative bacteria on sanitary and organoleptic qualities of an experimental soft smeared cheese (Munster type) was investigated in the presence of a defined surface starter cultures. Two strains, *Hafnia paralvei 920* and *Psychrobacter celer 91*, were added either each alone at 10^6 cfu/g of cheese or together in a ratio of $10^6/10^6$ or $10^6/10^4$ cfu/g to a suitable surface starter cultures composed of six bacteria and four yeasts. *H. alvei* and *P. celer* grew well in the

model reaching 8 or 9 log10 cfu/g at maximum respectively. Addition of *P. celer* and/or *H. paralvei* had no effect on yeast growth but had an effect on bacterial strains. It inhibit the growth of *A. arilaitensis, S. xylosus* and *S. equorum* at variable stage of ripening but not that of *C. flavescens* and *C. casei*. Both Gram-negative bacteria were active in different manners in modifying cheese quality when compared to control. Contrarily to *P. celer, H. paralvei* highly enhanced cadaverine and putrescine concentrations in cheese compared to control. *H. paralvei* enhanced also total volatile compounds production more than did *P. celer*. Sensory score (odor) was only slightly enhanced with any of the added Gram-negative bacteria. The co-addition of *P. celer* and *H. alvei* had a strong stimulating effect on cadaverine and putrescine production and also, to a lesser extent, on odor scores. These results reinforce the contribution of Gram-negative bacteria to the biogenic amine content and sensory characteristics of smear soft cheese.

Keywords: Smear cheese; Gram-negative bacteria; *Psychrobacter*; *Hafnia*; biodiversity; biogenic amine; sensory characteristics

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P3.16

Yeast microbial ecology, aroma profiles and safety features of wines from organic or biodynamic grapes in relation to starter addition

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The fermentation of grape juice into wine is a complex microbial reaction traditionally involving the sequential development of various species of yeasts, lactic acid bacteria.
Traditionally, wines have been produced by natural fermentations caused by the development of yeasts and lactic acid bacteria originating from the grapes and

winery equipment. Natural fermentations continue to be the main style of fermentation conducted in some countries although active dry yeasts are sometimes used in specific conditions when they are able to improve the fermentation process (Fleet et al., 1984; Rodríguez-Lerma et al., 2011). Wine quality is closely related to the microbial ecology of grape and fermentation as well as to the used of selected strains to guide the winemaking. In fact, wine aroma is the outcome of a complex of interactions among the substances from the grapes and those produced during fermentation and aging (Vernocchi et al., 2011). The importance of microbial species that develop during fermentation determine the types and concentrations of many substances that may contribute to the good fermentation, aroma and flavor characteristics, and also the safety of wine.
The main goal of this study was to characterize the yeast populations of musts from grapes produced from biodynamic or organic agriculture and monitoring their evolution during spontaneous and guided fermentations. Moreover, the physico-chemical and safety features as well as the volatile molecule profiles of wine in relation to grape production practices and to starter addition were investigated.
The physic-chemical characterizations of samples were performed on according to the Official EU Methods (EC, 2000). The yeast viable cell counts were performed by plate count methods by using selective growth media while the identification and typing of yeasts were performed by MALDI-TOF-MS Biotyper, which employed as analytical and typing expression profiling of yeast, yeast-like species and strain variants in order to achieve a microbial population characterization. The volatile molecule profiles were investigated by GC-MS solid phase microexctraction (SPME) (Vernocchi et al., 2011). Moreover, the survey on biogenic amine (BA) and ethyl carbamate (EC) concentrations of wines was conducted in according to Patrignani et al. (2012).
The results of MALDI-TOF-MS Biotyper showed significant differences in the yeast populations in the musts in relation to the grapes used (from organic and biodynamic agriculture) and starter addition. Moreover the MALDI-TOF-MS typing evidenced significant differences also at strain level.
The wine volatilome showed specific volatile molecule fingerprinting in relation to grape cultivation and winemaking (guided or spontaneous fermentations) and the presence in the wines obtained from spontaneous fermentations of volatile phenols having very low perception threshold. Regarding biogenic amines, histamine, spermine, spermidine and 2-phenylethylamine were never detected while tyramine, cadaverine, putrescine were present at very low concentrations in all the samples. Agmatine was the most abundant amine detected. However, the highest contents of total biogenic amines were detected in samples produced by guided fermentation suggesting that starter selection criteria should include also this feature.

Keywords: wine yeasts; wine volatile molecule profiles; wine safety features; organic and biodynamic grapes; yeast ecology

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P3.17

Exploring the sources of bacterial spoilers in beefsteaks by culture-independent high-throughput sequencing

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Microbial growth on meat to unacceptable levels contributes significantly to change meat structure, color and flavor and to determine meat spoilage (Gram et al., 2002). The types of microorganisms initially present in meat depend on several factors and multiple sources of contamination can be identified. Carcass contamination takes place during slaughtering by the animal endogenous microbiota, but also microbial contamination of tools and surfaces can contribute to the initial microbial load on carcasses (Yalcin et al., 2001). In addition, subsequent handling of meat in the operations of sectioning and portioning can determine further contamination (Aslam et al., 2004). All the above routes will potentially contribute to the initial structure of the microbiota of meat before the storage starts, but only a fraction of the initial population will grow and determine spoilage depending on the storage conditions (Nychas et al., 2008; Doulgeraki et al., 2012).
The aims of this study were to evaluate the microbial diversity in beefsteaks before and after aerobic storage at 4°C and to investigate the sources of microbial contamination, in order to ascertain if the beef steak microbiota originate from the carcass or if the environment where meat is handled and portioned has a resident microbiota that can contribute to contamination. Two separate carcass samplings in two different slaughterhouses were performed. Carcass swabs were taken directly at the slaughterhouses. The half carcass was followed during the production line and steaks from the beef cuts previously sampled were collected at the butchery after portioning. The steaks were analyzed at time zero and after one week of

refrigerated aerobic storage. Moreover, environmental swabs were collected at the butcher's shop. All samples were analyzed by culture-independent high-throughput sequencing of 16S rRNA gene amplicons.
The microbiota of carcass swabs was very complex, including more than 600 operational taxonomic units (OTUs) belonging to 15 different phyla. A significant association was found between beef microbiota and specific beef cuts ($P<0.01$) indicating that different cuts of the same carcass can influence the microbial contamination of beef. Despite the initially high complexity of the carcass microbiota, the steaks after aerobic storage at 4°C showed a dramatic decrease in microbial complexity. *Pseudomonas* sp. and *Brochothrix thermosphacta* were the main contaminants, and *Acinetobacter*, *Psychrobacter* and *Enterobacteriaceae* were also found. Comparing the relative abundance of OTUs in the different samples, it was shown that abundant OTUs in beefsteaks after storage occurred in the corresponding carcass. However, the abundance of these same OTUs clearly increased in environmental samples taken in the processing plant suggesting that spoilage-associated microbial species originate from carcasses, they are carried to the processing environment where the meat is handled and there they become a resident microbiota. Such microbiota is then further spread on meat when it is handled and it represents the starting microbial association wherefrom the most efficiently growing microbial species take over during storage and can cause spoilage.
An in depth analysis of meat microbiota along the meat chain can reveal sources and routes of meat contamination and can be useful to plan adequate measures for improving quality during meat storage.

Keywords: beef spoilage; meat contamination sources; beef carcass contamination; 16S rRNA pyrosequencing

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P3.18

Transformation and lateral transfer of plasmids in wild strains of *Escherichia coli*

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Lateral gene transfer between bacterial cells contributes to bacterial adaptation to various environments. In human environments, however, it results in the undesirable spread of pathogenic, antibiotic resistance, or artificially engineered genes. *Escherichia coli* is not assumed to be naturally transformable; it develops high genetic competence only under artificial conditions, e.g. exposure to high Ca^{2+} concentrations. However, several reports have shown that *E. coli* can express modest genetic competence in certain conditions that can arise in its environment (Baur et al., 1996; Baur et al., 1999; Maeda et al., 2003). These studies used only laboratory strains.
In this study, we sought to examine the ability of wild *E. coli* in transformation and lateral transfer of plasmids. We used 17 strains of ECOR (*Escherichia coli* collection of reference), which is a standard collection of wild *E. coli* strains. Natural transformation experiments revealed that several ECOR strains showed higher transformability compared with laboratory strains. Using a co-culture system of ECOR strains, we also found that lateral plasmid transfer occurred by a DNase-sensitive mechanism, suggesting the occurrence of transformation. These results suggest that wild *E. coli* strains differ from laboratory strains in their transformability.
(This work was supported by JSPS KAKENHI Grant Number 25292051)

Keywords: transformation; *Escherichia coli*; plasmid

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P3.19

Microbial dynamics and competition among lactic acid flora and foodborne pathogens in an Italian raw milk cheese

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Listeria monocytogenes is one of the most important foodborne pathogens because of its high fatality rates and its ability to survive in foods under stressful conditions (Warriner and Namvar, 2009). *Staphylococcus aureus* is considered one of the most important pathogen in foodborne intoxication (Le Loir et al., 2003). It's recognized that different strains of lactic acid bacteria (LAB) associated with food systems are capable of producing bacteriocins. Aim of this study was to evaluate the effect of LAB strains on the behavior of *L. monocytogenes* and *S. aureus* in the production of Fontina, an Italian PDO raw milk cheese produced in the Valle d'Aosta Region.
Two strains of *L. monocytogenes* and of *S. aureus* were used to contaminate 80-90 liter of milk produced in the Valle d'Aosta Region. Milk arrived at the experimental dairy plant of Istituto Zooprofilattico Sperimentale of Piemonte, Liguria and the Valle d'Aosta (IZSPLV) and six batches for each pathogen were set up. Raw milk was contaminated obtaining almost 3-4 Log CFU/ml for *L. monocytogenes* and 5-6 Log CFU/ml for *S. aureus*. Specific LAB starter cultures (*Streptococcus thermophilus*, *Lactococcus lactis*, *Lactobacillus delbrueckii lactis*) were used. Powder rennet was added and after curdling, the curd was cut and cooked raising the temperature up to 48°C. Curd was transferred in 40 cm diameter and 8 cm height mold and mechanically pressed for 8 hours. The cheese was immersed in 22-23% brine for 10 hours. Then it was transferred into a ripening room (+10°C, Relative Humidity 90%) where it stayed for 80 days. During milk processing and at 7, 14, 21, 28, 56 and 80 days of ripening the following analysis were performed: count of *L. monocytogenes* and *S. aureus* (in triplicate), mesophilic Lactococci, thermophilic Lactococci, mesophilic Lactobacilli; determination of pH and Aw.
The count of *L. monocytogenes* was about 3.0 Log CFU/ml in the contaminated

milk and reached 4.9 Log after brining and about 4.5 Log at the end of ripening. After brining, the LAB flora increased more than 8.0 Log CFU/g and remained constant till 80 days of ripening. The pH decrease, reaching a value of 5.4 at the end; the Aw was 0.98 until 14 days and then decreased up to 0.92 at 80 days. *S. aureus* decreased slightly from 5.5 to 5.1 Log CFU/ml after curdling, then increased until 6.6 after brining and decreased up to 3.6 Log at the end of ripening. The LAB flora increased, reaching more than 8.0 Log CFU/g. The pH was 5.6 at 7 days and remained almost stable at 80 days; the Aw was 0.98 in the first phases and decreased until 0.93 at the end.
The significant growth of LAB during first phases determined an inhibition effect against *L. monocytogenes* and *S. aureus*. Ripening temperature, the rapid reduction of pH and the competition due to LAB, that remained constantly high till the end of ripening, were the major factors implicated in the stop growing of two pathogens.

Keywords: Competition; lactic acid bacteria; *Listeria monocytogenes*; *Staphylococcus aureus*

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P3.20

High throughput DNA sequencing to profile microbial communities in high and low biogenic amine Cheddar-type cheeses

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Biogenic amines (BA) are low molecular weight compounds produced via microbial decarboxylation reactions and are often found in fermented food products, including cheese. Histamine, at levels as low as 20 mg/kg of cheese, are sufficient to produce a toxic effect in certain individuals. The objective of this novel study was to profile the microbial composition of cheeses with high and low levels of BA using 454 GS-FLX high throughput sequencing of 16S rRNA

amplicons. Results show a significant difference in species diversity between cheeses with high levels of BA and those with low or no detectable BA. Cheeses with high BA showed considerably greater diversity and contained *Streptococcus* (29%), *Lactobacillus* (19%), *Staphylococcus* (36%), *Thermus* (13%), *Vibrio* and *Psychrobacter* (1% each). Cheeses with low levels of BA had high levels of *Streptococcus* (94%) and *Lactobacillus* (5%) only. On the basis of these results it is proposed that variations in the activity/amount of the starter inoculum, as well as in levels of pH, salt and temperature, may allow spoilage bacteria that are usually competitively inhibited to grow to levels such that BA formation may occur.

Keywords: DNA sequencing; biogenic amines; cheese

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P3.21

Process and microbial diversity of French traditional organic sourdough and sourdough breadmaking

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Sourdough still represents an attractive image and method for breadmaking process in France despite its technological constraints. However, bakers practices - from wheat culture to bread - and their effects on bread quality are not enough documented. Moreover, sourdough yeast and lactic acid bacteria (LAB) constitute a reservoir for inter-specific and intra-specific diversity studies (Scheirlinck et al., 2007; Pocozzi et al., 2010) The aim of this project is (i) to investigate the diversity of the traditional SD breadmaking practices (ii) to study the SD microbial dynamic and diversity during SD bread-making and (iii) to examine the microbial

community at few months apart. This study is carried on with a network of 5 French traditional organic bakers or farmers-bakers.
To characterize the microbial dynamics during the bread making process, sampling were performed at 4 steps: chief sourdough, final leavened dough, dough after kneading, bread before baking. The final leavened dough was sampling a second time at few months apart. At each step, yeast and LAB were enumerated and identified with 16S or 28S rDNA gene sequence analysis. Moreover a TTGE approach had also been used to monitor the LAB dynamic.
For each baker, the LAB : yeasts ratios were stable during the bread making process but were different for some baker from the first to the second sampling showing a count variation. Nine and six species of yeasts and LAB, respectively, were identified. The major species of yeasts was different from one baker to another, except for 2 bakers who shared the same yeast community whereas *Lactobacillus sanfranciscensis*, the typical LAB sourdough, was detected for all bakers.
During the bread making process, the major LAB and yeast species stay the same while differences were observed for the minor species which the frequency varied.
From the first to the second sampling of final leavened dough, one baker had a variation of major yeast species whereas the LAB community changed for 3 bakers.
Future research prospects will be discussed concerning the intraspecific diversity of *L. sanfranciscensis* which are the major LAB species detected. In addition, to better understand the links between biodiversity *vs* bread-making practices, more sourdoughs will be studied with metagenomic approach.

Keywords: Lactic acid bacteria; yeast; sourdough; dynamic; bread making process

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P3.22

The microbial interactions in the truffle brûlé

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Truffle-grounds are complex environments where microbial interactions are crucial for the development of the precious truffles. Truffles are ectomycorrhizal fungi that produce hypogeous fruiting bodies belonging to the *Tuber* genus (Ascomycota, Pezizales). Among the *Tuber* species, the black truffle *T. melanosporum* is highly appreciated because of its special taste and smell. *T. melanosporum* grows in symbiosis with several oak species and hazelnut trees in Mediterranean countries such as France, Italy and the Iberian Peninsula, although plantations have been introduced in New Zealand, Australia, Israel and North America. The development of *T. melanosporum* is associated with the production of an area around the symbiotic plants that looks burned (commonly referred to by the French word brûlé), where the fruiting bodies of *T. melanosporum* usually are collected. The brûlé is an area devoid of vegetation and, because of this, is easy recognizable. In order to understand whether the scanty plant cover within the brûlé is related to a change in fungal biodiversity, soil samples from *T. melanosporum*/*Quercus pubescens* truffle-grounds located in Cahors, France, were analyzed inside and outside of the brûlé. Both denaturing gradient gel electrophoresis (DGGE) and molecular cloning of the internal transcribed spacer (ITS) marker were employed on soil DNA to obtain fungal profiles from nine truffle grounds and sequences from one selected truffle ground sampled in two years. *T. melanosporum* resulted to be the dominant fungus within the brûlé where the Basidiomycota ectomycorrhizal fungi decreased, indicating a competitive effect of *T. melanosporum* on the other ectomycorrhizal fungi (Napoli et al., 2010). This suggests that the dynamics of fungal populations are correlated to brûlé formation. In a parallel approach we have applied the high throughput 454 sequencing on the same truffle-grounds providing an experimental validation of the efficacy of the short ITS-1 and ITS-2 reads in the studying of specific environments, and confirming the competitive effect of *T. melanosporum* (Mello et al., 2012). Since changes in the vegetation and in the fungal communities are important for soil bacteria, two molecular techniques – DGGE and PhyloChip - were used to compare bacterial community structures between *T. melanosporum* brûlé's and surrounding soils. Both analyses detected differences in community composition inside the brûlé compared with outside. *Pseudomonads* and most

members of *Actinobacteria* had different abundances according to both DGGE and PhyloChip microarray analyses, suggesting a close relationship between these taxa and brûlé formation. Small differences in richness at the phylum level have been found in *Firmicutes* and *Cyanobacteria* that showed higher relative richness inside the brûlé (Mello et al., 2013).
Taken together these results highlight how a peculiar environment, such as a truffle-ground, can be dissected to unravel the microbial interactions occurring below the ground. Understanding the ecological role of brûlé associated microorganisms and how they interact with *T. melanosporum* will be the next step.

Keywords: *T. melanosporum*; brûlé; bacterial and fungal interactions; dominance

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P3.23

Inhibition of *Brochothrix thermosphacta* by *Lactococcus piscium* CNCM I-4031 in cooked/peeled shrimp as a function of salt, temperature and concentration of the bioprotective strain and incidence on the sensory quality

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Brochothrix thermosphacta is a major spoiling bacteria of meat and lightly preserved seafood products. *Lactococcus piscium* CNCM I-4031 has recently been isolated in our laboratory to inhibit its growth (Fall et al., 2010, 2012) and delay the sensory shelf-life of naturally contaminated cold-smoked salmon and cooked/peeled shrimp packed under modified atmosphere (Matamoros et al., 2009). The aim of this study was to model the inhibition of *B. thermosphacta* and the consequences on the sensory characteristics of cooked/peeled shrimp as a function of salt concentration (NaCl), temperature (T) and the initial concentration of the bioprotective strain (LogNi).
An experimental design of 15 experiments was set-up in real matrix to calculate the linear effect of each parameter, the quadratic effect of LogNi and the first order

interactions. Salt varied between 0.8 and 2.0 %, temperature 4 and 12°C and *L. piscium* 0 and 7 Log cfu/g. Frozen peeled shrimp (*Penaeus vanamei*) were cooked in boiling water containing different salt concentrations. They were inoculated with *B. thermosphacta* (1.6 Log cfu/g) and *L. piscium* (10% v/m) and stored under 50% CO_2/50% O_2 for 28 days. Sensory analysis were performed by 13 trained panellists and a quality indice was calculated. In all conditions, *B. thermosphacta* stopped its growth when *L. piscium* reached its maximum level, suggesting a Jameson effect.
T had a significant positive effect on the maximum growth rate (µmax) of *B. thermosphacta*. LogNi and LogNI2 had also a significant effect but µmax was lowered only when LogNi was higher than 10^{6-7} cfu/g. LogNi and LogNi2 had a major effect on the maximum number of *B. thermosphacta* (Nmax). With no *L. piscium*, Nmax reached 10^9 cfu/g whereas with 3.5 and 7 Log (cfu/g) of *L. piscium*, *B. thermosphacta* reached 10^6 and 10^4 cfu/g respectively. Salt had weak linear negative effect and T had no significant effect on Nmax. No effect was observed on the lag time. A model was proposed to predict the growth of *B. thermosphacta* as a function of the 3 parameters with R^2=0.938 and RMSE = 0.55 Log cfu/g.
LogNi was the only factor with a negative effect on the quality indice. Interestingly, the quality was improved even with 3.5 Log (cfu/g) of *L. piscium*, although *B. thermosphacta* reached a concentration at which sensory deterioration should have been noticed. This suggests that the improvement of shrimp quality may not be due to the inhibition of *B. thermosphacta* only and that metabiosis between *L. piscium* and *B. thermosphacta* may contribute to the to the protective effect.
The inhibitory effect of *L. piscium* on *B. thermosphacta* can be implemented in a secondary order multiplicative model. A high level of *L. piscium* is necessary to prevent *B. thermosphacta* growth but sensory quality is improved with lower concentrations.

Keywords: biopreservation; inhibition; modeling; *Lactococcus piscium*; *Brochothrix thermosphacta*

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P3.24

Dairy lactic acid bacteria producing different antimicrobial compounds as effective competitive exclusion factors

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Microbial composition of cheese surface is influenced by different factors such as pH, temperature, relative humidity, environment, milk quality, brine, ripening condition, frequency of washing during ripening. This complex ecosystem is characterized by different species of yeasts, moulds and bacteria. The interactions that occur during the ripening on the active rind cheese among these microbial groups are not well understood, however may be defined as a beneficial biofilm being responsible for the flavour and appearance of this type of cheese. Bacteria have the ability to interact with each other and with the environment. In food ecosystems, the prevalence of certain species will be determined by their relative initial level, the affinity for substrates, the substrate availability, the relative growth rate of the competing species at different temperatures, and the production of antimicrobial metabolites. In particular, Lactic Acid Bacteria (LAB) can produce a wide variety of antagonistic primary and secondary metabolites including organic acids, diacetyl, and bacteriocins, which are active peptides against related bacterial groups and also against food pathogens and/or spoilage microorganisms. In this study, LAB isolated from different traditional Italian cheeses surface (Taleggio, Gorgonzola, Casera Valtellina, Scimudin and Formaggio di Fossa) were analyzed for their ability to produce antimicrobial substances. LAB, were preliminary screened by an agar spot test and the well diffusion assays. PCR was also performed to search the presence of bacteriocin-encoding genes. Inhibitory activity against *Listeria monocytogenes* was detected for all isolates by the agar spot test. However, the number of positive strains was severely reduced, when the neutralized cell-free supernatants (CFS) were subjected to the well-diffusion assay, resulting much less inhibitory against *L. monocytogenes*. In addition, several LAB species showed and important antifungal activity. The preliminary characterization of the antimicrobial substances present in the CFS revealed high thermo stability, while the sensibility to the treatment with trypsin, proteinase K and papain was different and strain dependent. Based on our results we found 14 new bacteriocin-producing *Lactobacillus* strains, belonging to the species *L. curvatus* (8), *L. plantarum* (2), *L. rhamnosus* (1), *L. casei* (1), *L. acidophillus* (1) and *L. acidipiscis* (1). According to trycine–SDS–PAGE, these inhibitory active molecules are approximately 2.5-7 kDa in size. PCR-based analysis demonstrated a great

diversity among bacteriocin genes harbored by different microorganisms isolated from the same source. Interestingly, some strains carried bacteriocin structural genes usually related to different species, suggesting the occurrence of a horizontal gene transfer among bacterial populations in the same food matrix. The ability to produce different antimicrobial compounds, such as bacteriocins and/or low-molecular mass antimicrobial compounds, may be one of the critical characteristic for effective competitive exclusion. The newly identified bacteriocin-producing *Lactobacillus* strains from traditional cheeses may also be useful for designing starter cultures with protective properties.

Keywords: Lactic Acid Bacteria; inhibitory compounds; bacteriocins

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P3.25

Effect of a high pressure homogenization sub-lethal treatment on volatilome, membrane fatty acid composition and cell outermost structures of two probiotic strains

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High Pressure Homogenization (HPH) seems to have a great potential to improve some Lactic Acid Bacteria (LAB) functional properties when applied at sub-lethal levels. In previous works (Tabanelli et al., 2012 & 2013) we observed that 50 MPa HPH treatment did not affect LAB cell viability but could increase some technological and probiotic properties such as resistance to simulated gastric digestion in vitro and the level of IgA-producing cell in the mouse gut even if the responses varied according to the species and the characteristics of individual strains.
Thus, considering that some probiotic properties are related to cell wall, also principal target of HPH, the aim of this experimental work was to evaluate the effect of HPH on bacterial cell surface hydrophobicity and aggregation properties of two already-known LAB functional strains, isolated from Argentinean products:

Lactobacillus paracasei A13 and *Lactobacillus acidophilus* DRU (Burns et al., 2008). Moreover, the response mechanisms activated in cells exposed to HPH have been investigated by analyzing cell membrane fatty acid (FA) before and after the treatment. Afterwards, the studied strains were evaluated in their cellular morphological changes after HPH by using Trasmission Electronic Microscopy (TEM). For these purposes, strains were inoculated in PBS at levels ranging between 7.5-8 Log CFU/mL and treated at 50 MPa with a Lab scale homogenizer (Niro Soavi Spa, Parma, Italy). All the data were compared with control data obtained under the same conditions but without the pressure application. In addition, treated and control strains were inoculated also in buttermilk (acidified or not) stored at 4°C for 30 days in order to better understand the contribution of employed strains and technological process to the sample overall aroma by SPME-GC-MS technique.

Different results were achieved for the two strains. In fact, while *L. paracasei* A13 enhanced its cellular hydrophobicity and auto-aggregation capacity after HPH treatment, *L. acidophilus* DRU decreased these features after pressure stress. These modifications, involving cell outermost structures, were highlighted also by TEM analysis. In fact, a significant effect of pressure treatment on cell morphology and particularly on the cell wall and S-layer (in *L. acidophilus* DRU) was observed.

As far as the FA modulation, a significant decrease of the dihydrosterculic and lactobacillic acids and a significant increase of the principal branched FAs were detected for both the strains following the HPH treatment. The results showed that plasma membranes, their level of unsaturation and their composition are involved in response mechanisms adopted by microbial cells exposed to the sub-lethal HPH treatment.

SPME-GC-MS analysis evidenced that pressure stress was able to modify volatilome produced by the strains in buttermilk enhancing the production of positive molecules for the product sensorial profile.

Concluding, this work demonstrated that, although the response to pressure treatment was train-dependent, HPH could be exploited to enhance the quality and the functionality of probiotic products as well as to improve and/or differentiate the organoleptic properties of probiotic fermented milks.

Keywords: High Pressure Homogenization; Lactic Acid Bacteria; probiotics

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P3.26

Prokaryotic communities in patterned grounds in North-western Italian Alps: Influence of lithology on small-scale distribution

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Patterned Grounds (PG) are formed on permafrost soils as a result of cryoturbation. The mixing, heaving, and churning of soil that occurs during freeze-thaw cycles form stripes, circles, polygons and nets with and without visible surface textural sorting. They occupy small flat surfaces and show quite different morphologies in relation with the lithology of the parent material. The parent material lithology is important in the formation and development of different PG features, thanks to different resistance to physical weathering leading to sorted or non-sorted patterns (the latter often associated with easily weatherable materials). Although many studies have described PG features and their formative processes, the important role that they play in controlling soil properties has only recently been recognised and abundance and distribution of microorganisms has not yet been considered. Prokaryotic communities that develop in these ecosystems may play important roles in nutrient availability and stabilization of organic matter and therefore in plant colonization and ecosystem evolution.
In this study, we assessed small scale distribution of prokaryotes in PGs, in terms of abundance and diversity, from four areas in the Graian Alps (North-western Piedmont and Valle d'Aosta Region). Multiple sites were chosen in order to evaluate influence of different lithotypes on spatial distribution of microbial communities. Denaturing gradient gel electrophoresis (DGGE) and quantitative real-time PCR (qPCR) approaches were used to assess respectively molecular diversity and abundance of Bacteria and Archaea.
Results indicate that PGs are composed by different ecological niches that allow the growth of phylogenetically and metabolically diverse prokaryotic groups. Abundance of microbial population shows a clear spatial distribution that is correlated to organic matter and influenced by lithology of parent material. These first results seems to confirm the important role of prokaryotes in nutrient cycling but, above all, in evolution of PG soil ecosystems.

Keywords: patterned grounds; prokaryotic communities

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P3.27

Yeast strain competition during wine alcoholic fermentation

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Yeast strains were isolated form an alcoholic fermentation performed with grapes from Mas Sinén vineyard located in the AOC Priorat. After the grapes were crushed, the paste containing juice, skins and seeds was placed in a 2-litter fermenter where alcoholic fermentation was carried out. All the process was done in the laboratory under sterile conditions. Samples were taken every day and plated in YPD and Lysine media in order to recover in plates the different populations of yeast. DNA was extracted from either individual colonies or pellets and analysed. Colonies were analysed by RFLP-PCR of the ITS region according to Esteve-Zarzoso et al. (1999), and further sequencing of the D1/D2 region of the 26S rRNA gene was performed for the final identification of the isolates. 135 isolates were obtained and identified as *Hanseniaspora uvarum* (74 isolates), *Saccharomyces cerevisiae* (45 isolates), *Candida zemplinina* (9 isolates) and *Issatchenkia terricola* (5 isolates). Typification of the Non-Saccharomyces isolates at strain level was performed by the PCR-based method of tandem repeat tRNA proposed by Barquet et al. (2012). Typification of *Saccharomyces cerevisiae* strains was performed by delta elements analysis (Legras et al, 2003).
Nine different strains of *Hanseniaspora uvarum*, five from *Saccharomyces cerevisiae* and three of *Candida zemplinina* were identified. The different strains were analysed in alcoholic fermentations that were performed individually (each fermentation with a single strain), with intraspecific competence (all the strains of the same species together) and interspecific competence (strains form different species together). As expected, all the fermentations performed by *S. cerevisiae* finished, despite that some of them could be considered slow fermenters, yet the Non-Saccharomyces species were much slower and generally left residual sugars over 10 g/litter after 20 days of fermentation. *Candida zemplinina* left less sugar content than *Hanseniaspora uvarum*, with only one strain of *H. uvarum* being able to finish the fermentation. As expected, in mixed fermentations with *S. cerevisiae*, the fermentations finished and the strains of *S. cerevisiae* took over the fermentation. However, the results of recovery of Non-Saccharomyces strains at the end of fermentation were dependent both on the strain and species of Non-Saccharomyces as well as the relative proportion between the strains.
The present work is part of the WILDWNE project (EU contract 315065, FP7).

Keywords: *Saccharomyces cerevisiae*; *Hanseniaspora uvarum*; *Candida zemplinina*

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P3.28

Vitality of *Lactococcus lactis* throughout cheese ripening

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Some recent evidences support the probable presence, during cheese late ripening, of microbial species belonging to starter populations as *Lactococcus lactis*.
The detection and vitality of *L. lactis* starter cultures were investigated, by culture-dependent and -independent techniques, in commercial and artisanal ripened cheeses. Quantitative PCR (qPCR) and Reverse Transcriptase (RT)-qPCR protocols were optimized and standard curves were constructed from serially diluted cells of *L. lactis* in physiological solution and inoculated in grated cheese used as biological model.
Ten grams of each cheese sample were submitted to direct extraction of nucleic acids and qPCR and RT-qPCR protocols. In this way, a quantification of *L. lactis* populations was reached in terms of both *L. lactis* total cells (at DNA level) and metabolically active populations (at RNA level). The cheese samples resulted positive for the presence of active *L. lactis* populations by RT-qPCR, were submitted to traditional culture-dependent analysis on lactococci selective medium M17 agar, in order to check for *L. lactis* colonies. DNA extraction and *L. lactis* species-specific PCR were carried out on the colonies isolated in order to assign their belonging to this species. Culture-independent analysis highlighted the presence of metabolically active populations of *L. lactis* in most of the cheeses examined at advanced stage of ripening. On the contrary, traditional plating on selective medium underlined a low number of *L. lactis* colonies probably due to the presence of microbial cells viable and metabolically active but not cultivable (VNC), and to the microbial competition on selective medium. These results highlight, once again, the limitations due to culture-dependent approach and the possibility to overcome it by culture-independent method.

Further studies will be carried on the technological role of *L. lactis* in ripened cheeses.

Keywords: *Lactococcus lactis*; cheese; real-time PCR; RNA

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P3.29

Stress response to gas plasma treatments in *Salmonella Enteritidis*

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Currently, there are numerous forms and apparatus used in the food industry for decontamination of food. The cold plasma is one of the most promising technologies with respect to the inactivation of microorganisms, including endospores, viruses and fungi, without changing the product quality. Although of the successful applications of low temperature gas plasmas in the food decontamination, the fundamental nature of the interactions between plasma and microorganisms is to a large extent unknown. A detailed knowledge of these interactions is essential for the development of new as well as for the enhancement of established plasma-treatment procedures. In this study, the effects of cold plasma treatments on the mechanisms of stress and resistance acquired by microorganisms to be eliminated by this process, especially in pathogenic bacteria such as Salmonella was investigating. For this, the behavior of the different strains of *S. enteritidis*, including three mutants (*S. enteritidis* lacking *rpo*S, *omp*R e *dps*) and their mechanisms of action when treated with cold plasma was observed. The survival of the wild- type and attenuated strains was determined by bacterial counts in different times, and the protein expression was evaluated by Multidimensional protein identification technology (MudPIT).

Keywords: *S. enteritidis*; stress; MudPIT

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P3.30

Wine mixed fermentations as a tool to study micro-ecological interactions

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Wine must is a ecological niche in which different yeast species succeed one another within a limited time frame. In mixed fermentations, selected non-*Saccharomyces* yeast species are introduced in the musts in high concentrations in combination with *S. cerevisiae* yeasts strains. Because of the different sensitivity toward waste products (most notably ethanol) different population dynamics can be obtained and studied. The evolution of the microbial populations as well as the changes in the chemical parameters can be carefully monitored throughout the fermentation process; also, although other yeast species are present in the must, their concentration is constantly kept at low levels: this makes their contribution negligible. In *Kluyveromyces marxianus*/*Saccharomyces cerevisiae* mixed fermentations, both yeast species can tolerate ethanol accumulation, therefore a simple competition scenario can be obtained. In *Candida zemplinina*/*Saccharomyces cerevisiae* mixed fermentations, because of the higher sensitivity toward ethanol of the first yeast species, a more complex situation is produced. The possibility of changing the initial concentration and the time of inoculation (co-inoculation or sequential inoculation), can be exploited to add a further element of complexity. Therefore we propose to utilize wine mixed fermentations to study interactions between different yeast.

Keywords: wine mixed fermentations; *S. cerevisiae*; *K. marxianus*; *C. zemplinina*

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P3.31

Study on the dominance of starter strains during craft beer bottle fermented

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In the production process of the craft beer is usually planned a refermentation step in bottle. This refermentation phases could be carried out with or without yeast starter inoculation. An investigation on *Saccharomyces cerevisiae* biodiversity of artisanal beer indicated that a wide yeast strain variability was found (Cocolin et al., 2011). This high variability highlighted the necessity for better investigations of *Saccharomyces cerevisiae* population dynamics during artisanal brewing. The yeast strain that carried out the bottle fermentation strongly influence the sensory and analytical profile of beer both producing volatile compounds and yeast autolysis products. In its turn, autolysis can lead to the excretion in beer of intracellular compounds as amino acids, peptides, nucleotides, fatty acids and enzymes, which may affect the flavor profile (Masschelein, 1986).
The aim of this study is to assess the dominance of inoculated starter strain after the bottling by molecular characterization of the isolated yeast strains , using analysis of the inter-δ regions. The capability of a yeast strain to dominate the refermentation process is very important for the production of a beer with desired and constant sensory characteristics. For this reason, the sensory and analytical profile of beer were also evaluated. The results obtained from the molecular characterization showed the clear dominance of *Saccharomyces cerevisiae* strain used for the second fermentation in the bottle. Generally the dominance varied from 80% to 95% but in some cases, achieved 100%. As regards the analytical profile of beer, the strain used influence both volatile compounds and sensory analysis.

Keywords: craft beer; dominance; refermentation

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P3.32

Inducing yeasts dominance in the presence of multiple environmental stress to cream off new robust candidates for lignocellulosic bioethanol production

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Increasing attention has been recently devoted to the production of bioethanol from lignocellulosic biomass. However, lignocellulose is expensive to process because of the need for costly pre-treatments and large dosages of commercial enzymes. Moreover, lignocellulose pre-treatment results in the formation of inhibitors affecting the following fermentation phase. Although ethanol production from pre-treated lignocellulose has been widely described (Hamelinck et al., 2005), only limited efforts have been spent on selecting yeasts able to both tolerate inhibitors and ferment sugars (Lindén et al., 1991; Favaro et al., 2012).
This study aimed at the isolation, characterization and selection of robust yeasts suitable for the lignocellulose-to-bioethanol route. While the majority of related researches has focused on isolating yeasts from first generation bioethanol and/or wine industrial plants, this work specifically targets to yeasts capable of exhibiting their dominance once challenged by multiple environmental stress all at once. To this purpose, grape marcs were selected as extreme environment because of its limited nutrients, exposure to solar radiation, temperature fluctuations and ethanol (Favaro et al., 2013a).
Grape marcs, collected, immediately after crushing, from a winery, were left at 30 and 40 °C for seven days, afterwards samples have been incubated at both temperatures in fermenting bottles containing 100 mL YNB (Yeast Nitrogen Base) broth supplemented with 100 g/L glucose, 50 g/L xylose and a cocktail of inhibitors (aliphatic acids, furans and aldehydes). Microbial biomass fluctuations together with ethanol and by-products concentrations have been monitored by means of microbiological techniques and HPLC (High Performance Liquid Chromatography) analysis. As soon as ethanol productions and biomass concentrations reached reasonable levels, samples of each fermenting bottles were aseptically transferred in fresh YNB formulated with the same sugars composition but with higher levels of inhibitors.
After three month experimental activities, the evolved yeast populations were able to grow quite fast in the presence of elevated inhibitors concentrations exhibiting promising ethanol yields. Moreover, microbiological quantitative and qualitative

analyses, performed at the beginning of the experiment and periodically throughout all the transfers, gave a fascinating picture of population dynamics as well as of yeast species dominance in such hostile environment.
At 30 °C, the majority of the isolates capable of withstanding osmotic, ethanol and inhibitors stress belonged to *Saccharomyces cerevisiae* while, at 40 °C, the dominant species revealed to be *Issatchenkia orientalis*. On the other hand, when higher inhibitor levels were added, lower consistency of *I. orientalis* was detected in favour of *S. cerevisiae*. The isolated yeasts were then screened for their fermentative abilities in minimal medium as well as for their inhibitors tolerance as described by Favaro et al. (2013b). Interestingly, many *S. cerevisiae* strains exhibited very high ethanol yields also at 40 °C and displayed strong abilities to tolerate weak acids, furans and aldehydes.
The preliminary results of this study indicate that the obtained microbial collection is a promising platform towards the development of robust and efficiently fermenting microbes suitable for the industrial processing of lignocellulosic biomass into ethanol.

Keywords: lignocellulosic bioethanol; inhibitors; robustness; yeasts dominance; selection

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P3.33

Yeast ecology of twelve vineyards of Barbera within Monferrato wine area and selection of autochthonous *Saccharomyces cerevisiae* strains

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The conversion of grape must to wine is a complex biochemical process mediated by the extracellular and intracellular activities of several yeast strains. Wine

Fermentations are ecologically complex and involve successional evolution of both species and strains within each species.
The apiculate yeasts, having low fermentation activity and belonging to genera *Kloeckera* and *Hanseniaspora*, and other genera such as *Candida* and *Pichia*, carry out the first period of the spontaneous fermentations.
As the fermentation progresses, the non-*Saccharomyces* species successively die off, leaving *Saccharomyces cerevisiae* to dominate and complete the fermentation.
Despite the fact that there is a succession of yeast genera and species involved in the spontaneous fermentation, only a few *S. cerevisiae* strains dominate the fermentation. This fact is a result of a natural selection during the spontaneous fermentation, in which the *S. cerevisiae* strains replace each other along the fermentation process. The diversity of yeast species correlates with the formation of secondary products of fermentation, such as acetaldehyde, ethylacetate and higher alcohols.
The aim of this research was the study of indigenous yeasts' ecology isolated from grape harvest till complete alcoholic fermentation of Barbera cultivar grapes from 12 vineyards in the Asti-Monferrato area, Northwest of Italy.
A total of 1100 yeast colonies, 157 on grapes and 943 during fermentations, were isolated from Wallerstein Laboratory Nutrient (WLN) agar. All *S. cerevisiae* isolates were simultaneously identified and characterized applying interdelta analysis, and the non-Saccharomyces yeast population was identified via the PCR-RFLP of the ITS1 5.8S rDNA-ITS4 region. Six different yeast species were found: *S. cerevisiae*, *Hanseniaspora* uvarum , *Candida zemplinina* , *Torulaspora delbruecki* and *Pichia anomala.*
H. uvarum appeared in major proportions at the beginning and at the mid of fermentation, otherwise in the last part of fermentations *Saccharomyces* was present mostly. In the first phase of alcoholic fermentations *C. zemplinina* , *T. delbruecki* and *P. anomala* were isolated as well, but with a lower frequency.
H. uvarum, *C. zemplinina* and *T. delbruecki* represented about 80%, 10% and 5% of the total *non-Saccharomyces* population isolated, respectively.
This activity carried out in the 2012 harvest was useful for the constitution of a collection of indigenous yeast strains, including *S. cerevisiae* and *non-Saccharomyces* species, of enological interest. This collection represents a source of wild yeasts, among of which it is possible to select indigenous starters able to maintain the specific organoleptic characteristics of Barbera wine.
This study is part of the EU project WildWine: Multi-strain indigenous yeast and bacteria starter for wild-ferment wine production, funded in the VII FP under grant agreement n. 315065.

Keywords: yeast; wine; grape; wild-fermentation; *Saccharomyces cerevisiae*

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P3.34

Genomic analyses indicate that the NRPS machinery is intrinsic in the *Bacillus subtilis* group

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Several bacterial species of the *Bacillus* genus, produce cyclic lipopeptides (LPs) through the activity of Non Ribosomal Peptide Synthetases (NRPS). LPs have antimicrobial, cytotoxic, antitumor and surfactant activities. The proposed primary mode of action of these compounds is a pore formation in membrane, causing an imbalance in transmembrane ion fluxes and concomitant cell death. From an evolutionary point of view, it is proposed that LPs have an important role in antagonism toward other microorganisms such as bacteria, fungi and viruses through lytic and growth-inhibitory activities, as well as postingestional defensive mechanisms against predators such as protozoa. The presence of NRPS in the genome of *Bacillus* species can thus significantly increase their fitness.
The real level of diffusion of NRPS genes in *Bacillus* species is however still partly unknown. The present work was thus carried out to evaluate whether non ribosomal peptide synthesis is intrinsic in the *Bacillus subtilis* group, and whether each strain can harbor more than one NRPS gene. Furthermore, the study was conducted in order to assess the level of phylogenetic similarity of NRPS sequences.
All available sequences coding for the synthesis of the cyclic lipopeptide belonging to the surfactin group, plipastatin-fengycin group and iturin group (including iturin, mycosubtilin and bacillomycin) were retrieved, focusing on *Bacillus subtilis*, *B. licheniformis* and *B. amilolyquefaciens*. These selected sequences were used to create an *in silico* pseudomolecule which was aligned against all available annotated genomes and all draft genomes of several *Bacillus species* (*B.amyloliquefaciens*, *B.atrophaeus*, *B.licheniformis*, *B.mojavensis*, *B.subtilis*, *B.vallismortis*, *B.pumilus*). Analyses results show that all the strains belonging to *Bacillus subtilis* group harbor at least one operon encoding for NRPS responsible of the surfactin-like LPs synthesis. Since NRPS are currently used at regulatory level to identify hemolytic and cytotoxic *Bacillus* species, results will also support a potential refinement of the risk assessment approach.

Keywords: *Bacillus subtilis* group; non ribosomal peptide synthetase

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P3.35

Bacterial and enzymatic activity diversity of *Arundo donax* lignocellulosic biomass

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In 2001 about 97% of the world's liquid transportation fuels were derived from petroleum (Mielenz, 2001). Governments have initiated extensive research into the large scale production of alternative liquid transportation fuels from renewable resources to reduce the reliance on fossil fuels and the hikes of their price. The lignocellulosic biomass is used as biofuel to achieve this purpose, since it is composed for 75% of polysaccharides which constitute a source of fermentable sugars. Lignocellulosic biomass include agricultural waste such as corn stover, bagasse, wood, grass, and dedicated energy crops such as miscanthus, switchgrass, *Arundo donax* (Ask et al., 2012). Has emerged that enzymatic hydrolysis of plant carbohydrates is the most prominent eco-technology for the degradation of biomass. The aim of this study was to evaluate the bacterial diversity of *A. donax* lignocellulosic biomass during natural biodegradation and to isolate microorganisms of biotechnological interest for the application in bioenergetic systems.
The raw vegetable biomass was composed by chipped wood from *Arundo donax* processed to degradation under natural conditions (open field and underwood). The *A. donax* lignocellulosic biomass were analyzed to evaluate the microbial diversity by high-throughput sequencing of 16S rRNA gene. The microbiota of lignocellulosic biomass including about 50 classes. *Actinobacteria*, *Sphingobacteria*, *Bacilli*, *Alphaproteobacteria* and *Gammaprobacteria* were the dominant classes.
Moreover, the two different drifts were used to obtain isolate colonies by using selective media for cellulolytic microorganisms containing carboxymethylcellulose (CMC) or Avicel as sole carbon source. About 90 microorganisms were isolated and assayed for different enzymatic activities such as endo- and exo-cellulase, xylanase, cellobiase, pectinase and ligninase to establish the number of putative degrading bacteria showing multifunctional activities. Fourteen cellulolytic strains showed an Indices of Relative Enzyme Activity (ICMC) values > 13 and the presence of different enzymatic activities (from four to five). The isolates were identified by 16S rRNA gene sequence analysis. Moreover, a phylogenetic tree was generated from the distance data using the neighbor-joining method in MEGA4 Program. For this analysis 16S rRNA gene of related type strains of

different genera were included in the data set. Seven genera and eleven different species were identified. *Bacillus* spp. was the genera most presented with the species *B. amyloliquefaciens*, *B. licheniformis* and *B. subtilis*. The other species belonged to the genera *Isoptericola*, *Cellulosimicrobium*, *Microbacterium*, *Pantoea*, *Curtobacterium* and *Shingobacterium*.
These results improve the knowledge on microbial diversity of poorly known environments such as lignocellulosic biomass and demonstrate the effectiveness of selective ecological strategy to isolate new degrading bacteria as source of enzymes to use in bioethanol production.
This work was supported by Project EnerbioChem, PON01_01966, funded in the frame of Operative National Programme Research and Competitiveness 2007–2013 D. D. Prot. n. 01/Ric. 18.1.2010.

Keywords: cellulolytic bacteria; microbial diversity; enzyme activities; *Arundo donax*

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P3.36

The presence of *Staphylococcus equorum* in cheeses

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Staphylococcus equorum is a well-known starter culture in dry fermented sausages where it has important roles in colour and flavour formation. However, many studies report that it is also found in various cheeses during the ripening period. From a preliminary study of a blue-veined raw milk cheese (Stichelton) community by PCR-Denaturing Gradient Gel Electrophoresis analysis of the V3 rDNA region, *Staphylococcus equorum* was found in the crust of a ripening cheese pre-vein piercing and in all parts of the post-vein piercing samples. However, this bacterium was not detected in the fully ripened cheese. The presence of *Staphylococcus equorum* in Stichelton, Stilton, Danish Blue, and Reblochon cheeses was detected by a species-specific PCR assay targeting the *Sod*A gene of coagulase negative Staphylococci isolated on Mannitol Salt agar. Sequencing of

the 16S rDNA region was used to confirm isolates were *Staphylococcus equorum*. The results showed that from 82 isolates, 7 isolates from Stilton core, 5 isolates from Stilton crust, 8 isolates from Danish Blue, 4 isolates from Reblochon core, and 2 isolates from Reblochon crust were confirmed as *Staphylococcus equorum*. However no potential isolates of this bacterium were detected from Stichelton cheese confirming the results of the original PCR DGGE study. The interaction between a mixed *Lactococcus lactis* starter and *Staphylococcus equorum* in a cheese model is being investigated to determine if *Staphylococcus equorum* survives early fermentation or whether its presence is due to contamination during ripening as suggested by the PCR DGGE studies.

Keywords: *Sod*A gene; *Staphylococcus equorum*; cheese

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P3.37

Mobilome of *Oenococcus oeni*: a role for the supremacy of the strains in wine?

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Oenococcus oeni is the key lactic acid bacterium involved in malolactic fermentation (MLF), a secondary fermentation that improve the quality of wine. Strains belonging to this species are capable to survive, proliferate and dominate in this complex ecosystem, despite the harsh winemaking conditions (e.g., low pH, high level of ethanol, presence of sulfites, nutrient restriction) and the strong competition among several microorganisms. This adaptability could be linked to the genome plasticity of *O. oeni*, due to the lack of the mismatch repair system which leads to a high level of mutation frequency and horizontal gene transfer (HGT) (Marcobal et al., 2008; Bon et al., 2009). A recent study of Favier et al. (2012) has reported the presence of two large plasmids (18.3 and 21.9 kb) in some *O. oeni* strains, most of them used as starter cultures to promote MLF. Sequence analysis of these plasmids has revealed the presence of several hypothetical proteins, and also of transposases, besides genes with a predicted function. In addition, one of the two plasmids, pOENI-1v2, had been previously reported by Piffanelli et al. (2010) in the strain KM383 from Amarone, a wine characterized by a very high ethanol content (15% v/v). The results shown by these authors give an indication that these plasmids could enhance the genetic flexibility of the strains, thus contributing to their fitness and supremacy in wine.
However, studies on the possible relationships among fitness, dominance and

mobilome, represented by plasmids and mobile elements, are very scanty, especially for oenological bacteria. Therefore, in this study, we investigated the distribution of the two described plasmids and some genes present therein in a collection of 27 *O. oeni* strains, including a subcollection of 13 strains isolated during a single spontaneous MLF of Amarone wine (Zapparoli et al., 2012).
The PCR screening for the nickase revealed that such gene was present in the 42% of strains isolated from Amarone wine, whereas it was not found in the strains isolated from other wines. This observation represents a further evidence that some plasmids could effectively contribute to bacterial fitness, especially in a hostile environment such as Amarone wine.
As regards the analysis of other plasmid-related genes, particular attention was paid to transposases. Indeed, in literature, a genomic island of *O. oeni* PSU-1 has been described as almost identical to a genomic region of *Lactobacillus plantarum* WCFS1 (Bon et al., 2009); in the flanking regions we observed the presence of a transposase highly related to sequences present in *Lactobacillus helveticus*, *Pediococcus claussenii*, both in genome and plasmid, and partially in plasmid pNP40 of *Lactococcus lactis*.
These findings indicate an important role of transposases as target for recombination and integration of plasmids in *O. oeni* genome. Therefore, the mobilome could be a major driving force in HGT among bacterial strains, made possible by microbial interactions in the shared ecological niche. Further studies regarding the expression and translation of plasmid-related genes are needed, to understand their physiological role in the adaptation, activity and dominance of strains in wine ecosystem.

Keywords: *Oenococcus oeni*; plasmids; mobilome; Amarone wine

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P3.38

Assessment of aerobic and respiratory metabolism in *Lactobacillus casei* group

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Lactobacillus casei, *Lb. paracasei* and *Lb. rhamnosus* are industrially important species of lactic acid bacteria (LAB), used as probiotics or as starter/adjunctive cultures in the production of many fermented and functional foods. As other LAB, these species respond to harmful conditions activating general or specific stress response mechanisms, which can improve cell survival and strain performances (Corcoran et al., 2008). The growth behaviour and type of metabolism affect the robustness to stresses and recently, several studies have demonstrated that the aerobic/respiratory promoting conditions (presence of O_2 , hemin and/or menaquinone in the substrate) induce in some LAB species (*Lactococcus lactis*, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Lb. plantarum*) helpful traits for industrial applications (Pedersen et al., 2012). However, with exception of *Lb. plantarum*, reports on the aerobic/respiratory pathway in other species of *Lactobacillus* genus are rare (Brooijmans et al., 2009).
Previously (Zotta et al., 2013 submitted), we investigated the ability of 184 strains belonging to *Lb. casei* group to grow in presence of air. In this study, we evaluated the effect of anaerobiosis (AN; static cultivation), aerobiosis (AE; cultivation with shaking, air) and respiration (RS; cultivation with shaking, air, supplementation with hemin and menaquinone, cofactors for the activation of electron transport chain, ETC) on 60 selected strains. Increases in OD_{650} , pH values and production of H_2O_2 were measured after 16 h and 24 h of incubation at 37°C. A new rapid approach based on the reduction rates of resazurin (change from blue oxidised form to colourless reduced form) was optimized and used to evaluate the O_2 uptake by AN, AE and RS cells in aerated buffer. On 5 selected strains, the O_2 consumption was also measured with a polarographic electrode to confirm the adequacy of resazurin assay. Moreover, on the same strains, the activities of O_2 -related enzymes POX (pyruvate oxidase), NOX (NADH oxidase) and NPR (NADH peroxidase) were measured at both 25°C and 37°C.
Our results confirm that respiratory conditions significantly improved the growth of *Lb. casei* group. Several strains (especially belonging to *Lb. rhamnosus* and isolates from human sources) exhibited the common traits (concurrent increase of

OD_{650} and pH values as well the capability to consume O_2 in aerated buffer) of aerobic or respiratory pathway. A good correlation in O_2 uptake was found between resazurin assay and polarographic measurement, and the new optimized method proved to be fast and effective for testing a large number of strains. As expected, the activity of POX, NOX and NPR were higher in the presence of O_2 and, mainly, with hemin and menaquinone supplementation and the enzymes were differently affected by temperature.
We confirmed the ability of some strains of *Lb. casei* group to perform a respiratory metabolism and the benefits for growth and robustness of the strains. Further studies, to evaluate the effect of respiration on technological, probiotic and stress tolerance traits, are needed to improve the fitness of the strains.

Keywords: *Lactobacillus casei* group; aerobic metabolism; respiration; resazurin

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P3.39

Lactic acid bacteria isolated from artisanal cheese and their potential probiotic activities

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The present study aims to evaluate the probiotic potential of lactic acid bacteria (LAB) isolated from a hard cheese, produced in North of Italy, in which artisanal whey starter cultures are used. Samples were collected from whey and milk to the twelfth ripening month, from three different productions. A total of 620 isolates were grouped by PCR-RSA and then identified at specie level by the16S rRNA gene sequencing. During all the period followed, the presence of *Lactobacillus helveticus*, *L. delbrueckii*, *L. fermentum*, *L. pontis*, *L. rhamnosus*, *L. reuteri* and *L. gasseri* was detected with a predominance of *L. helveticus* and *L. rhamnosus*

showing respectively 41% and 53% of the total isolates. *L. helveticus* was present at the beginning, whereas *L. rhamnosus* was related with the last ripening months. The probiotic potentials of all the isolates were then subjected to an in vitro screening. The in vitro tests included survival in simulated gastric and pancreatic digestion, autoaggregation and hydrophobicity properties. Sixteen isolates with promising probiotic properties were selected. These isolates belong to *L. helveticus* (6), *L. rhamnosus* (9) and *L. fermentum* (1).
Due to the autochthonous origin of the strains, their use as starter cultures may contribute to improving natural fermentation and the nutritional characteristics of the cheese studied. However, final selection would also depend on their real in vivo probiotic properties as well as on other technological characteristics.

Keywords: LAB; cheese; probiotic activities

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P3.40

Diversity in tolerance to different physicochemical stresses in bacteria of the *L. casei* group

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Numerous bacterial strains belonging to species of the *L. casei* group are used as starter or adjunct cultures for their technological role in food and beverages and for their probiotic characteristics (Oberg et al., 2011; Argyri et al., 2013). These bacteria must be able to resist to the different adverse conditions encountered in industrial production, handling and storage by freeze-drying or freezing. Moreover, probiotic strains must retain viability during transit in the gastrointestinal tract. The stress conditions to which these bacteria are exposed include heating and cooling, acidity, bile salts and high osmolarity (van de Guchte et al., 2002; Tsakalidou and Papadimitriou 2011). The exploration of their stress tolerance is fundamental to warrant their positive effects. However, to date, these aspects were studied for a few strains already in use as commercial cultures, whereas scarce knowledge on the general trends within this taxonomic group is available.

In this work 184 strains of lactobacilli, belonging to *L. casei* group, were characterized to study the ability to withstand different stressful conditions. One hundred and five *L. paracasei*, 71 *L. rhamnosus* and 8 *L. casei* strains isolated from different sources (dairy and meat products, sourdoughs, wine, beverages, plant material, and human) and geographical regions were screened for their ability to resist to acidity, bile salts, NaCl and to grow after refrigeration and freezing. The strains were assayed for their ability to survive in acidic environments (pH 1.5 and 2.5 for 2h at 37°C); to grow at different pHs (3.5, 4.5, 5.5 and 6.5), in presence of 2%, 4% and 6% NaCl and of 1.5% bile salts. Cold shock tolerance was evaluated by exposure to both refrigeration and freezing at -20°C.
Results highlighted that none of the strains was able to grow at pH 1.5 and 2.5 and that only 3.3% and 61.4% of the strains survived after 2h of incubation at 37°C at these pH values. All the strains survived in presence of 1.5% bile salts after 24h of incubation at 37°C. Furthermore, 56% strains evidenced a good ability to growth in this condition. The species *L. paracasei* and *L. rhamnosus* showed the highest variability on this respect, compared to *L. casei*. The strains showing the best bile salts tolerance were isolated from wine, human faeces and cheeses. Regarding low pH tolerance, most of the strains showed a low ability to grow at pH 3.5 with the exception of five *L. rhamnosus* and one *L. casei* strains isolated from wine, four *L. paracasei* strains isolated from dairy products, human body, wine and fermented beverages. At pH 4.5 and 5.5 almost all the strains showed a good capacity to growth. A strain specific growth behaviour was observed after refrigeration and freezing. Generally, a prolonged lag-phase was registered.
Results highlighted a noticeable diversity in the ability of the strains to withstand harsh growth conditions. For some parameters this ability varied according to the source of isolation. Strains adapted to some ecological niches showed better stress tolerance and more interesting features for being industrially exploited.

Keywords: stress tolerance; *L. casei* group; acidity and high osmolarity; bile salt; cold shock

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P3.41

Interactions between *Saccharomyces* and Non-*Saccharomyces* species during wine alcoholic fermentation

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An alcoholic fermentation was followed by culture-dependent and independent methods. Grapes were taken from Ferrer-Bobet winery located in the AOC Priorat. After the grapes were crushed, the paste containing juice, skins and seeds was placed in a 2-litter fermenter where alcoholic fermentation was carried out. All the process was done in the laboratory under sterile conditions. Samples were taken every day and processed in two different ways. On one hand there were plated in YPD and Lysine media in order to recover in plates the different populations of yeast. On the other hand, the solution was centrifuged and the pellet was frozen until culture-independent analysis was performed. DNA was extracted from either individual colonies or pellets and analysed. Colonies were analysed by RFLP-PCR of the ITS region according to Esteve-Zarzoso et al. (1999), and pellets were analysed by quantitative PCR according to Hierro et al. (2006) and by massive sequencing. D1/D2 region of the 26S rRNA gene was sequenced for the final identification of the isolates. The yeast populations were low according both culture-dependent and independent methods, ranging from 10^5 to 10^7 cells or cfu/ml, depending on the evolution of the alcoholic fermentation. Until half of the fermentation, most of the plate-recovered colonies belonged to Non-*Saccharomyces* yeasts, mostly *Hanseniaspora uvarum* and *Candida zemplinina*. These results were confirmed by quantitative PCR, where *H. uvarum* and *C. zemplinina* reached the highest level up to mid fermentation. In the latter stages of fermentation S. cerevisiae took over the fermentation.

These results show that the Non-*Saccharomyces* yeast can perform and lead the fermentation at least until well-advanced stages and S. cerevisiae is the yeast that takes over at the end. However, in this case H. uvarum remained at very high population level until the end of fermentation by culture-independent methods, although no colonies were recovered in plates. The main yeast species were the same by both methods, except in the last stages of fermentation, where *H. uvarum* was present, yet it was not recovered in plates. In contrast, this fact was not observed with *C. zemplinina*. Thus, the interaction between S. cerevisiae and Non-Saccharomyces species is species-specific and does not affect all the Non-Saccharomyces species in the same fashion.

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Keywords: *Candida zemplinia*; *Hanseniaspora uvarum*; Priorat

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P3.42

Survey and isolation of lysogenic phages in 72 strains of *Escherichia coli* collection of reference (ECOR)

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Escherichia coli collection of reference (ECOR) (Ochman and Selander, 1984) is a standard collection of wild *E. coli* strains, which are considered to represent the range of genotypic variation in the wild *E. coli* strains as a whole. Although they are widely used for experiments to understand the physiology and behavior of wild *E. coli*, their genetical features including accessory DNAs have been studied insufficiently (Nilsson et al., 2004; Riley and Gordon, 1992).
In this study, we have surveyed the presence of lysogenic phages in ECOR strains under inducing and non-inducing conditions. Culture supernatants of each ECOR strain were examined for their lytic activity against a laboratory *E. coli* strain.
We found that 36 strains can produce active phages, and among them 14 strains were newly found to possess lysogenic phages. We have also identified a phage derived from an ECOR strain, MSU52, as a lambdoid phage, which shows homology to some known phages.
(This work was supported by JSPS KAKENHI Grant Number 24650488, 25292051)

Keywords: *Escherichia coli*; bacteriophage; lambdoid phage

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P3.43

Molecular identification and characterization of yeasts, LAB and AAB strains isolated from cocoa beans fermentation

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Cocoa beans fermentation is a determining technological phase in the transformation of raw cocoa beans into chocolate. It consists in a natural microbiological transformation of pulp and seeds arising from the pods of *Theobroma cacao* involving activity of yeasts, lactic acid bacteria (LAB) and acid acetic bacteria (AAB). This fermentation process is weakly studied due to the uncontrolled environmental conditions.
In the present study two different spontaneous cocoa bean fermentation were carried out in West Africa, heap and box fermentation. Microbiological sampling was performed for both fermentation systems at first day and after 1, 2, 4 and 6 days. Samples were studied using a culture-dependent microbiological method and identified by molecular techniques. Identification was carried out by using Repetitive Element Palindromic-PCR (rep-PCR) and Denaturant Gradient Gel Electrophoresis (DGGE). Rep-PCR and DGGE profiles were analyzed using cluster analysis. One representative strain for each cluster was identified sequencing 16S and 26S rRNA gene for bacteria and yeasts, respectively. Cluster analysis was performed using Pearson correlation index. Furthermore cluster analysis was used to evaluate the intraspecific biodiversity of predominant species of each microbial group.
Saccharomyces cerevisiae, *Issatchenkia orientalis*, *Lactobacillus fermentum*, *Acetobacter syzygii* and *Acetobacter pasteurianus* were the prevailing species in the box fermentation. The most abundant species concerning the heap fermentation, as revealed by molecular fingerprints, were *S. cerevisiae*, *Pichia galeiformis* and *Pichia guillermondii* as yeasts, *L. fermentum* as LAB and *A. pasteurianus* and *Acetobacter lovaniensis* as AAB. These species were isolated from the beginning to the end of fermentation, therefore, they were even the most involved in the process. *S. cerevisiae*, *A. pasteurianus* and *L. fermentum* showed a high biodiversity within the species. Strains grouped according to the type of fermentation, box and heap. In conclusion, the results obtained showed that

fermentation of cocoa beans is a very complex process with variations in yeasts, LAB and AAB compositions. In this work we gave a better idea of the microbial dynamics during the cocoa beans fermentation, a process so far weakly studied.

Keywords: cocoa beans fermentation; identification; characterization

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P3.44

Heterogeneity of growth response in *Lactobacillus casei* group

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The *Lactobacillus casei* group includes three closely related species, *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus* (Felis and Dellaglio, 2007), involved in different food and health-related applications. Recently, many researches have been addressed on the identification and genotypic characterization of strains belonging to this group (Huang and Lee, 2009; 2011), but few studies have considered their physiological heterogeneity in response to different growing conditions, especially in a large number of isolates.
We investigated the capability and the diversity to cope with different growth conditions (sub-optimal temperature, presence of oxygen, hemin and ROS generator compounds) in 184 strains of *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus*, isolated from several ecological niches and geographical origins. Additionally, the shift towards aerobic or respiratory lifestyle has been considered in these species for the first time.
All strains were grown at 10, 35, 37 (optimal T°C of growth; control), 40, 42, 45, 48, 49 and 50°C, to evaluate the maximum temperature of growth (T_{max}) and screened for their capability to grow (OD_{450} and pH values after 16h and 42h at 37°C) in anaerobiosis (AN, static cultivation), aerobiosis (AE, with agitation) and heme-supplemented aerobiosis (AEH, with agitation). The presence of catalase was qualitatively assayed on AN, AE and AEH cultures, while the tolerance of H_2O_2 (1 and 2 mM), pyrogallol (25 and 50 mM) and menadione (1.5 and 2 mM) was evaluated on AEH cells. The survival to different (from 0.88 to 0.0017 M) H_2O_2 concentrations was further investigated in the 4 strains of *Lb. casei* that exhibited a catalase-like activity.

A wide heterogeneity was found within the *Lb. casei* group. Most of *Lb. rhamnosus* strains grew at T°C above 45°C and a large number of isolates grew at 48-49°C. With few exceptions, in *Lb. paracasei* the values of T_{max} did not exceed 45°C. Strains from human sources had the highest (up to 49°C) range of T_{max}, while those isolated from wine and sourdoughs exhibited the lowest adaptation to high temperatures.
The presence of oxygen increased the OD_{450} of *Lb. casei* and *Lb. paracasei* at both incubation times, while heme-supplementation offered a net growth gain during long-term cultivation. The growth of *Lb. rhamnosus* was not significantly affected by aerobic conditions.
With the exception of 4 isolates of *Lb. casei*, showing a catalase-like activity (even if they lack *kat* gene), none of the strains were able to survive to H_2O_2. Several strains of *Lb. casei* and *Lb. paracasei* tolerated menadione and, most pyrogallol. The species *Lb. rhamnosus* showed the lowest tolerance of superoxide generators, probably because they lack of *sod* gene.
This study allowed the identification and selection of strains with unique properties that deserve further investigation. Moreover, the respiratory pathway may confer several physiological and metabolic advantages also in *Lb. casei* group and the exploitation of oxygen-tolerant phenotypes could be useful for the development of starter and probiotic cultures as for other LAB species (Pedersen et al., 2012).

Keywords: *Lactobacillus casei*; growth heterogeneity; oxidative stress

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P3.45

Yeast microflora during spontaneous fermentations of 'Nero di Troia' wines

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In order to investigate the yeast microflora during spontaneous fermentations of 'Nero di Troia' wines, a panel of strains representing natural diversity was selected. Technological characterization of selected *Saccharomyces cerevisiae* and non-*Saccharomyces* strains was perform with the aim to design multi-strain autochthonous starter cultures and increase the 'unique' qualities of 'Nero di Troia' wines. The yeast strains were identified using restriction pattern analysis of the internal transcribed spacer region (5.8S-ITS), sequence of the internal transcribed spacer region (5.8S-ITS), species-specific primers, and interdelta analysis for *Saccharomyces cerevisiae* strains characterization. The first step of technological characterization (e.g., killer activity, H2S production, fermentation kinetics in synthetic medium and in must, cytofluorometric analysis) led us to select the most promising *S. cerevisiae* and non-*Saccharomyces* strains, mainly belong to *Hanseniaspora* and *Candida* species, and to assess the performance of possible co-inoculation approaches. However, using two classical strategies of inoculum and planned to promote the non *Saccharomyces* 'expression', a strong competition took place with some of the non-*Saccharomyces* strains, compromising an efficient alcoholic fermentation. An effective development of the *S. cerevisiae* strains was only reached when the concentration of the non-*Saccharomyces* strains in the co-inoculation approaches was strongly reduced. Our observations suggest the presence of "robust" competitors between non-*Saccharomyces* strains of oenological importance that may negatively affect wine fermentation.

Keywords: *Saccharomyces cerevisiae*; non-*Saccharomyces*; wine; *Candida*; *Hanseniaspora*

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P3.46

Biotechnological and Safety Aspects of Wild Enterococci Strains Isolated from Italian Traditional Malga-Cheese

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Malga-cheese is made from raw cow's milk in small scale on-farm dairies located at least 1400 meters above the sea level in Trentino, an alpine province of Northern Italy, following traditional and artisanal technologies. The aim of this study was to characterize *Enterococcus* strains which leads the first step of fermentation of Malga-cheese, focusing on technological and safety properties in order to evaluate their potential as dairy starter cultures. One-hundred fourteen *Enterococcus spp.* strains were isolated from 36 samples of Malga-cheese after 24 hours of ripening. All bacterial isolates were identified by partial sequencing of 16S rRNA gene and species-specific PCR. *Enterococcus faecalis* was the dominant species (52% of the isolates); *Ec. faecium* (25%), *Ec. italicus* (14%), *Ec. durans* (5%) were also found. The isolates were clustered into 79 biotypes by RAPD-PCR (Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction) performed with two primers. One isolate representative for each biotype was tested for the ability to inhibit coliforms growth, to grow in presence of bile salts (BS), to hydrolyse BS and subjected to further safety tests: haemolysis assay, biogenic amines production and growth in the presence of antibiotics (penicillin, erythromycin, vancomycin, chloramphenicol and tetracycline). In particular, the vancomycin resistance was verified both by plating on M17 agar supplemented with 4 µg/ml vancomycin (concentration suggested by the European Food Safety Authority - EFSA), and also by *vanA* and *vanB* genes-specific PCR (Dutka-Malen et al., 1995). A multiplex PCR for the simultaneous detection of five virulence genes (*asa1*, *cylA*, *esp*, *gelE* and *hyl*; Vankerckhoven et al., 2004), and the presence of CRISPR1 locus was also performed.

Thirty-seven (34 *Ec. faecalis*, 1 *Ec. faecium*, 1 *Ec. durans* and 1 *Ec. italicus*) out of the 79 tested biotypes showed an inhibitory effect against coliforms growth. The coliforms inhibitory activity of the most efficacious strain *E. faecalis*, was confirmed *in vitro* in a laboratory produced cheese. Fourteen biotypes were able to grow on M17 added with BS and 2 *Ec. faecium* strains hydrolyzed BS. Putrescine and cadaverine were the most commonly detected biogenic amines (produced by 28 and 25 isolates, respectively), tyramine was produced by 15 and histamine by 11 isolates. Almost all the 79 tested biotypes showed a resistance to one or more antibiotics: only 8 (4 *Ec. faecium*, 1 *Ec. faecalis*, 1 *Ec. durans*, 2 *Ec. italicus*) of them were sensitive to each of the tested antibiotic. *VanA* and *vanB* genes were

detected in 13 biotypes, but only 5 of these were able to grow in the presence of vancomycin (4 μg/ml). Seventy-one percent of the tested strains had at least one virulence gene and in 77% the CRISPR1 *locus* was also detected. Only three *Ec. faecalis* strains, free from virulence genes, were able to inhibit the growth of coliforms. This study confirmed that enterococci could be exploited and proposed as safe in adjunct or protective cultures for dairy production.

Keywords: Malga-Cheese; entorococci; safety aspects

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P3.47

Development of an ad hoc natural whey starter culture for the production of Vastedda della valle del Belìce cheese

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This work was aimed to characterize the autochthonous lactic acid bacteria (LAB) of PDO Vastedda della valle del Belìce cheeses, produced in several dairy factories, for the development of an *ad* hoc starter culture preparation for the production of this cheese. To this purpose, winter and spring productions were analysed, in order to isolate LAB adapted to perform the fermentation at low temperatures. Plate counts showed the total microbial counts (TMC) till levels of almost 10^9 CFU g^{-1} and all cheese samples were dominated by coccus LAB. Not all samples were positive for the presence of enterobacteria, but when they were found their concentrations were at similar levels in both seasons. All colonies with different morphological appearance were isolated and differentiated on the basis of phenotypic characteristics and by randomly amplified polymorphic DNA (RAPD)-PCR analysis. A total of 65 strains were considered and subjected to the genotypic analysis by means of 16S rRNA gene sequencing, which identified 13 LAB species

belonging to five genera (Enterococcus, Lactobacillus, Lactococcus, Leuconostoc and Streptococcus). The species most frequently found were *Streptococcus macedonicus*, *Streptococcus thermophilus*, *Lactococcus lactis* and *Leuconostoc mesenteroides*. The 65 strains were investigated in vitro for their general dairy aptitudes and some strains of the above four species showed technological traits relevant to act as starter strains for this cheese production.
Among those strains, 12 LAB *(Lactobacillus delbrueckii* PON79, PON256 and PON405, *Lactococcus lactis* PON36, PON46 and PON203, *Leuconostoc mesenteroides* PON169, PON259 and PON559, *Streptococcus thermophilus* PON3, PON120 and PON261) were used in different combinations [all strains belonging to each species in triple combinations (*Lb, lactobacilli; Lc, lactococci; Ln, leunostocs; St, streptococci),* all thermophilic strains *(Lb-St,* lactobacilli and streptococci) and all mesophilic strains (Lc-Ln, lactococci and leuconostocs)] to produce experimental cheese by means of a dairy pilot plant. The different bacterial combinations (final concentration of approximately 10^7 CFU/mL) were tested in different conditions: 1, after growth in the optimal synthetic media, re-suspended in Ringer's solution and inoculated in pasteurised ewes' milk; 2, after growth in whey-based medium (WBM) and inoculated in pasteurised ewes' milk; 3, after growth in WBM and inoculated in raw ewes' milk. Plate counts and RAPD analysis were applied to monitor the bacterial evolution during the different trials, while pH was measured to follow their acidifying activities. All lactococci and leuconostocs were able to perform the rapid acidification of the curd in winter conditions; the experimentation was carried in February with 10°C as minimum ambient temperature registered in the room where the curd were left to acidify. A sensory evaluation of the resulting cheeses, obtained after stretching of the acidified curds, indicated the cheeses processed with lactococci in single and multiple combinations as those well appreciated by the judges. These cheeses were then subjected to the analysis of the volatile organic compounds carried out by gas chromatography coupled with mass spectrometry (GC/MS) which clearly showed substantial differences with the control cheese.

Keywords: Lactic acid bacteria; natural whey starter culture; pilot plant; raw ewes' milk cheeses; typicality

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P3.48

Antagonistic impact of lactic acid bacteria isolated from environment

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In the era of targeted trend of returning to nature, consumers are looking for less and less processed food without chemical compounds or preservatives. Resignation of adding substances with properties that inhibit the development of undesirable microflora is not possible for safety reasons. The consequences of such action would result in decreasing the stability of the products, but also by increased incidence of food poisoning. In the face of this trend to replace chemical preservatives maintenance of natural methods, selected strains of bacteria that contains relevant activity, may be worth mentioning alternative. Lactic acid bacteria are microorganisms commonly used in industry, especially in the production of a wide range of fermented products. These are bacteria which have GRAS status (generally recognized as safe), which means that they are safe for the host. Numerous clinical studies have shown the impact of health promoting lactic acid bacteria for both the animal and human. In addition, lactic acid bacteria produce a number of metabolites, such as lactic acid, acetic acid, bacteriocins and hydrogen peroxide. However, in most of the
examples the impact is dedicated to a small group of pathogenic microorganisms (Gwiazdowska and Trojanowska 2005; Kučerová et al. 2006). So researchers are looking for lactic acid bacteria, which will be characterized by extremely wide spectrum of antagonistic.
The aim of the research is to determine the antibacterial activity of strains of lactic acid bacteria. As an indicator microorganisms selected microorganisms which are characterized by very high causative agent of diseases of the digestive system of animals and humans: Aeromonas hydrophila, Campylobacter jejuni, Clostridium botulinum, Escherichia coli, Listeria monocytogenes, Salmonella enteritidis, Salmonella typhimurium, Shigella flexneri, Staphylococcus aureus and Yersinia enterocolitica.
Object of the study were 48 isolates assigned to the group of lactic acid bacteria originating from two corn silages, alfalfa silage and two haylages. The isolates were considered to be LAB according to their morphology, catalase negative characteristic and lactic acid production.
Antibacterial activity of bacteria was determined using the well diffusion method. Incubation proceeded for 24 hours at a temperature suitable to the optimal parameters for the growth of various pathogenic bacteria.

Studies have confirmed a strong antibacterial properties of 35% of strains. Strains of bacteria that will be characterized as the most efficient and at the same time will maximize the antibacterial impact towards selected pathogenic bacteria were selected for further study. The result of research is to develop multidirectional biopreparation containing in its composition of selected strains of LAB.

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P3.49

Study on microbial population dynamics during PDO Fontina maturation in relation to different stages of lactation

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Fontina is a full-fat semi-cooked washed-rind PDO cheese which is traditionally made in Aosta Valley.
The management of cattle farms in Aosta Valley calls for seasonal migration to high pastures during the summer and the concentration of calving during the winter months. This organization of animal husbandry, common to many mountain dairy cattle farms, means that the seasonality of the calving influences average milk composition. Essentially, three phases of lactation (post partum, oestrus and early gestation) can be identified, based on the physiology of the cattle, that seem to affect the activity of microbial populations and the cheese-making aptitude of the milk. The aim of the study was to investigate growth dynamics in microbial populations during cheese maturation in relation to the different lactation phases. Cheese production was monitored 3 times in 2 different dairies for each of the 3 phases. The following samples were collected for each production: milk before and after addition of starter cultures and cheese wedges in various stages of ripeness. The methodological approach followed exploited both traditional approaches based on isolations on selective microbiological media, and culture-independent molecular methods such as PCR-DGGE, using total RNA extracted from the samples. Acidification curves after starter culture inoculation were also determined in milk during the different stages.

The time required for the acidification process to begin increases significantly during the cows' oestrus period. The increase in this time interval may facilitate the development of bacteria which do not favour cheese production and so explain the qualitative differences found in cheeses made in the three periods.
RT-PCR-DGGE highlighted the predominance of *Lactobacillus delbrueckii* and *Streptococcus thermophilus* starter cultures during the fermentation and ripening of all the productions analyzed. On the contrary, the starter *Lactococcus lactis* was detected only in a few samples. Despite the predominance of starter cultures, in a few milk and cheese samples, authochtonous microbiota was found belonging to the species *Enterococcus faecium*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus zeae*, *Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Corynebacterium variabile*. Finally, DGGE profiles were compared by cluster analysis and the dendrograms obtained showed that both the variables, different lactation phases and different dairy farms, affected the presence of the metabolically active microbiota.

Keywords: Fontina PDO; RT-PCR-DGGE; lactation; microbial dynamics

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P3.50

Volatile molecule profiles and sulphur compounds in Trebbiano wines obtained by different *Saccharomyces cerevisiae* strains

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Saccharomyces cerevisiae has being employed since ancient time to produce fermented beverages. Despite ethanol, during alcoholic fermentation, it is able to produce many low molecular weight compounds such as aldehydes, ketons, organic acids and also sulphur compounds (Dubourdieu et al., 2006). The balance between these compounds forms the organoleptic "fingerprinting" of the products. On the other hands, sulphur products are of great importance and able to affect the overall wine aroma. Among sulphur molecules, dimethyl sulphur (DMS), dimethyl disulphur (DMDS), methanethiol (MESH) and thiols play an important role also affected by their concentrations (Ugliano et al., 2009). In wines, the presence of sulphur compounds is the resulting of several contributions such as yeast metabolism, the grape varieties and the malo-lactic fermentation. However, also

the process conditions and the must physico-chemical characteristics can affect the sulphur compound concentrations. Thus, the characterization of the *Saccharomyces cerevisiae* strains has a great importance also on the basis of their ability to produce sulphur compounds. However, this strain ability needs to be evaluated together with the overall metabolic profile since the wine aroma is related to the complex equilibria among the different compounds. In this perspective, principal aim of this experimental research was the evaluation of the volatile profiles, throughout GC/MS technique coupled with solid phase micro extraction (SPME), of wines obtained throughout the fermentation of 10 strains of *Saccharomyces cerevisiae*, gifted of good oenological properties. In addition also the production of sulphur compounds was evaluated by using a gas-chromatograph coupled with a Flame Photometric Detector. Specifically, the ten strains were inoculated in Trebbiano musts and the fermentations were monitored for 20 days. The yeast cell loads were monitored thought fermentation while at the end the analyses of volatile and sulphur compounds as well as aminoacid concentrations were investigated. Moreover, also the physico-chemical characteristics of the wines and their electronic nose profiles were evaluated.
The data showed that the wine volatile and sulphur profiles obtained was strain dependent and consequence of complex interactions which take place in the wine systems. Among the sulphur compound, methanethiol, dimethyl disulphur, trimethyl sulphur, 3-methylthio-propanol, ethyl-3-methylthio-propanoate and 4-isopropylthio-phenol were detected in the samples at different concentrations in relation to the *Saccharomyces cerevisiae* strain employed. The application of the GC/MS-SPME technique allowed the identification and quantification of more than 40 molecules belonging to several classes of compounds and mainly to aldehydes, ketons, esters, alcohols, organic acids and terpenic alcohols. However, also in this case, this analysis showed specific profiles in relation to the S. cerevisiae strain used. Regarding the detected electronic nose profiles, the ten wines obtained were grouped in three clusters on the basis of the affinity with the sensors employed .

Keywords: *Saccharomyces cerevisiae*; wine; sulphur compounds; GC-MS volatile profiles; electronic nose profiles

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P3.51

Evaluation of different processing measures to resist aflatoxin contamination of maize products in Nigeria

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Fungal deterioration of stored seeds and grains is a chronic problem in the Nigeria storage system because of the tropical hot and humid climate (Adhikari et al., 1994).
In this study we determined the different processing methods that can reduce aflatoxin contamination of maize products in the country.
Yellow Maize samples were collected from some villages in Ondo State Nigeria and were screened for the presence of aflatoxin. Some samples were naturally contaminated with toxigenic moulds (Benford et al., 2001) while spore suspensions of toxigenic *Aspergillus flavus* were inoculated into the uncontaminated samples to assess the effect of different processing technique on the level of aflatoxin of the maize products. The maize samples were sorted, washed and soaked in cold water and naturally fermented for 24, 48, and 72 hours. Thereafter milled, sieved and boiled for 10, 20 and 30 minutes to make gruel.
All the processing techniques used reduced the spore's level of the toxigenic *Aspergillus flavus*. The highest reduction was observed in the samples fermented for 72 hours and boiled for 20 minutes 75% of maize collected from the villages show high level of aflatoxin contamination. Aflatoxin B1 occurred more frequently in all the maize samples. Most of the total samples were found to be contaminated with the aflatoxin level even more than 30 ppb (recommended value). Aflatoxins were detected in the simples in a range (20-90) ug/kg levels this was evaluated using Tin layer chromatographic technique.
Fermentation and boiling at 20 minutes of maize is an effective control measure during processing to reduce aflatoxin.

Keywords: toxigenic *Aspergillus flavus*; contamination; processing measures

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P3.52

Characterization of yeast flora of 'Hurma' olives grown in Karaburun Peninsula in Turkey

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Olive is an important agricultural product. 'Hurma' olive, especially grown in Karaburun Peninsula of Izmir, Turkey, has a special characteristic of losing its bitterness throughout its maturation period. Therefore, this type of olive does not require further processing steps for debittering and is more preferable for consumers who have high tension problems than debittered and brined olive products containing salt. According to small number of studies in literature, it was stated that a fungus called Phoma olea is the reason for this phenomena with the help of climactic conditions (Buzcu, 1969; Kalogereas,1932).
Until present, the characterization of yeasts associated with table olives has mainly been made through cultural (biochemical and morphological) methods, using the taxonomic keys given in the literature (Looder, 1970, Barnett et al., 1990). More recently, a considerable development in the identification and classification of yeasts has come with the introduction of molecular techniques, which are rapid, easy and more precise for yeast identification. Previous results have demonstrated that the complex internal transcribed spacer (ITS) regions (non-coding and variable) and the 5.8S rRNA gene (coding and conserved) are useful in measuring close fungus phylogenetic relationships since they exhibit greater interspecific differences (Kurtzman, 1992).
The purpose of this study was to identify yeast flora of Hurma olive and leaves during its debittering phase using cultural (biochemical and morphological) and molecular methods. In addition to Hurma olives, another traditional olive, namely "Gemlik" and leaves of both olive types were also investigated for yeast flora. For this aim, olive and olive leave samples were collected from trees during the maturation period which lasted about 8 weeks between October-December 2011. After isolation and purification of suspicious yeast colonies using yeast selective agar media, microscopic morphological properties were investigated under phase contrast microscope. As a result, totally 48 yeast isolates from 29 Hurma olives, 9 Hurma olive leaves, 3 Gemlik olives and 7 Gemlik olive leaves were identified by

cultural (morphological, biochemically with API 20C AUX test kit) and molecular methods (ITS PCR). The most frequently isolated genera are Candida, Cryptococcus, Rhodotorula, Stephanoascus and Trichosporan.
To the best of our knowledge this is the first study in literature identifying and comparing the yeast flora of Hurma olives possibly responsible for debittering by itself, Gemlik olives and olive leaves. This study also allows comparison of cultural and culture-independent molecular methods for identification of yeasts isolated from olive fruits and leaves.

Keywords: Hurma olive; yeast; characterization; molecular; API

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P3.53

Evaluation of the evolution of autochthonous acid lactic bacteria isolated from water buffalo Mozzarella cheese

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The water buffalo Mozzarella cheese, a typical Italian cheese, has been recently introduced in the thriving Brazilian market with good acceptance by its consumers. Traditionally, it is produced with raw buffalo milk added with natural whey starter culture (NWSC) from the previous day of manufacture. The NWSC consists of autochthonous acid lactic bacteria (LAB) from raw milk that play an important role in the sensory properties of the cheese. In Brazil, this cheese is usually produced with the addition of commercial starter cultures in pasteurized milk. Nowadays there are hardly any studies about the autochthonous LAB involved in the

manufacturing process of this product. Thus, the aim of this study was to evaluate the diversity and evolution of the autochthonous LAB isolated from water buffalo Mozzarella cheese produced with raw milk in the cheese processing. Samples were collected during the steps of manufacture: raw milk, curd and stretching. The cheese and its solution of maintenance samples were collected after being produced. The analysis was repeated after 14 and 28 days of storage. The process was repeated for 3 times. The isolation of mesophiles (MRS medium at 30ºC) and thermophiles (M17 medium at 42ºC), the morphological characterization by Gram staining and the catalase test were carried out. It was obtained 171 isolated cultures that showed characteristics of LAB. Their diversity was evaluated using the genotypic RAPD-PCR and they were identified by 16S rRNA gene sequencing. Most of the isolates showed cocci shaped bacteria, while rod shaped ones were more prevalent in the sample collected during stretching. The cultures were clustered into 61 different clusters by RAPD-PCR considering 85% of similarity. One representative strain of each cluster was identified. The cocci shaped bacteria were identified as *Enterococcus sp*. (41.52%), *Enterococcus faecalis* (0.58%), *Leuconostoc mesenteroides* (23.39%), *Leuconostoc citreum* (2.9%), *Streptococcus thermophilus* (2.33%), *Lactococcus lactis* (2.33%) and *Lactococcus garvieae* (0.58%) and the rod shaped bacteria were identified as *Lactobacillus fermentum* (6.3%), *Lactobacillus casei* (12.28%), *Lactobacillus delbrueckii* subsp. bulgaricus (6.43%) and *Lactobacillus helveticus* (1.16%).

The *Enterococcus sp*. were dominant in all samples collected except for the samples of raw milk and curd, where *Lc. mesenteroids* and *Lb. casei* were majority, respectively. The RAPD-PCR technique showed that some strains isolated from the raw milk remained during the manufacturing process and in the cheese.

The identification and the biotyping of isolated lactic cultures can help on the understanding of population dynamics and dominancy of LAB present in cheese. Thus, future studies may relate these characteristics with cheese quality.

Keywords: RAPD-PCR; 16S rRNA gene sequencing; population dynamics

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P3.54

Diversity and evolution of yeast in fermented dairy beverage production

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Despite the simplicity of the manufacturing process, technological innovations and the availability of quality assurance programs, the manufacturing process of dairy beverages may be subject to numerous sources of contamination when basic hygiene conditions are not met, resulting in undesirable changes in the product. The aims of this study was to characterize the diversity and evolution of the yeast microbiota of dairy beverage samples collected during the processing steps of fermented dairy beverages, as well as the final product at different times of storage. The counting the total lactic acid bacteria and the enumeration of yeast were count in 10 steps of production of fermented dairy beverage of 2 industries (A and B) in 3 different periods. Yeast species were identified by biochemical and morphologic assay. A high population of yeasts was found in all samples collected from dairy B. *Candida krusei*, *Candida pseudotropicalis*, *Candida guilliermondii* and *Rhodotorula glutinis* were isolated from the samples from dairy A. During the manufacturing process, these microorganisms were no longer found after the addition of the starter culture. The dairy B samples showed contamination up to 60 days of storage. The identified species were *C. krusei*, *C. pseudotropicalis*, *C. guilliermondii*, *Candida tropicalis* and *Geotrichum candidum*.

Keywords: dairy products; microbiota; yeasts; *Candida* sp.; *Geotrichum* sp

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P4.1

Study of the character "strain competition" among wild *Saccharomyces cerevisiae* isolated from Sangiovese grapes

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Although a low frequency is reported for *Saccharomyces* species on the grapes, the predominance of *S. cerevisiae* in fermentations has led to its recognition as the principal wine yeast. However, the inoculated starter culture must compete with indigenous yeasts (Barrajón et al., 2009; Capece et al., 2011, 2012) and, consequently, also wines produced by addition of starter cultures are the products of mixed fermentation. The sequential strains and species evolution throughout alcoholic fermentation is mainly determined by susceptibilities to the increasing concentration of ethanol, other factors, such as fermentation temperature, dissolved oxygen content, killer factor, quorum-sensing molecules and spatial density influences, can affect the competitive interaction between yeast species and strains (Perez-Nevado et al., 2006). However, little is known regarding how these factors might affect the dominance and succession of individual species and strains within the total population (Zott et al., 2008). The aim of present work was to verify if a native selected strain of Saccharomyces cerevisiae, resulted dominant during spontaneous fermentations for two consecutive years, dominated also during inoculated mixed fermentations. The analysis of interdelta region (primer pair δ2/δ12) revealed the existence of six different biotypes among 160 *S. cerevisiae* isolates, obtained from spontaneous fermentations of Sangiovese grapes collected in one vineyard (same rows) during four harvest seasons (2006-2010). Only one biotype (indicated with "A") was found for two consecutive vintages; S. cerevisiae isolated during 2006 vintage exhibited all the same biotype, whereas among yeasts isolated during the last sampling four different profiles were found. The six *S. cerevisiae* strains were tested in inoculated fermentations at laboratory scale of *Sangiovese* grape must as single and co-cultures. In all the co-cultures the strain "A", dominant for two consecutive years, was inoculated in combination with the other five strains. The molecular monitoring of fermentative processes was performed by intedelta analysis of yeast colonies isolated from mixed fermentations. The strain "A" was dominant at different level (from 65 to 90%) in almost all the fermentations (except in one case). The wines obtained from single and mixed inoculated fermentations were analyzed for the content of volatile compounds correlated to wine flavor, such as acetaldehyde, n-propanol, isobutanol,

amyl alcohols, ethyl acetate, acetic acid, terpenic compounds, other alcohols, ethyl acetates and esters, volatile fatty acids. Significant differences between wines by single and mixed starters were detected for almost all the compounds analyzed. However, in each mixed fermentations, no statistically significant differences between wine produced by A and mixed starters were found. The analysis of metabolic compounds of single and mixed-cultures wines revealed that the influence of strain "A" on composition of mixed-cultures wines was prevalent in comparison to the other strain included in the mixed starter, confirming the dominance character of this strain. In conclusion, the strain "A" possesses characteristics able to confer it a competitive advantage against the other strains because it resulted a good "competitor" not only among natural yeast population, but it was also capable to dominate mixed fermentations at lab scale.

Keywords: *Saccharomyces cerevisiae*; co-cultures fermentations; strain competition

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P4.2

Saccharomyces cerevisiae and *Hanseniaspora uvarum* interaction during the alcoholic fermentation

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Non-*Saccharomyces* yeasts are predominant on the grapes and the early stages of winemaking (Zott et al., 2008). During the alcoholic fermentation, *Saccharomyces* has a clear ecological advantage over the non-*Saccharomyces* yeast mainly due to the production of ethanol and heat via fermentation (Goddard 2008). The greater competitiveness of *S. cerevisiae* has also been attributed to cell-to-cell contact interactions, to the secretion of toxic compounds and a more quickly and efficiently nitrogen used (Nissen et al., 2003, Albergaria et al., 2010). Species

belonging to the *Hanseniaspora* genus, and specifically to *Hanseniaspora uvarum* was reported to be dominant among the wine non-*Saccharomyces* (Zott et al., 2008). Due to the prevalence of that species among the yeast community, we attempted to study the interactions between *S. cerevisiae* and *Hanseniaspora uvarum* using a binary system in mixed cultures (synthetic must media). According to previous studies (Perez-Nevado et al., 2006), our results show a phenomenon of early death for *Hanseniaspora uvarum* in mixed cultures with *S. cerevisiae* after 100 hours, which was accentuated when the inoculum rate of *S. cerevisiae* was higher. In a contradictory way, the *Hanseniaspora uvarum* dynamic established by quantitative PCR shows a population remaining stable at 105 cells/mL during the whole process. The early death phenomenon was not observed in an experimentation separating the two yeast species cultures physically while assuring a homogeneous media (Transwell® system). This phenomenon seems to be due to a cell-to-cell contact mechanism as it was previously described (Nissen et al., 2003) and not to a change in media, toxic compound or nutritive factors. The cell-to-cell interaction seems to induce a change of the physiological state of *Hanseniaspora uvarum* thus explaining our controversial result: lost of the ability to grow in agar plates and stable high population level obtained by quantitative PCR.

Keywords: *Saccharomyces cerevisiae*; *Hanseniaspora uvarum*; early death; cell-to-cell interaction

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P4.3

Biosurfactants and Tyrosol as quorum sensing molecules affecting PGPR biofilm formation on Biochar

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Soil and rhizosphere amendment with biochar might be considered a tool to improve fertility, crop production and plant responses to climate changes. In some studies, plant-growth promoting rhizobacteria (PGPR) have been found to increase after biochar addition (Kolton et al., 2011), and moreover novel biofilm carriers have been developed by carbonization of plant biomass.
It is clear, however, that physical proprieties of biochar and its adsorption capacity, of different soil adsorbates (i.e. phenols, aromatic alcohol, toxins, autoregolatory molecules and biosurfactants), have to be believed relevant in determining how biochar affects soil microbiota. Specifically some quorum-sensing signal compounds (QS), such as tyrosol and glycolipids (Chrzanowski et al., 2012) may be adsorbed on biochar influencing signal molecule longevity, stability and accumulation. Additionally certain social-microbiological phenomena, like QS, swarming and biofilm formation, in several bacterial species, are connected instead of operating separately (Daniels et al., 2004). For these reasons local accumulations of QS (at microniche level) on biochar or charcoal, may affect biofilm formation and surface establishment of PGPR on this material.
The objectives of this study were to elucidate the relationship between sessile growth on charcoal, and QS signaling for some PGPR strains from different bacterial genera: *Pseudomonas* spp., *Burkholderia* spp. and *Azospirillum* spp. Experiments were carried out *in vitro* and glycolipids produced by *P. aeruginosa* AP02-1 were obtained and considered as QS compounds to evaluate their activities in bacterial macroscopic swarming experiments and biofilm growths on charcoal. Also tyrosol, a relevant QS molecule, able to control the communication in fungal species, was assessed. For *Burkholderia* spp. and *P. fluorescens* (15A), exhibiting plant growth promotion activities on *Tamarix* spp. and tomato, the results highlighted that rhamnolipids synthesized by *P. aeruginosa* and a commercially available anionic surfactant, were both able to improve cellular swarming. Moreover rhamnolipidic addition, on hydrophobic plastic and charcoal, impacted the formation of mature biofilms, as estimated by XTT assay. For *Azospirillum brasilense*, on the contrary, the rhamnolipidic presence on charcoal did not change

biofilm productions and additionally the swarming motility improving, by biosurfactants, was observed only in some phenotypic variants selected in chemostat under nutrient limitations.
The results also indicated that the combined adsorption, by charcoal, of rhamnolipids and tyrosol, at lower concentrations, affected biofilm establishment in some PGPR strains.

Keywords: biofilm; quorum sensing; biosurfactant; charcoal; PGPR

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P4.4

Microbial signal molecules: effect on the growth kinetic parameters of the bacteria *Serratia liquefaciens* and *Pseudomonas putida*

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The present study aimed to evaluate the effect of acylated homoserine lactones (AHLs) on the growth kinetic parameters, lag phase duration (lag) and maximum specific growth rate (μmax), of two bacteria dominating in a wide variety of foods, *Pseudomonas putida* and *Serratia liquefaciens*. Although, these bacteria are associated with the production of signal molecules involved in intra- and inter-species communication, only a few studies have focused on the bacterial response to exogenous signals present in a complex microbial environment. Therefore, aliquots of cell-free culture extracts (CFCE; 20, 50 and 100% v/v) from the AHL-producing strain *Hafnia alvei* 718 (CFCEAHL), and different concentrations (2, 5 and 10μM) of synthetic AHLs, N-3-oxo-hexanoyl homoserine lactone (OC6), were transferred to growth media inoculated with 103 CFU/mL of *Ps. putida* or *Ser. liquefaciens* culture, and held at 4 and 10°C. Cell-free culture extracts from the AHL-lacking mutant *H. alvei* 718 halI (CFCEMUT) and growth medium (0% v/v CFCE) were served as controls. Culture samples were taken at periodic time intervals during growth and tested for AHL induction using the biosensor

Agrobacterium tumefaciens A136 on a well diffusion assay. Microbial data (CFU) were transformed to log10 values, fitted using the primary model of Baranyi and Roberts', and the kinetic parameters, μmax and lag, were estimated. AHL induction was calculated as the ratio of induction diameter of the test sample to the assays' control (positive) sample. The tested *Ps. putida* strain was unable to produce detectable AHL signals, whereas *Ser. liquefaciens* produced AHLs. The presence of CFCEAHL and CFCEMUT affected the growth of the tested bacteria, whereas the presence of synthetic AHLs did not significantly influence bacterial growth, compared to 0% v/v CFCE treatment. Generally, the addition of 20 and 50% CFCEs reduced μmax and increased lag phase duration ($P<0.05$), while higher concentrations (100% CFCEs) prevented bacterial growth. It was observed that in the presence of 20, 50 and 100% CFCE containing AHLs the growth rate was increased for *Ser. liquefaciens* compared to control samples (i.e. CFCEMUT) at 10°C. No significant differences ($P>0.05$) were observed among all other tested cases. Results indicate that the growth of a bacterium may be affected by the presence of AHL signals and/ or other bacterial metabolites existing in CFCEs. The ability of a bacterium to produce or not similar AHL signals may influence growth as well.

Keywords: communication; acylated homoserine lactones; bacterial growth

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P4.5

Anti quorum sensing activity of polyphenol extracts from two Brassicaceae of Southern Italy

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The plant kingdom is source of medicines and contributes extensively to the development of pharmaceuticals. Many plants have co-evolved and established accurately regulated symbiotic or syntrophic associations with bacteria; for this reason, it may not be too surprising that higher organisms are capable of perceiving and responding to these molecules. Current literature estimates 10% of all terrestrial flowering plants on earth are used by different communities in treating diseases, however, only around 1% have gained recognition and validation. Thus, phytochemicals can represent the richest reservoir of novel therapeutics (Lewis and Ausubel, 2006). Several QS-inhibiting phytochemicals, such as polyphenols, are

capable to affect biofilm formation in some bacteria. Brassicaceae are a family of vegetables widely diffused in the world, however, the widest area of biodiversity is represented, in terms of species, by the Mediterranean countries. Numerous surveys highlighted their potential importance as a source of antibacterial substances, contributing these health-related properties to phenolics, flavonoids, glucosinolates and relative analogues. In the Campania region (Southern Italy) two Brassica species are mainly present as typical products: rape broccoli "Friariello" (*Brassica rapa* var *rapa*) and "Torzella" (*Brassica oleracea* var *acephala*). We evaluated the quorum sensing inhibiting activity of the ethanolic extracts of Friariello and Torzella leaves,using the Chromobacterium violaceum a Gram-negative water and soil microorganism, which phenotypic response to AHLs is the production of a variety of factors, including antibiotics, proteases and mainly of violacein, a water-insoluble purple pigment. Rape broccoli Friariello and Torzella were collected from the same experimental farm located in Acerra, Italy. Edible portions were homogenised with ethanol and incubated at 4°C overnight. After centrifugation, supernatant was collected, dried and re-suspended in 3 ml of sterile deionised water. Total phenolics were spectrophotometrically determined at λ= 760 nm with the Folin–Ciocalteu reagent. The disc diffusion method was employed to detect the anti-QS activity of the ethanolic extract from Brassicaceae. In this test, bacterial growth inhibition would result in a clear halo around the disc, while a positive result of quorum sensing inhibition would result in a turbid halo harbouring the pigmentless bacterial cells of Chromobacterium violaceum. Cultures of (*C. violaceum* DSM 30191 were prepared by incubating the bacteria in Nutrient broth for 16-18 h at 30 °C (Fratianni et al.,2011). Different amount of polyphenol extracts (from 1 to 150 µg) were added to *C. violaceum* inoculated Nutrient agar plates (0.1 ml per plate), which were incubated at 30°C for 24 h. Both extracts exhibited Anti Quorum Sensing (AQS) activity, causing the inhibition of the violacein production. Polyphenol profile, evaluated by UPLC-DAD, showed a little bit different composition between the two extracts: for example rutin and apigenin were found in the extract of Torzella. The presence of a specific biomolecule in a vegetal extract may, in some cases, affect its biological activity; in our case, the presence of other biomolecules common to the two extracts (chlorogenic, ferulic and caffeic acids, naringenin, catechin, epicatechin), although not present at the same concentration, probably exerted in synergistic way AQS activity.

Keywords: polyphenols; antiquorum sensing; Brassicaceae

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P4.6

Development of labeled *Saccharomyces cerevisiae* strains for monitoring mixed cultures during wine fermentation

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Interaction relationships (competition, inhibition, mutualism, quorum sensing…) between microorganisms in their natural environment are a key field for modern microbiology. The grape must constitutes a complex and changing ecosystem in which numerous microbial interactions occur. During the first stages of the alcoholic fermentation, yeast populations coexisting in the grape must are particularly affected by the rapid development of *Saccharomyces cerevisiae*. Although they have a slower growth rate, other species (non-*Saccharomyces*) are able to grow in grape juice. Some of them, such as *Torulaspora delbrueckii*, have a particular interest for winemaking as they positively impact the chemical composition of wine and may enhance the wine flavor (Cabrera et al., 1988; Viana et al., 2008). The industrial yeast market, which, until recently, exclusively focused on *S.cerevisiae*, now offers *S.cerevisiae*- non-*Saccharomyces* (including *T. delbrueckii*) multi-starters. The development of these new mixed industrial starters requires a better understanding of the interaction mechanisms between yeast populations.

To study yeast interaction in various environmental conditions, the existing methods to monitoring the populations have some limits. Quantitative PCR, frequently used to rapidly tract different species, fails to give reliable data on the physiological state of yeast populations. Cultural methods on selective media are time consuming and do not allow isolation of viable but not cultivable microorganisms. Here we propose to develop yeast strains labeled with a fluorescent protein allowing species discriminating in a mixed and their physiological state monitoring by flow cytometry.

As a preliminary work, labeled laboratory strains expressing constitutively the YFP or CFP proteins (DeLuna et al., 2010) were used. With this material we validated a flow cytometry protocol for tracking growth, viability and fluorescence intensity. Pure cultures were carried out in different media including natural grape must. In such conditions, most living cells are fluorescent during exponential phase. In contrast, during the stationary phase, the proportion of labeled strains as well as fluorescence intensity significantly decreased. Our results show that sample oxygenation before flow cytometry analysis increased both proportion of live

fluorescent cells and fluorescence intensity and allowing the recovery of more than 80% of fluorescent cells at the end of the alcoholic fermentation. Competition experiments were carried out to measure the effect of fluorescent protein on fitness. When mixed together, YFP and CFP strains have a similar fitness values. In contrast when labeled strains are mixed with wild type strains, the proportion of fluorescent cells is stable for the first ten generations and finally decreases. This result suggests that the constitutive expression of the fluorescent protein has physiological consequences on labeled laboratory yeast especially on growth rate. All these data prompted us to construct by genetic engineering labeled strains with the genetic background of wine starters and expressing constitutively YFP and CFP proteins.
Finally, mixed cultures with non-*Saccharomyces* species were carried out paving the way for rapid monitoring of complex cultures and investigating yeast interaction in natural grape must.

Keywords: Wine; yeast interactions; monitoring populations; labeled yeast

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P4.7

Yeast interactions in controlled mixed wine fermentation

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The use of selected strains of *Saccharomyces cerevisiae* is widely diffused in all winemaking area and it is one of the most technological advances in winemaking.
In recent years the use of non-*Saccharomyces* wine yeast species in mixed cultures together with *S. cerevisiae* to improve wine quality has been suggested as a way of taking advantage of spontaneous fermentations without running the risks of stuck fermentations or wine spoilage (Ciani et al., 2010). Non- *Saccharomyces* yeast species can persist during the various stages of fermentation and they give some beneficial or negative characters to the final product. Several studies showed that the use of selected non-*Saccharomyces* wine yeasts in multistarter fermentation may have a positive impact on metabolic and aroma complessity of the final

product.
In the present work we will investigate on the interactions among some selected non-*Saccharomyces* species and a starter strain of *S. cerevisiae*. A pilot scale multistarter fermentations of *Lachancea thermotolerans*, *Pichia kluyveri*, *Starmerella bombicola*, *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* together with *Saccharomyces cerevisiae* were studied. The biomass evolution and the fermentation behavior of these mixed cultures as well as the analytical profiles (main enological compounds, volatile compounds) of the resulting wines were evaluated. Results showed that different non-*Saccharomyces* yeast species differently influence the *Saccharomyces cerevisiae* starter strain. Likewise, *S. cerevisiae* starter strain strongly influence the biomass evolution and the fermentation behavior of non-*Saccharomyces* yeasts. The chemical and sensory analysis showed significantly differences among the resulting wine. In particular, all mixed fermentations were characterized by a significant increase in isoamyl acetate, phenyl ethylacetate and 2-phenyl ethanol.

Keywords: non-Saccharomyces; mixed fermentation; winemaking

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P4.8

Influence of N-acyl homoserine lactones on growth and enzyme formation of *Pseudomonas* in pasteurized milk

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Heat-stable enzymatic activity of *Pseudomonas* leads to the deterioration of ultra high temperature milk and other milk products during storage. These products are often manufactured using intermediate products. Species of the genus *Pseudomonas*, which produce heat-stable enzymes, are part of the natural microbial flora of raw milk. They can grow at low temperatures and reach high cell numbers in raw milk. *N*-acyl homoserine lactones (AHLs) are produced for the intra-species cell-to-cell communication during growth of *Pseudomonas*. Production of peptidases is mediated via quorum sensing using AHL signaling. The formation and activity of peptidases can only be measured at high cell numbers and high AHL amounts. For the production of intermediate products

pasteurization (i.e. 72-75°C, 15-30 s) is commonly applied to milk in dairies in order to inactivate the psychrotolerant microbial flora. AHLs are still detected in pasteurized milk although the corresponding microorganisms are already inactivated by the heat treatment. Little information is available on whether the remaining heat resistant AHLs can influence the growth and enzyme formation of microorganisms that enter the pasteurized milk as recontaminants. Therefore, the aim of this study was to examine growth and peptidase formation of a *Pseudomonas* ssp. strain in milk supplemented with AHLs. In addition, the effect of quorum sensing inhibitors was also examined.
Thermized milk and broth were inoculated with a *Pseudomonas* strain producing heat stable peptidases, which was previously isolated from raw milk. Growth as well as enzyme formation using the azocasein assay were monitored under absence and presence of AHLs (*N*-hexanoyl-DL-homoserine lactone) as well as cinnamon aldehyde, an inhibitor of the quorum sensing system. Additionally, pasteurized milk containing AHLs was recontaminated and growth and peptidase formation kinetics were obtained.
First results will be discussed providing an insight into growth and enzyme production characteristics of *Pseudomonas* during storage of milk with the aim of maintaining a higher storage stability of milk products.

Keywords: *Pseudomonas*; milk; AHL; spoilage

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P4.9

Biofilm formation and interactions by endophytic bacteria

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Over 99% of microorganisms on Earth live within biopolymers, called biofilm. The formation of biofilms is a prerequisite for the existence of all microbial aggregates as an essential step in the survival of bacterial populations. Biofilm is a phenotype under control of Quorum Sensing. QS allows bacteria to maintain cell-cell communication and regulate the expression of specific genes in response to changes in cell population density. Bacteria produce biofilm when they detect a certain amount of small molecules, produced by their ones, called autoinducers (Donlan 2002; Branda et al, 2005). Two QS processes have been described for Gram-negative bacteria: the production and release of AI-1 molecules, involved in intra-species communication - generally N-acyl homoserine lactone (AHL) - and

AI-2 molecules, associated with inter-species interactions. Bacteria detect the accumulation of signal molecules, and, above a certain threshold concentration, these signals are present in sufficient amount to enable similar transcriptional effectors to activate silent genes. This alters their cell-density dependent gene expression and therefore their behavior (Waters et al., 2005).
Biofilm matrix is formed by extracellular polymeric substances (EPS). EPS contain polysaccharides, as well as proteins, nucleic acids, lipids and humic substances. Functions of EPS comprise the mediation of the initial attachment of cells to different surfaces and protection against environmental stress and dehydration (Sutherland 2001).The biodiversity of plant-associated microbial species is a basic condition to establish plant fitness in each environment.
Azospirillum brasilense, Burkholderia ambifaria, Gluconacetobacter diazotrophicus and *Herbaspirillum seropedicae* are autoinducers producing endophytes. These bacteria live inside the plant and in the rhizosphere. Everyone synthesize at list one type of AHL. They are able to fix atmospheric nitrogen, produce plant growth stimulating substances, such as auxins and protect the host plant from pathogens. It has been shown in previous researches that these plant beneficial microorganisms are of interest for application in agriculture either as biofertilizers or as for phytoremediation agents, to reduce or to substitute agrochemicals (Botta et al., 2013).
Effective stimulation of plant growth, biological fixation of atmospheric nitrogen and its supply to the host depend, however, on an efficient colonization and, in conclusion, in a positive chemical exchange among the microbial community and the host plant (Botta et al., 2013).
In the present research the four bacteria mentioned above were tested for biofilm formation in PDB medium in pure and mixed culture at different concentration and at 24-48 hours of incubation. Composition of EPS for H. seropedicae was studied and SEM images of biofilm were taken.
The results obtained show that gene expression encoding for biofilm formation is not universal at the same cell densities. All bacteria are able to synthesize biofilm. The main biofilm producer is H. seropedicae which induces biofilm formation in all tested strains.

Keywords: biofilm; EPS; autoinducer; endophyte; quorum sensing

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P4.10

C. zemplinina: genetic biodiversity and extracellular hydrolytic enzymes production

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Nowadays, the potential use of non-*Saccharomyces* yeasts in combination with *Saccharomyces cerevisiae* could be an important strategy to mimic the spontaneous fermentations without the risk of arrest and at the same time to increase the complexity of the wine. This application has stimulated the interest of how these non-*Saccharomyces* yeasts can contribute to the final organoleptic properties of the wines. In this context, the aim of this study was to analyze how the extracellular enzymatic activities of *Candida zemplinina* can influence the wine aroma. The screening presented here is a first approach to explore the potential of this yeast to enhance the analytical composition of the wine. In this study, we investigated the physiological and genetic biodiversity of different *C. zemplinina* isolates isolated from grapes, musts and during the fermentation process of diverse varieties of grapes. The molecular identification of the isolates was investigated by RFLP analysis of the 5.8S ITS rDNA region and with denaturating gradient gel electrophoresis (DGGE). Physiological analysis included ethanol, SO_2 and glucose tolerance, production of extracellular esterase, b–glucosidase, pectinase, protease and sulphite reductase activity. All the tests were carried out on agar plates with only exceptions of the esterase activity, ethanol, SO_2 and glucose tolerance that were calculated by measuring OD on microplates. Genetic biodiversity was evaluated with SAU – PCR with primers SAG1 and SCA. The results obtained from the analysis of these isolates, has demonstrated the potential production of extracellular enzymes during the first stages of the wine fermentation. In particular, β – glucosidase activity was observed in 5 % of the isolates, which was not inhibited in present of 5 g/L of glucose in the medium. Pectinolytic activity was not found in any isolates of *C. zemplinina*, indicating a little influence of this yeast to the pectin must (de)composition. It is important to highlight that 77 % of the strains were identified to have a protease activity. The semi quantitative production of H_2S has demonstrated that the 96% of the isolates were produced a medium amount while only 4% produced H_2S in low levels, revealing a species homogeneity. The results of this study, opens new applications of *C. zemplinina* to make the organoleptic profiles of the wines more complex thanks to the enzymatic activities that this species possess. Therefore, future research should be focused on the exploitation of this yeast in combination with *S. cerevisiae*, in order to better

understand the production of these enzymes during the fermentation process.

Keywords: *Candida zemplinina*; hydrolytic enzymes; wine; fermentation; biodiversity

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P4.11

An innovative tool reveals interaction mechanisms among yeast populations

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Alcoholic fermentation (AF) of grape must is a complex bioprocess, involving sequential succession of several yeast communities: the early stage of AF is dominated by non-*Saccharomyces* yeasts (*Hanseniaspora sp.*, *Candida sp.*, etc.) that are gradually replaced by *Saccharomyces* species that completes the fermentation. AF is thus a pertinent model for studying yeast-yeast interactions in a complex environment from a fundamental point of view, and offers the opportunity to investigate the biotechnological impact of yeast interactions on wine quality. Indeed, previous studies showed that the high prevalence of non-*Saccharomyces* yeasts during the early stage of the AF has major (potentially positive) consequence on the aromatic composition and sensory properties of wine. As a result, in the past decade, the industrial yeast market developed multi-starters associating non-*Saccharomyces* yeast to enhance wine aromatic complexity with one *S. cerevisiae* strain allowing full achievement of the fermentation. However, the development of these new mixed industrial starters requires a better understanding of the interaction mechanisms between yeast populations in order to optimize wine quality.
For this purpose, a new double-compartment fermentor was designed, allowing: (1) physical separation of two yeast populations, (2) homogeneity of the culture medium in both compartments, (3) fermentation kinetics monitored by weight loss due to CO_2 release, and (4) independent monitoring of growth kinetics in the two compartments. This tool was used to compare mixed inoculations of *Saccharomyces. cerevisiae* / *Torulaspora delbrueckii* with and without physical separation. Our results revealed that physical contact/proximity between *S. cerevisiae* and *T. delbrueckii* induced an early death of *T. delbrueckii*, a phenomenon previously described by Nissen's team and attributed to a cell–cell contact mechanism (Nissen and Arneborg, 2003; Nissen et al., 2003). In contrast,

when physically separated from *S. cerevisiae*, *T. delbrueckii* maintained its viability. The viable state of *T. delbrueckii* had a marked impact on *S. cerevisiae* which presented delayed growth and presented longer viability. These findings resemble the "quorum sensing" mechanism described in prokaryotes, although the "messenger" molecules remain to be identified.
The double fermentor is thus a powerful tool for studying yeast-yeast interactions and provided evidence for the occurrence of cell–cell contact/proximity phenomenon, as well as a quorum sensing-like mechanism in winemaking conditions.

Keywords: *Saccharomyces cerevisiae*; *Torulaspora delbrueckii*; wine fermentation; cel-cell contact; quorum sensing

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P4.12

Effect of membrane permeabilizers on Acylated Homoserine lactone-based Quorum sensing of a Gram positive bacteria

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Group-based behaviour amongst bacteria involves the diffusion and sufficient accumulation of the autoinducer, a phenomenon which is commonly referred to as Quorum sensing (QS) (Dilanji et al., 2012). The most widely studied autoinducers are the Acylated Homoserine Lactones (AHLs), which until now are found to be exclusively present in Gram negative microbes (Galloway et al., 2011). AHL-based QS was detected in a novel strain of Gram positive bacteria from sea water. The isolate belonging to the *Exiguobacterium* genera was found to produce c3-oxo-octanoyl homoserine lactone (OOHL) as its cognate AHL. Its genetic system was characterized to contain LuxR and LuxI homolog (ExgR and ExgI respectively).
Since diffusion of AHLs can be affected by the surrounding bacterial membrane (Boyer and Wisniewski-Dyé, 2009), membrane permeabilizers could have a direct effect on AHL accumulation, its subsequent autoproduction and ultimately on the related QS phenotype. Membrane permeabilizers namely PolymixinB, Sodium

dodecyl sulphate (SDS), Polyethyleneglycol (PEG) and Ethylene diamine-tetraacetic acid (EDTA) are tested at their subMIC (Minimum Inhibitory Concentration) concentrations. EDTA was found increase AHL production in this strain to by 40%. The effect of EDTA on the surrounding bacterial membrane is observed by Scanning Electron Microscopy.

Keywords: Acylated Homoserine Lactone; quorum sensing; Gram positive; Exiguobacterium; membrane permeabilizers

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P5.1

Use of lactic-acid bacteria to inhibit growth of fungi typical contaminants of bakery products

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Fungal spoilage of bakery products causes conspicuous direct losses and it is considered a threat to consumers' health, due to the fungus' ability to produce very hazardous mycotoxins. In the bakery industry, chemical preservatives (i.e. calcium propionate) are commonly used, however, during the last few years, to meet the consumer's demands, producers choose to rely on natural methods of biopreservation. Lactic-acid bacteria (LAB), with a long history of use as biopreservation agents, show good perspective of application also in the bakery industry. Actually they represent, together with yeasts, the dominant microbial component in sourdough, the leavening agent used in the traditional bread making process. During fermentation process, LAB produce antimicrobial metabolites, such as organic acids and small proteinaceous compounds externally secreted, known as bacteriocins.The aim of this study was to investigate the potential of LAB in the prevention of fungal growth. Twenty-one strains of *Lactobacillus plantarum* isolated from sourdoughs for the production of Italian Panettone, 3 strains of *Lb. plantarum*, 1 *Lb. sakei* and 4 *Lactococcus lactis* subsp. *cremoris* isolated from other food matrices were grown in SDB (Sourdough Broth) medium, after 24 hours the resulting fermentation products (FP) were filtered and tested against 18 fungal strains, common contaminants of bakery products. Microdilution tests were performed and propagules germination and mycelial growth were monitored by optical density for 6 days. At least 30% of the selected bacterial strains had a prolonged inhibitory activity towards more than 70% of the fungal strains. Some fungal strains (i.e. *Penicillium* spp.) resulted particularly resistant, but not completely insensible to LAB antimicrobial compounds. In order to investigate the possible mode of action of LAB, in a second series of microdilution tests, the fungal growth was monitored both in FB, as the previous tests, and in FB in which the action of organic acids and bacteriocins was neutralized by rising the pH or by adding the proteolytic enzyme proteinase k, respectively. Analyzing the fungal growth rate, we observed that the inhibitory action was lost in altered conditions, proving that it was probably linked to the presence of organic acids and/or bacteriocins in the growth media. In conclusion, the obtained results provide new perspectives for a possible use of LAB's antimicrobial compounds to control

fungal contaminants and extend the shelf-life of bakery products.

Keywords: Lactic-acid bacteria; fungal spoilage; bakery products; bacteriocins

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P5.2

Survival of *Listeria monocyt*ogenes and *Salmonella* spp. in Cacciatore and Felino, two Italian fermented sausages representing short- and long- ripening times

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Fermented sausages are generally considered safe. However, some notable outbreaks associated with fermented foods have occurred (Adams and Mitchell, 2002). Depending on manufacturing conditions, foodborne pathogens may survive at the end of the process. Moreover, foodborne pathogens may adapt to the dynamically changing environment of the fermented sausages allowing better survival. The objective was to evaluate the survival potential of *Listeria monocytogenes* and *Salmonella* spp. in Cacciatore (fermentation-ripening 20 days) and Felino (fermentation-ripening 40 days) fermented sausages.
Sausages batter was inoculated with a five-strains cocktail of *L. monocytogenes* or *Salmonella* spp. (approximately 10^5-10^6 cfu/g) and their survival was monitored at specific time points during sausages manufacturing. For each pathogen and sausage, four different batches were prepared at different times. Kinetic parameters (inactivation rate, kmax in day-1; and time needed for 1 log reduction of the pathogen, D-value in days) were calculated after fitting kinetic behavior models and selecting the best model based on statistical indices. Model fitting was performed using GraphPad Prism 5. Significant factors (pH and aw) for *L. monocytogenes* and *Salmonella* spp. inactivation were assessed by multiple regression using SPSS v15.1.
L. monocytogenes and *Salmonella* spp. survival in both sausages followed a log-linear trend. Inactivation of *L. monocytogenes* during manufacturing of Cacciatore

(kmax=0.04, D-value=52) and Felino (kmax=0.02, D-value=111) was relatively small (only 0.4 log cfu/g). On the other hand, *Salmonella* spp. displayed faster inactivation (kmax=0.13 and 0.09, D-value=18 and 24, total inactivation=1.1 and 1.6 log cfu/g for Cacciatore and Felino, respectively). Multiple regression showed that aw was a significant parameter ($p<0.05$) for *Salmonella* spp. inactivation in both products, explaining 60-70% of the variance observed in the data. For *L. monocytogenes*, aw was significant ($p=0.002$) during its inactivation in Felino (40% of explained variance) whereas pH ($p=0.003$) during its inactivation in Cacciatore (50% of explained variance). A part (10-15%) of the remaining unexplained variance was attributed to the applied temperature.
Salmonella spp. proved to be more sensitive than *L. monocytogenes*. Both pathogens, however, survived relatively well as result of the conditions (pH, aw and fermentation temperature) prevailing during manufacturing of the sausages. Water activity proved to be a key factor. Quantitative analysis of the data originating from challenge tests may provide critical information on which combinations of the process parameters would potentially lead to better control of the pathogens. Moreover, the analysis clearly identified factors that need to be validated experimentally, i.e. Felino characteristics lie within the range that may support growth of *L. monocytogenes* according to EC regulation 2073/2005 and its amendment 1441/2007.
This work was supported by the EU project LisGenOmics (People, MC-CIG, 7th Fram. Prog., PCIG09-GA-2011-293406).

Keywords: *Listeria monocytogenes*; *Salmonella*; fermented sausages; survival

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P5.3

Antimicrobial activity of *Metschnikowia pulcherrima* in winemaking

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Metschnikowia pulcherrima occurs naturally on flowers and fruits such as grape berry. Some strains are effective as biocontrol agents against pathogens of fruits (El-Ghaouth et al., 1998; Janisiewicz et al., 2001; Santos et al., 2004). In this study, five different strains of *M. pulcherrima* were evaluated for inhibition of

growth of the main oenological yeast involved in winemaking process. The effective antagonistic actions of *M. pulcherrima* strains was showed on *Pichia*, *Candida*, *Hanseniaspora*, *Kluyveromyces*, *Saccharomycodes*, *Torulaspora* and *Brettanomyces/Dekkera* strains, while *Saccharomyces cerevisiae*, the main species of wine fermentation, does not seem to be affected by *M. pulcherrima*. Interestingly, in fermentation trials carried out in grape juice, *M. pulcherrima* showed a wide and effective antimicrobial actions on undesired wild spoilage yeasts, such as *Brettanomyces/Dekkera* yeasts. In fermentation trials with mixed culture of three species, *M. pulcherrima* confirmed the antimicrobial action against *Brettamomyces bruxellensis* without any negative influence on *S. cerevisiae*. These data further support the potential use of selected *M. pulcherrima* strains in controlled multistarter fermentations with *S. cerevisiae* starter cultures.
Preliminary investigations in the mode of action of the *M. pulcherrima* excluded the killer phenomenon, showing the involvement of pulcherriminic acid during antimicrobial activity. This study shows that although all *M. pulcherrima* strains assayed produce the pulcherrimin pigment, the antimicrobial activities showed specific and significant variations among the different yeasts tested.

Keywords: Antimicrobial activity; winemaking; *Metschnikowia pulcherrima*

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P5.4

Antagonistic effect of endophytic bacteria against pathogenic fungi of *Lycopersicon esculentum*: *Fusarium oxysporum* f. sp. *radici*, *Fusarium oxysporum* f. sp. *lycopersici* and *Pythium ultimum*

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Plants offer large and diverse niches for microorganisms, bacteria and fungi, in particular. Bacteria which live in the rhizosphere benefit from root exudates, but some of them are capable of entering the plant as endophytes, not causing harm and establishing a mutualistic association with the host plant (Compant et al.,

2010). These bacteria can stimulate plant growth and manage soil and plant health. In spite of their different ecological niches, free-living rhizobacteria and endophytic bacteria use some of the same mechanisms to promote plant growth and control phytopathogens (Benhamou et al., 1998; Bashan et al., 1998; Botta et al., 2013).

Agricultural production intensified over the past few decades, but producers became more and more dependent on agrochemicals. However, the increasing utilization of chemical inputs causes several negative effects, i.e., development of pathogen resistance and negative environmental impacts. Furthermore, the growing cost of pesticides and consumer demand for pesticide-free food has led to a search for substitutes for these products.

Biological control is thus being considered as an alternative or a supplemental way to reduce the utilization of agrochemicals (Welbaum et al., 2004).

Aim of this work was to analyze the antagonistic capability towards some pathogenic fungi of four endophytic bacteria selected for their capability of fixing atmospheric nitrogen and stimulating plant-growth: *Azospirillum brasilense*, *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Burkholderia ambifaria*. Tomato plant - *Lycopersicon esculentum* cultivar Sanmarzano - was chosen because its relevance among the Italian crops. It needs high nitrogen, phosphate and potassium inputs, and is frequently affected by attack of the three pathogenic fungi tested in this work: *Fusarium oxysporum* f. sp. *radici*, *Fusarium oxysporum* f. sp. *lycopersici* and *Pythium ultimum*.

Antibiosis tests for individual bacteria against each single pathogens show the inhibition effects of individual bacterial against each of the tested pathogen.

In a second experiment, tomato seeds were inoculated with the bacterial mix, both in the presence and in the absence of the single pathogenic fungus.

After 30 days-growth in greenhouse, tomato plants were analyzed for biometric parameters. All microorganism, both bacteria and pathogenic fungi, colonized tomato plant. The experiment demonstrates the effectiveness of the bacterial inoculum on the tomato plant; endophytes exert inhibitory actions in a different way against the three pathogens.

Keywords: endophytic bacteria; tomato plant; pathogenic fungi

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P5.5

Control of tyramine production by *Enterococcus faecalis in vitro* through the use of *Lactococcus lactis* bioprotective cultures.

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Together with the histamine, tyramine is the biogenic amine with the most dramatic acute affects. Tyramine intoxication is also known as "cheese reaction" because it can be accumulated in high amounts in cheeses and other fermented foods. Tyramine formation is the result of bacterial decarboxylation of tyrosine that is carried out by several microorganisms, including strains of lactic acid bacteria (LAB) belonging to different genera and species (Coton and Coton, 2009; La Gioia et al., 2011). However, the major responsible for its production are usually enterococci which can be present during ripening. In order to contrast the metabolic activity of these microorganisms several strategies can be adopted. Among these, the use of competitive microflora able to limit enterococci growth, can be proposed. It is well known that lactococci can produce several bacteriocins (Beshkova and Frengova, 2012), including nisine, which can exert antimicrobial activity against a wide spectrum of bacterial species.
In this work the competition between three *Lactococcus lactis* strains, isolated from raw milk, and the tyraminogenic strain *Enterococcus faecalis* EF37 (La Gioia et al., 2011; Gardini et al., 2001) has been studied. The three *L. lactis* strains were chosen for the different inhibiting potential against several Lactic Acid Bacteria shown in the screening phase. These strains were inoculated at a level of about 6 Log CFU/ml in BHI Broth added with 0.5% (w/v) of tyrosine in the presence of different inoculums of *E. faecalis* EF37 (2, 3, 4, 5, 6 Log CFU/ml). The broth was incubated at 30°C (± 1) and plate counts were performed after 0, 8, 24, 48, 72, 96 hours using selective conditions for the two species. At the same time, the samples were analyzed for the tyramine concentrations with HPLC method. Also the growth and the tyramine production of *E. faecalis* cultured alone were monitored: all the samples, independently of the initial inoculums level, reached the stationary phase (about 9 Log CFU/ml) within 24 hours when the maximum amounts of tyramine were already accumulated (about 300 mg/l).
The strains *L. lactis* N44 resulted the most effective in reducing the decarboxylase activity of *E. faecalis* Ef37. In fact, when the tyraminogenic producer was

inoculated at 2 Log CFU/ml, its maximum growth was lower than 6 Log CFU/ml and, consequently, the tyramine detected resulted less than 20 mg/l during all the incubation period. Also when the *E. faecalis* EF37 inoculum level was 3 Log CFU/ml the growth potential was reduced together with the tyramine amount (less than 100 mg/l). The strains N63 did not show any competition activity while *L. lactis* N17 was able to partially reduce the maximum tyramine amount (lower than 100 mg/l) only in the presence of an initial *E. faecalis* inoculum of 2 Log CFU/ml. Given the encouraging results obtained in this in vitro experiment, the study of this dynamics in real food systems could be taken into consideration. In fact, the usual concentration of enterococci present in fermented food at the beginning of fermentation and ripening period can be controlled by the activity of bioprotective *L. lactis* strains that can be effective in significantly reducing the tyramine concentration in food.

Keywords: *Lactococcus lactis*; *Enterococcus faecalis*; tyramine; bioprotective cultures

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P5.6

Selection of *Pseudomonas* able to produce fluorescent pigments

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The genus Pseudomonas, particularly the group of fluorescent *Pseudomonas* are able to produce a range of secondary metabolites. Fluorescent *Pseudomonas*, when growing under iron limited conditions, produce siderophores, a low molecular weight (< 10 KD) iron chelating compounds. Although iron is the fourth most common element in the earth's crust, siderophores are required to solubilize precipitated iron. We are performing an assessment of environmental conditions for the efficient production of siderophores by benefic bacteria, in the presence of copper. In the culture supernatants of medium B of King, which has been used to identify bacterial cells that produce a soluble fluorescent pigment called pyoverdin

(formerly called fluorescein) which is a type of siderophore (Cox et al., 1985), the accumulation of pigments was determined at 400 nm (Carrillo-Castañeda, et al., 2005). A group of strains isolated from different sources are being characterized according to their ability to grow on different culture media. The isolates grew on the medium B of King, 28% grow well on a minimal medium (RMin) (Carrillo-Castañeda, et al., 1988) and 22% on an iron deficient minimal medium (RMin – Fe). In the supernatants of bacterial cultures developed in the RMin culture medium, 17% of the isolates showed spectrophotometer readings at 400 nm over 0.3 (strains A7, A9 and A9m) being the highest value of 0.43 (A9m) while in 44% of the supernatants of cultures grown in the medium Rmin-Fe (A7, A9, A9m, Avm), exhibited higher readings being 0.71 the highest value recorded. In both culture media, the level of development and production of pigments was not directly related. Most of these strains promote the rate of Fe3+ transport and thus alleviate the problem of iron unavailability in plants and exhibit antagonistic activity that interferes the growth of target pathogenic microorganisms.

Keywords: Siderophores; pigments; culture growth

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P5.7

Evaluation of fungitoxic effects of *Ageratum conyzoides* Linn

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A. conyzoides is allelopathic plant and have many antifungal compounds which can be used for the treatment of certain fungal species. The present study was designed to evaluate the antifungal activity of aqueous extract of different plant parts of *A. conyzoides* (Inflorescence, leaf, stem and root) was determined against the mycelial growth and biomass production of *Fusarium solani* (Mart.) Sacc. The cause of Fusarial wilts of Egg plant (*Solanum melongena* L.) A measured reduction in F. solani biomass was observed due to aqueous extracts of different concentrations. Leaf extract proved to be more effective as compared to the other parts in the flask experiment. Further pot experiment was conducted for more

conspicuous results. It was noticed that as the concentration of leaf residue was increased from lower to higher i-e 2%-6% in the soil the disease incidence decreased significantly. The present study revealed that *A. conyzoides* have potential to control the growth of F. solani.

Keywords: *Ageratum conyzoides*; antifungal activity; aqueous extract; *Fusarium solani*; mycelial growth

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P5.8

Microbial ecology during ripening of a Minas Padrão cheese produced with raw goat milk and a *Lactococcus lactis* nisin Z producer

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Minas Padrão cheese is one of the most popular cheeses in Brazil, and a typical food product from Minas Gerais State. It is usually produced with pasteurized cow milk, and the addition of alternative ingredients can enhance its value and quality. In such context, Minas Padrão cheese produced with goat milk would be an interesting alternative for this producing sector. This study aimed the characterization of the changes in the microbiota of Minas Padrão cheese made with raw goat milk during ripening, and the interference of an added starter culture capable of producing nisin Z (a *Lactococcus lactis* strain, previously isolated from raw goat milk). Minas Padrão cheese was produced with raw goat milk added (Treatment 1, T1) or not (Treatment 2, T2) with a nisin Z producer *Lactococcus lactis* strain. Cheeses were ripened during 60 days, and samples were collected at beginning of ripening (Day 0), at every 5 days until 30 days of ripening, and after 60 days of ripening. In each sampling, pH was measured and microbiological analyses were conducted (aerobes, coliforms, *E. coli*, coagulase positive and negative staphylococci, *Enterococcus*, mesophilic and termophilic cocci and rods, and yeast and moulds). The experiment was conducted in three repetitions and the results for T1 and T2 were compared. pH values from T1 and T2 cheeses did not present relevant differences during all steps of ripening, ranging from 5.3 to 6.1. Similar results were observed for the tested microbial populations, except for coagulase positive staphylococci. This group was kept controlled at 2.9 to 3.4 log CFU/g in T1 cheeses until 30 days of ripening, and after 60 days it was not detect. In counterpart, coagulase positive staphylococci in T2 cheeses ranged from 5.3 to

4.9 log CFU/g until 30 days of ripening, and finished at 3 log CFU/g after 60 days. Considering the similar pH variation in both treatments, the bacteriocin production is the most probable cause of the decrease and control of coagulase positive staphylococci in T1 cheeses. The nisin Z producer *Lactococcus lactis* strain was previously characterized as possessing bacteriocingenic activity against *Staphylococcus aureus*, confirming this inhibitory interaction. It was demonstrated the bacteriocinogenic activity of the *Lactococcus lactis* strain against coagulase positive staphylococci, representing an alternative biopreservative tool in the Minas Padrão cheese produced with raw goat milk.

Keywords: Minas Padrão cheese; *Lactococcus lactis*; Nisin Z; coagulase positive staphylococci

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P5.9

Influence of rhizobacterial volatiles on soil-born fungi

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Soil is one of the major habitats of bacteria and fungi. In this arena their interactions are part of a communication network that keeps microhabitats in balance (Effmert et al., 2012). Many rhizobacteria inhabiting here are classified as plant growth promoting rhizobacteria (PGPR) and they are potentially useful for biological control because of their capability to suppress soil-borne plant diseases (Whipps et al. 2001; Lugtenberg and Kamilova et al. 2009). Prominent mediator molecules of this biological control are inorganic and organic microbial volatile compounds. These volatiles are low molecular mass compounds with high vapor pressures, low boiling points, and a lipophilic character (Effmert et al., 2012). For example Serratia sp. 4Rx13, a gram-negative γ-proteobacteria synthesizes and emits volatiles like β-Phenylethanol, Dimethyldisulfide, Dimethyltrisulfide, Methanol, Sodorifen (Kai et al., 2007 and 2010) which influence the growth of plant pathogenic and non-pathogenic fungi an in-vitro dual-culture system.
Further investigations using the plant-pathogenic fungi Rhizoctonia solani, Sclerotinia sclerotiorum and Phoma eupyrena and the non-pathogenic fungus Neurospora crassa were done to explore the effects of bacterial volatiles of Serratia sp. 4Rx13 on the fungal growth and fungal enzyme activities. In in-vitro dual-culture systems overnight cultures of the bacteria were applied on one side of a bipartite Petri dish, on the other side of the dish a mycelium plaque of the fungi

were placed. This experimental design only allowed volatiles to move from one side to the other side of the Petri dish. The effects of volatiles were quantified by measuring radial growth and biomass of these fungi. All four fungi showed growth inhibition when exposed to bacterial volatiles of Serratia sp. 4Rx13. In addition, these fungi showed increased catalase activity which could indicate for reactive oxygen species (ROS) activities. Furthermore, Rhizoctonia solani and Sclerotinia sclerotiorum did not show lipid peroxidation activity while Phoma eupyrena and Neurospora crassa showed an increase in lipid peroxidation which indicated cell-membrane stress.

Keywords: Rhizobacteria; fungi; volatiles; growth inhibition; ROS

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P5.10

Antimicrobial peptides from *Saccharomyces cerevisiae* induce physiological changes in *Hanseniaspora guilliermondii*

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Saccharomyces cerevisiae secretes antimicrobial peptides (AMPs) during alcoholic fermentation that are active against other wine-related yeasts (e.g. *Hanseniaspora guilliermondii*) (Albergaria et al., 2010) and bacteria (e.g. *Oenococcus oeni*) (Osborne and Edwards, 2007). In the present study we assessed the physiological changes induced by those AMPs on sensitive yeast cells of *Hanseniaspora guilliermondii*, namely membrane permeability and intracellular pH (pHi) alterations. Membrane permeability was evaluated by staining cells with propidium iodide (PI) and pHi by the fluorescence ratio imaging microscopy (FRIM) technique (Guldfeldt and Arneborg, 1998). Results showed that after 20 min of incubation with inhibitory concentrations of AMPs, the average pHi of cells

dropped from 6.5 to 5.4. After 8 h of incubation, 32% of the cells had lost their ΔpH (=pHi-pHext) and after 24 h that percentage rose to 77%. The culturability (plating) and viability (PI staining) of the sensitive yeast cells also decreased in the presence of the AMPs. After 24 h of exposure to AMPs, 61% of the cells were dead (PI-stained) and the number of viable cells fell from 1×105 to 1.5 CFU/ml, which means that virtually all cells (99.999%) became unculturable but a sub-population of 39% of cells remained in a viable but non-culturable (VBNC) state. However, those VBNC cells were able to recover their culturability after incubation at optimal growth conditions. Our study revealed that the mode of action of these AMPs seems to be primarily targeted to the cell membranes, reducing their permeability and preventing cells to maintain pH homeostasis.
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Keywords: pH homeostasis; fluorescence ratio imaging microscopy (FRIM); intracellular pH (pHi); membrane permeability; mode of action of AMPs

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P5.11

Class I integrons as a bioindicator in freshwater channels in Northern China

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The emergence and spread of antibiotic-resistance is of general concern worldwide. To understand the causal factors driving these processes, we need to identify antibiotic resistant determinants in the environment. Such systems can be both as a source and reservoir of resistance genes. The broader environmental prevalence of antibiotic resistance genes is in general poorly studied. Class I

integrons are genetic elements that serve as hubs for many and diverse antibiotic resistance genes in the hospital and have been studied in detail They were chosen as biomarker for antibiotic resistance. We analyzed as a case study environmental samples from the city of Zhangye (Gansu province, China), with a population of 1,200,000 inhabitants, located within a big oasis surrounded by the Gobi desert and Qilianshan Mountains. The city area is characterized by the widespread presence of Phragmites australis (Cav.) Trin (common reed), that covers all the channels and the ponds within the urban area, the industrial zones and the surrounding agricultural land.
Twenty samples of P. australis root-associated sediments were collected from flowing water of wastewater treatment channels, as well as industrial, urban, agricultural area and in the reed stand National Park located south of Zhangye. Chemical analyses of the concentration of heavy metals and nutrients have also been done. Highly polluted sediments were found in samples from the industrial areas, where abnormal concentrations of zinc, arsenic, cadmium and lead, in particular, have been recorded. Our results show the presence of integrons void of gene cassettes in all sampling sites, and a higher presence of integrons carrying various gene cassettes in the urban and agricultural area. The gene cassettes are currently being characterized. Moreover, we did not detect the presence of class I integrons and relative gene cassettes from the samples of the National Park. Our preliminary results indicate that class I integrons could be a promising biomarker in highly complex freshwater environments affected by a broad spectrum of pollutants.

Keywords: Class I integrons; rhizobacteria; bioindication; reed; freshwater

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P5.12

Development and validation of a chemically defined medium used for studying foodborne bacterial-fungal interactions

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The use of protective cultures as natural preservatives has gained increasing interest in the food industry due to many consumers' preference for less chemical

preservatives. Lactic acid bacteria and dairy propionic acid bacteria are important organisms in some fermented foods and can, alone or in combination, be used as natural preservatives to control spoilage by food-borne fungi. The mechanisms behind antifungal activity of protective cultures are still not fully elucidated, but a synergistic action of several microbial metabolites is believed to take place. Although various antifungal compounds have been described, a major problem in the identification and ranking of the antifungal compounds is the complexity of many food matrices and their interaction with microbial activity. In order to reduce the matrix noise a chemically defined microbial interaction medium (CDMIM) was developed supporting the growth of commercialized protective cultures (*Lactobacillus paracasei* and *Propionibacterium freudenreichii* subsp. *shermanii*) and indicator fungi (*Penicillium spp*., *Rhodotorula mucilaginosa*, and *Debaromyces hansenii*). Ferment of protective cultures grown in CDMIM inhibited the growth of the indicator fungi with results comparable to a milk based medium. The use of CDMIM will allow a better differentiation for future chemical characterization of mixed metabolites. The pros and cons of using CDMIM will be discussed.

Keywords: protective culture; antifungal; microbial interaction; chemically defined medium

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P5.13

Yeasts as biological agents to control moulds and filamentous fungi: study in vitro and in vivo

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Moulds colonization annually destroys large amounts of vegetables, fruit and cereal grain during cultivation, harvest, handling, transport and post-harvest storage (Druvefors et al., 2005). In conventional agriculture, the control of these pathogens mainly is managed treating the crops with chemical fungicides. In view of a progressive reduction or elimination of chemical fungicides the use of natural treatments are desirable in particular in organic agriculture where the use of chemical compound are not permitted. Among the natural procedures of pest control, the use of antagonistic microorganisms, which may counteract the mould and/of pathogen filamentous fungi growth, have been suggested. In particular the yeasts such as *Candida oleophila*, *Cryptococcus albidus*, *Wickerhamomyces*

anomalus, *Pichia angusta*, *Pichia membranifaciens*, *Debaryomyces hansenii*, *Metschnikowia pulcherrima*, are used in the biological control (Janisiewicz and Korsten, 2002). In addition to the inhibitory activity of some yeasts, other physical-chemical factors such as content of nutrients in the natural matrix, oxygen concentration, ethanol and ethyl acetate production, play an important role in microbial interactions during the biological control (Bleve et al., 2006).
In this investigation we evaluated the interaction between several killer yeasts and some filamentous fungi that generally colonize mature fruits, such as *Botrytis*, *Penicillium*, *Monilia* and *Aspergillus* strains. Preliminarily, it was developed a simple in vitro assay to detect the couples, killer yeast-mould. Unsin this method, a total of 42 killer yeasts were screened to select strains with antimicrobial activity. In a second step, ten selected strains were tested for their effective inhibitory activity againsts moulds in vivo assay on grapes, lemons, oranges and strawberries. In strawberry fruits, the evaluation was carried out also in pre and post-harvest phase.
Results indicated that the best antagonistic activity was exhibited by *Wickerhamomyces anomalus* and *Metschnikowia pulcherrima* species that produced a significant reduction of gray and green moulds after in vivo fruits infection.

Keywords: Mould; Botrytis; killer yeasts; fruit

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P5.14

Microbial interactions in food model systems: In situ antilisterial activity of mundticin KS producing strains

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It is known that microbial interactions are important for the success of food

fermentations, as well as for the establishment of the safety of the final products. In this study, we characterized extensively three enterococci (WFE3, WFE20 and WFE31) of flour origin, which were found to be active against *Listeria monocytogenes*, in order to investigate on their ability to produce bacteriocins in complex food model systems for their future industrial/food applications. The identification of the three bacterial isolates was performed by means of a combined 16S rRNA gene sequencing and multiplex PCR approach. Two isolates belonged unequivocally to the species *E. mundtii*, while one isolate could not be allotted into any described *Enterococcus* species. The randomly amplified polymorphic DNA (RAPD) analysis recognized three distinct strains. The supernatants were mainly active against *Listeria* spp., but some lactic acid bacteria were also inhibited. The proteinaceous nature of the three supernatants was detected after treatment with proteinase K, protease B and trypsin. The bacteriocins were found to be heat resistant, stable in a large pH range and in presence of ethanol. The bacteriocins were not adsorbed onto the surface of the producer cells and their mode of action was bactericidal. The production of bacteriocins was higher at neutral pHs and temperatures in the range 30-37°C. The active supernatants did not show cytotoxicity and the three strains were susceptible to the action of common antibiotics. The genetic characterization of the bacteriocin genes showed that all three strains produced mundticin KS. In order to evaluate the effect of different food components on the inhibitory activity of the *E. mundtii* strains, five food model systems were prepared from fresh vegetables, cereals, cheeses, meats and fishes. The broths were inoculated first singly with the strains WFE3, WFE20 and WFE31 at a final concentration of approximately 10^6 CFU ml-1. We observed that the three strains produced mundticin KS in all the five food model systems. Then the efficacy of the three *E. mundtii* strains was evaluated *in situ* against *L. monocytogenes*. The five model systems obtained from different food matrices were inoculated singly with one of the bacteriocin producing *E. mundtii* strains and *L. monocytogenes* ATCC 19114 added at approximately 10^4 CFU mL^{-1}. The *in situ* antilisterial activities of the strains WFE3, WFE20 and WFE31 were quantitatively different.

Keywords: bacteriocins; *Enterococcus mundtii*; food model systems; in situ activity; *Listeria monocytogenes*; Mundticin KS

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P5.15

Yersinia enterocolitica behavior in the presence of the bacterivorous *Acanthamoeba castellanii*

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Free-living protozoa are unicellular, eukaryotic micro-organisms ubiquitous in natural aquatic and terrestrial environments and present in diverse anthropogenic and food related habitats. Free-living protozoa are bacterial predators, but some bacteria are able to evade protozoan uptake and/or digestion, turning the protozoon into a reservoir, shelter, vector or virulence training ground. *Yersinia enterocolitica* is the third most reported foodborne pathogen in Europe and is associated with the consumption of raw or insufficiently heated pork.
In vitro cocultivation assays were set up to test if *Y. enterocolitica* is resistant to predation by the protozoon *Acanthamoeba castellanii*. Furthermore, we assessed if environmental factors and bacteria specific characteristics influence this interaction. Therefore, four *Y. enterocolitica* strains with different virulence properties (absence or presence of the *Yersinia* Virulence plasmid *pYV*) and different serotypes (4/O:3 and 2:O9) were cocultivated with *A. castellanii*.
The four *Y. enterocolitica* strains resisted predation by A. castellanii for at least 14 days, irrespective of medium (nutrient rich/poor) and temperature (7, 25 and 37°C) used. Moreover, association with A. *castellanii* enhanced the survival of the *Yersinia* strains under nutrient rich conditions at 25°C. Factors excreted by one *Y. enterocolitica* strain showed a temperature-dependent permeabilizing effect on the protozoa. Long-term intraprotozoan survival of *Y. enterocolitica* was dependent on nutrient availability and temperature, with up to 2.8 log cfu/ml bacteria surviving intracellular at 7°C for at least four days in nutrient rich medium. Transmission electron microscopy revealed that intra-amoebal yersiniae were located in the amoebal cytosol.
As *Yersinia* and *Acanthamoeba* share similar ecological niches, this interaction suggests a role of free-living protozoa in the ecology and epidemiology of *Y. enterocolitica*.

Keywords: *Yersinia*; protozoa; predation

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P5.16

Study of the inhibition of *Campylobacter jejuni* NCTC11168 by *Lactobacillus salivarius* SMXD51 in coculture and virulence assays

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Lactobacillus salivarius has gained attention as a promising probiotic species that influences cytokine profiles and modulates cellular responses to pathogenic challenge. In a recent study, we isolated *L. salivarius* SMXD51 from the cecum of a Tunisian poultry (Messaoudi et al., 2011). This strain was found to produce a bacteriocin-like substance active against *Campylobacter jejuni*, known as the major bacterial foodborne pathogen in humans and a common commensal of poultry. The bacteriocin, salivaricin SMXD51, was purified and characterized (Messaoudi et al., 2012). In addition to *Campylobacter*, salivaricin SMXD51 showed inhibition against a number of other foodborne pathogens, such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella*, without affecting lactic acid bacteria growth.
So in order to get more information about this interesting strain, the research was, in a first step, to study the interaction at 37 °C between *L. salivarius* SMXD51 and *C. jejuni* NCTC 11168 in mid model BHI (Brain Heart Infusion) buffered to pH 7. The coculture at 37°C was followed for 56 hours, repeated three times independently and a control culture with *C. jejuni* alone has been also achieved. Every 6 hours, the enumeration of *L. salivarius* was performed on MRS medium incubated at 30°C and the one of *C. jejuni* at 42°C on selective medium Karmali incubated in modified atmosphere (10% CO_2, 5% O2 et 85% N2) This study was conducted with different ratios between *L. salivarius* and *C. jejuni* (1: 10, 1: 100, 1: 1000 and 1: 10000). For the ratio 1: 10, it was not observed significant inhibition. For the ratio of 1:100, there is a strong inhibition of the order of 4 decimal reductions of the population of *C. jejuni* in the presence of *L. salivarius* SMXD51. At the ratio of 1: 10000, growth of *C. jejuni* is again reduced of the order of 2 decimal reductions of the population after 24 hours of culture. The pH remains stable and evolves only about 0.3 units over the 56 hours of coculture. Given these results, the study was continued by performing coculture at low temperature (8°C) to simulate the conditions of preservation of food. For ratios 1:10 and 1:100, the growth of *C. jejuni* is not affected by the presence of *L. salivarius* SMXD51. Whereas, for the ratio of 1:1000, the population of *C. jejuni* is reduced by 1.5 log CFU/ml after 24 hours of coculture in the presence of *L.*

salivarius.
Adhesion and invasion abilities of *C. jejuni* was examined in presence of *L. salivarius* SMXD51 and revealed that the lactic acid bacterium adheres to Ht-29 cells at a minimum level of 105 cells/ml, which is close to commercial probiotic strains.
These findings demonstrated that *L. salivarius* SMXD51 could be a reliable candidate for future use as probiotic in chicken feed supplement. Further investigations will be conducted in animal experiments to assess the in vivo efficacy of the strain.

Keywords: *Lactobacillus salivarius*; *Campylobacter*; bioprotection; bacteriocin

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P5.17

Inactivation of planktonic and sessile *Bacillus cereus* cells by enterocin AS-48 singly or in combination with biocides

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Enterocin AS-48 is a cyclic antibacterial peptide with a broad spectrum of bactericidal activity (Abriouel et al., 2010). This bacteriocin is being considered a strong candidate as a food preservative. In the present study, the efficacy of AS-48 was tested under different ecological and physiological situations, eg. planktonic versus sessile cells or vegetative cells versus endospores. Enterocin AS-48 was tested singly or in combination with biocides on a cocktail of six *Bacillus cereus* strains in planktonic state and in biofilms formed on polystyrene microtiter plates. The biocides tested were quaternary ammonium compounds (benzalkonium chloride, cetrimide, hexadecylpyridinium chloride), bisphenols (triclosan), biguanides (chlorhexidine), polyguanides (polyhexamethylen guanidinium chloride) and oxidizing agents (commercial sanitizers P3 oxonia and P3 topax 66). The numbers of survivors were determined after 60 min incubation with biocides or the biocide-bacteriocin combinations. Addition of enterocin AS-48 (25 mg/l) increased the inactivation of planktonic cells by the quaternary ammonium

compounds, bisphenols, biguanines and polyguanine. Increased inactivation of the bacilli was also observed for the combination of enterocin AS-48 with P3 oxonia, but not by P3 topax 66 commercial disinfectants. In the sessile state, the bacilli were more resistant to biocides and also to the bacteriocin-biocide combinations. Hexadecylpyridium chloride was the most active biocide on biofilms in the single treatments. In the combined treatments with 50 mg/l bacteriocin, hexadecylpyridinium, polyhexamethylen guanidinium chloride and P3 oxonia achieved complete inactivation of bacilli populations. P3 topax 66 showed the lowest performance among all treatments tested, either singly or in combination with bacteriocin. A cocktail of endospores was challenged with biocides and enterocin AS-48 for 60 min at temperatures of 22ºC, 40ºC, and 60ºC. Enterocin AS-48 did not significantly ($p > 0.05$) reduce viable counts or increase the lethal effect of biocides. However, treatments at 60ºC with benzalkonium chloride, hexadecypyridinium or P3 oxonia achieved complete inactivation of bacterial endospores, both singly and in combination with bacteriocin. Significant reductions of viable counts (1 to 2 log cycles) were also obtained for some treatments with cetrimide, triclosan or polyhexamethylen guanidinium chloride, but not for chlorhexidine or P3 topax 66. Polystyrene surfaces dosed with enterocin AS-48 (25 or 50 mg/l) remained free of detectable bacilli from 2 to 24 h after being inoculated with a cocktail of endospores, but stainless steel surfaces dosed with 50 mg/l bacteriocin did not prevent bacterial growth from endospores. Results from this study illustrate the influence of ecological conditions on the efficacy of bacteriocins and suggest that enterocin AS-48 could be applied as enhancer of biocide activity against planktonic and sessile *B. cereus* cells.

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P5.18

Biocontrol ability of food-isolated yeast strains against phytopathogenic molds

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The postharvest diseases caused by phytopathogenic molds are usually controlled by chemical fungicides. However, resistance to this compounds which often occurs in the biologically dynamic fungal population and growing public health and environmental concerns have resulted in the development of safer, effective

alternative strategies to control postharvest fungal diseases, especially for fruits, such as citrus fruits, whose peels are widely used by confectioners and often have high concentrations of pesticide residues. Several reports have proposed the use of microbial antagonists for controlling the postharvest fruit infections caused by molds. Among the different biocontrol agents, yeasts deserve particular attention, as their activity does not generally depend on the production of environmental and mammalian toxic metabolites.

The biocontrol abilities of *Saccharomyces cerevisiae* and *Wickeramomyces anomalus* strains have been recently proved to be correlated with killer phenotype (Platania et al., 2012; Lima et al., 2013), while *M. pulcherrima* and *A. pullulans* mainly compete for nutrients or produce volatile organic compounds (VOCs). In particular, the competition of *Metschnikowia pulcherrima* for iron has been recently believed to play a significant role in antagonistic interactions (Saravanakumar et al., 2008), while the biocontrol activity of *Aerobasidium pullulans* mainly includes nutrient competition (Bencheqroun et al., 2007), and production of glucanase, chitinase and extracellular proteases (Zhang et al., 2012).

Strains belonging to the species *S. cerevisiae, W. anomalus, M. pulcherrima* and *A. pullulans*, isolated from different food sources, were tested in vitro as biocontrol agents against the post-harvest pathogenic molds *Penicillium digitatum*, *Aspergillus flavus*and *Botrytis cinerea*. All yeast strains, tested on different growth media, demonstrated antifungal activity against *A. flavus*, *B. cinerea* and *P. digitatum*, but at a different level depending on species and medium.

Killer strains of *S. cerevisiae* and *W. anomalus* evidenced the highest biocontrol activity on PDA and YPDA media, as demonstrated by larger inhibition halos.

The possible involvement of competition for iron, as the mechanism of action of *M. pulcherrima* and *A. pullulans* was hypothesized, as their biocontrol efficacy was higher in experimental assays performed in iron limited conditions. In particular, *M. pulcherrima* produced wider inhibition zone against pathogens and lower amounts of red pigment in low iron amendments.

The understanding of the mode of action is a key step for developing appropriate commercial formulations and application methods.

Keywords: *Saccharomyces cerevisiae; Wickeramomyces anomalus; Aerobasidium pullulans; Metschnikowia pulcherrima*

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P6.1

Salivary microbiota and metabolome of celiac children: an integrated multi-omics approaches

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Celiac disease (CD), one of the most common food intolerances worldwide, is characterized by small intestinal mucosal injury and malabsorption in genetically predisposed individuals (Wieser et al., 2012). The intestinal inflammation is caused by gluten, proteins which are widely contained in the Western diet. Numerous and diverse microorganisms colonize the human oral cavity as commensals. The number of bacterial species identified exceeds 700 (Dewhirst et al., 2010) and the oral microbiome can represent a novel and rich source of gluten degrading enzymes. This study aimed at investigating the salivary microbiota and metabolome of 15 celiac disease children under gluten-free diet (treated celiac disease, T-CD) and 15 non-celiac children (HC). Salivary microbiota was characterized through an integrate approach of culture-dependent and -independent methods. Culture-dependent was carried out using various selective media for total anaerobe and aerobe bacteria, Clostridiaceae, Eubacteriaceae, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, Bacteroidaceae, Porphyromonadaceae, Prevotellaceae, Rikenellaceae, Enterobacteriaceae and Bifidobacteriaceae. Culture-independent approach was carried out by Bacterial tag-encoded FLX-titanium amplicon pyrosequencing (bTEFAP) and Denaturing Gradient Gel Electrophoresis (DGGE). Metabolically active bacteria were determined by sequencing of 16S rRNA. Differences in microbial communities between T-CD and HC groups were investigated using the phylogeny-based unweighted Unifrac distance metric. The catabolic profiles of the saliva was characterized by using BIOLOG 96-well Eco-Microplates (Crecchio et al.,2004). The metabolome of T-CD and HC children was studied using saliva samples which were analyzed by Gas-chromatography mass spectrometry/solid-phase microextraction (GC-MS/SPME). Multivariate statistical analysis (Principal component analysis; Permut-MatrixEN analysis and Canonical discriminant Analysis of Principal coordinates) were performed on data sets resulting from combinations of the microbial community and metabolic data. Based on the results found in this study, the gluten-free diet lasting at least two years did not completely restore the salivary microbiota and, consequently, the metabolome of CD children. The main differences between salivary microbiota and metabolome of T-CD and HC could be considered as markers for CD.

Keywords: salivary microbiota; pyrosequencing; celiac disease; microbial community

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P6.2

Synthesis of bioactive peptides with cancer preventive, antioxidant, and anti-hypertensive activities by selected lactic acid bacteria

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Cereal foods are important elements of the daily diet, providing mainly carbohydrates, but also proteins, dietary fibres and vitamins. It has been shown that sourdough fermentation may affect the functional features of cereal baked goods, decreasing the glycaemic response of bread, improving the properties of the dietary fibre complex and increasing the uptake of minerals, vitamins and phytochemicals. Microbial metabolism during sourdough fermentation may also produce bioactive compounds, such as peptides and amino acid derivatives (e.g., gamma-amino butyric acid) with various functionalities, and potentially prebiotic exo-polysaccharides. The potential of sourdough lactic acid bacteria to release lunasin during fermentation of cereal and non-conventional flours was exploited. Lunasin is a 43-amino acid peptide, corresponding to the small subunit peptide (Gm2S-1) of 2S soy albumin, implicated in cell-cycle control and suppression of carcinogenesis. Recently, lunasin was isolated also in some cereal and pseudo-cereal grains. Compared to not fermented doughs, the concentration of lunasin increased up to 2-4 times after fermentation. Besides the presence of the entire lunasin sequence, fragments containing the immune-reactive epitope RGDDDDDDDDD were found (Rizzello et al., 2012). The capacity of selected lactic acid bacteria to release antioxidant peptides was shown during fermentation of various cereal flours. The highest radical scavenging activity was found for whole-wheat, spelt, rye and kamut sourdoughs. Almost the same results were found for the inhibition of the linoleic acid autoxidation. Twenty-five peptides, consisting of 8 to 57 amino acid residues, were identified in the active fractions from sourdough water-soluble extracts and all sequences shared compositional

features which are typical of antioxidant peptides. All the purified fractions, showed ex vivo antioxidant activity on mouse fibroblasts artificially subjected to oxidative stress (Coda et al., 2013). Lactic acid bacteria selected for proteolytic activity were used for wheat and rye fermentation with the aim to produce anti-hypertensive peptides. A strong ACE-inhibitory activity was found fermenting flours under semi-liquid conditions (dough yield 330) and, especially by using whole-wheat flour. Fourteen peptides, not reported as ACE-inhibitory previously, were identified from the water/salt-soluble extract of wholemeal wheat sourdough (IC50 0.19-0.54 mg/ml). The major part of the identified peptides contained the well-known antihypertensive epitope VAP (Rizzello et al., 2008). The role of lactic acid bacteria in the improvement of nutritional value and quality of food is essential. In this sense, new perspectives are opening in relation to the production of innovative functional foods.

Keywords: functional food; bioactive peptides; lactic acid bacteria

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P6.3

Grapevine plant growth promoting microbiome: a new sustainable approach to protect vineyards during drought

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The progression of arid soils in many Mediterranean regions is posing serious challenges on agriculture sustainability and is dramatically affecting the management of several crops, including grapevine. In several wine production areas, drought hampers grapevine growth and development, negatively affecting fruit quality and yields (Chaves et al., 2010; Cias et al., 2005). The demand of environmentally friendly approaches in viticulture is therefore increasing, to reduce water waste and the use of chemical fertilizers, contributing to preserve biodiversity and natural resources. While several studies demonstrated that plant-associated bacteria provide important services to the host plant (Marasco et al., 2012; Fernandez et al., 2012), the contribution of grape root-associated microbiome to plant adaptation to water stress has been poorly studied. Besides, little is known about whether environmental factors and pedoclimatic conditions may influence the plant growth promoting (PGP) potential of the root-associated bacteria. To address these issues, the diversity and PGP potential of the bacterial assemblages associated with the root system of different grapevine cultivars were assessed by culture dependent and independent approaches in three Mediterranean environments, along an aridity macro-transect encompassing Italy, Tunisia and Egypt. According to Denaturing Gradient Gel Electrophoresis (DGGE) fingerprint applied to the 16S rRNA gene, the structure of endosphere and rhizosphere bacterial communities was highly diverse ($p=0.03$) and strictly related to cultivar/latitudinal/climatic effects. Bacterial communities associated to grapevines cultivated in Tunisia and Egypt shared a higher degree of similarity ($p<0.05$) compared to those associated to plants from the Italian vineyard. A similar distribution effect was observed also in the cultivable fraction of grapevine-associated bacteria, based on the phylogenetic identification of a microbial collection of about 800 bacterial strains. Noteworthy, 23% of the strains presented multiple stress resistance capabilities and *in vitro* PGP activities, including auxin synthesis, phosphate solubilisation and ammonia production. Interestingly, the three different environmental settings analysed exhibited a similar percentage of bacteria showing the same multiple PGP traits. This occurrence indicates a strong functional homeostasis in beneficial bacteria associated with grape root, independent from the cultivar and site of origin. In order to explore bacterial contribution to the improvement of grapevine resistance to drought, eight isolates were further assayed *in vivo*. In greenhouse experiments with drought-challenged grapevines, the selected PGP strains showed to increase shoot and leaf biomass and shoot length, with an enhanced effect for the rootstocks having the highest sensitivity to water stress. A higher photosynthetic activity was, moreover,

recorded in all the bacterized plants under water stress conditions. Three isolates were further assayed for grape growth promotion in outdoor conditions, exhibiting the ability to stimulate a more extended root system. The overall results indicate that grape roots are a reservoir of beneficial bacteria, showing similar growth promotion traits and able to contribute to grape adaptation to drought.

Keywords: grapevine microbioma; plant growth romoting bacteria; drought stress

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P6.4

Biodiversity and beneficial activity of indigenous yeasts for nutritional improvement of traditional african fermented foods

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Ogi, mawè, gowé and tchoukoutou are traditional fermented food from Benin, West Africa. They are the results of spontaneous fermentations mainly involving lactic acid bacteria (LAB) and yeasts. Besides being important in the fermentation of foods and beverages, yeasts have shown numerous beneficial effects on human health e.g. improvement of bioavailability of minerals through the hydrolysis of phytate by a class of phosphatases called phytases. Yeast diversity occurring in the four final products, as sold at local markets, and yeast dynamics during the fermentation processes of mawè and tchoukoutou were investigated using a combination of culture-dependent and –independent methods. A total of 413 isolates were collected. All of them were identified by PCR-Denaturing Gradient Gel Electrophoresis (DGGE) followed by sequencing of the D1/D2 domain of the 26S rRNA gene. The predominant yeast species identified were grouped by rep-PCR. Direct analysis of total DNA and RNA was performed by DGGE. In the four final products, *Candida krusei* was the yeast most frequently isolated with

strongest predominance in the maize based products. Other yeasts present at equal or lower incidence were detected. The DGGE analysis on the DNA directly extracted from the food matrices demonstrated the presence of two species, not detected by the culture-based approach. During mawè fermentation, a yeast succession took place that lead to a final population comprising *Saccharomyces cerevisiae*, *C. krusei*and *Kluyveromyces marxianus*. The yeast populations dominating fermentation of tchoukoutou were found to consist of *S. cerevisiae*, almost exclusively. Other yeast species were detected in the early stages of fermentation. The direct analysis at DNA and RNA level in case of mawè did not reveal any other species but those already identified by culture-based analysis. On the other hand, for tchoukoutou, four species were identified that were not detected by culture-based approach. Isolates were assessed for phytase activity and survival on gastric and pancreatic juices with the aim of finding high phytase-active yeast strains able to survive the passage through the gastrointestinal human tract. Tested strains belonged to eleven species: *C. krusei*, *Candida tropicalis*, *Candida glabrata*, *Candida rugosa*, *Clavispora lusitaniae*, *Debaryomyces nepalensis*, *Hanseniaspora guillermondii*, *K. marxianus*, *Pichia farinosa*, *S. cerevisiae*, and *Wickerhamomyces anomalus*. All strains were first screened for phosphatase production on YNB (without phosphate) agar plates supplemented with yeast extract and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). On the basis of the initial screening, 93 strains were selected and tested for specific phytase activity. The screening was carried out using two growth-based tests, solid and liquid. Selected strains were also subjected to a simulation of human digestion process. Survival rate was assessed after 2h of growth in synthetic gastric juice and 4h in pancreatic juice. In the present study yeast strains with potential beneficial nutritional properties have been identified to be included in starter cultures for the production of traditional fermented food from Benin.

Keywords: african fermented food; yeasts; phytase; PCR-DGGE

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P6.5

Biodiversity, structure and role of rhizosphere bacterial communities in a deglaciated alpine environment

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Glacier forefields are landscape characterized by primary successions where the interaction between microbial communities, pioneer plants and soil neo-genesis involves the development from bare substrate to complex plant communities after the ice cover retreats. In a young soil, characterized by a high fraction of coarse and grained mineral texture, the interactions between the indigenous bacterial community and the pioneer plant community may alleviate abiotic stressors such as lack of water and nutrients. In our research area in the Mazia Valley (South Tyrol), plant colonization was evolved since 1840, when the glacier began to retreat. Three distinct floristic communities, at two different time points of glacier retreat and along an entire growing season were studied. ARISA and 16S rRNA gene pyrosequencing (83,000 OTUs) were applied to both metagenomic DNA and RNA, in order to assess the diversity of the overall bacterial taxa and of the active bacterial fraction. The free-living nitrogen-fixing bacterial community was monitored by nifH gene pyrosequencing and qPCR of both metagenomic DNA and RNA. Finally the presence and diversity of other key-players of the nitrogen cycle was assessed by specific PCR-DGGE of 16S rRNA gene and by specific functional gene sequencing.
The community structures of both the overall and active bacterial communities appeared to be affected by the growing season and the soil age. Proteobacteria, Actinobacteria, Acidobacteria were the most representative phyla. Active Proteobacteria were less represented in the early growing season. In the older moraine, Acidobacteria were much more active in July than in October in both the transects. Planctomycetes were recorded even though they were less active in early and late seasons in younger soil. The characterization of the *nifH* gene and other genes involved in the nitrogen cycle diversity is also discussed.

Keywords: bacterial community structure; pioneer plants; nifH; bacterial diversity; pyrosequencing

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P6.6

Plant-microbes positive interaction enhance the carbon sequestration potential in *Gmelina arborea* forest seedlings

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Every living organism in the world depends on the association of its neighbor to uphold life. Most of this relationship is created by chemical indication exchanged between the host and the symbionts. Rhizosphere release plenty numbers of chemical compounds that are attracting the beneficial microorganisms. A large number of literature are available about the plant rhizosphere and Plant Growth Promoting (PGP) activities in agricultural crops. Till now no studies are reported in enhancing carbon sequestration through plant microbe interaction in forest seedlings. The present study aims to assess the carbon sequestration potential of *Gmelina arborea* using plant growth promoting actinomycetes in Nanmangalam Reserve Forest, Chennai, India. In this study four different treatments were subjected; (1) PGPR+ Inorganic fertilizer, (2) Inorganic fertilizer (3) PGPR and (4) Control. The PGPR (*Strptomyces* sp.) was isolated from Nanmangalam Reserve Forest. Streptomyces have the capability to produce, Indole Acetic Acid (IAA), Siderophore,ammonia, total nitrogen and mineral solubilization (Phosphate, Zinc, Silicate) in laboratory analysis and showed negative for Hydrogen cyanide (HCN) production. *Streptomyces* sp. with inorganic fertilizer showed a high statistical significance difference. The plant N, P, K content has increased 26.1%, 20.17.08% and 21.84% respectively. Inorganic fertilized seedling with *Streptomyces* increased the nutrient content in *Streptomyces* sp. alone showed the less significant impact. Compared to control PGPR with inorganic fertilizer showed 3 folds increased carbon sequestration potential in *Gmelina arborea*.

Keywords: plant-microbe; carbon sequestration; PGPR

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P6.7

Occurrence and diversity of bacteria intimately associated with spores of geographically different Glomeromycota isolates by PCR-DGGE analysis

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The development and performance of mycorrhizal symbioses may be positively affected by bacteria living in the mycorrhizosphere, which show different functional abilities, such as the promotion of spore germination and hyphal growth, the production of antibiotics providing protection against phytopathogens, the supply of nutrients and growth factors (Frey-Klett et al., 2007; Giovannetti et al., 2010). Different taxa of microbes were found associated with spores and sporocarps of arbuscular mycorrhizal fungi (AMF) (Filippi et al., 1998; Roesti et al., 2005), a group of beneficial symbionts which live in association with the roots of most land plants and play a key role in soil fertility and plant nutrition (Smith and Read, 2008). The occurrence of spore-associated bacteria was reported for a few number of AMF species, while little is known on the structure and function of bacterial populations living in association with spores of conspecific isolates originated from geographically different sites. In this work we analysed three strains of *Glomus mosseae* isolated from Arizona-USA, Indiana-USA, United Kingdom-EU, two strains of *Glomus intraradices* isolated from France-EU and Italy-EU and one strain of *Glomus coronatum* isolated from Italy-EU, in order to investigate the occurrence and diversity of bacteria strictly associated with their spores. AMF spores were sieved from pot culture soil, washed and vigorously shaken 15 times in sterile water. The rinsing water of the first and 15th washings, and the homogenized spores, were analysed using PCR of 16S rDNA V3 - V5 region and denaturing gradient gel electrophoresis (DGGE). Different DGGE profiles were obtained from the different AMF species and isolates, showing diverse spore-associated bacterial communities. Different banding patterns could also be observed when rinsing water and homogenized spores from a single isolate were compared. Some DGGE bands were found in spore homogenates but not in spore washings, which suggests the intimate nature of some fungal-bacterial associations. The sequence analysis of the major DGGE bands revealed the

occurrence of bacterial sequences homologous to species of the genera *Rhizobium, Sinorhizobium, Penibacillus, Geobacillus, Bacillus, Ralstonia, Propionibacterium, Pseudomonas, Ideonella, Massilia, Arthrobacter, Streptomyces*. Further studies will be aimed at revealing the diverse ecological and functional roles played by bacteria associated with AMF spores.

Keywords: mycorrhizosphere; arbuscular mycorrhizal fungi; spore-associated bacteria; PCR-DGGE; bacterial diversity

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P6.8

DNA-based molecular characterization of fungi isolated 'Hurma' olive

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Turkey is an important table olive producing country with diverse olive (*Olea europaea L.*) varieties, most of them are consumed in Turkey without exportation. Among these varieties, 'Hurma' olives grown mainly in Karaburun Peninsula differ from other varieties that fully ripen on the tree before harvesting and loose its bitterness caused by phenolic compounds especially oleuropein. Thus, they can be directly consumed when they are harvested and they do not require to undergone debittering process to make them edible by removing their bitterness components. It was stated in limited literature that the debittering phenomena during maturation period occurs by a fungus called *Phoma olea* with the help of climatic conditions. Besides, in small number of studies stated that similar olive varieties were grown in Greece and Tunisia (Jemai et al., 2009; Panagou, 2006).
In olive fruits, the microbiota is mainly constituted of non-*Saccharomyces* yeasts, lactic bacteria and filamentous fungi. However, with regard to fungi, there are few references in the literature about mould biodiversity in fresh olives and the data available only report on fungal diseases and toxic metabolites produced by certain

Aspergillus species (González-Lamothe et al., 2002; Leontopoulos et al., 2003). Traditionally, the identification of fungi has mainly been made through morphological and physiological parameters. Currently, in addition to classical methods, molecular techniques have been used in classification, such as the sequencing of β-tubulin and calmodulin genes, ribosomal RNA genes (rDNA) and their flanking internal transcribed spacers (ITS1–5.8S–ITS2 rDNA region). Previous results have shown that the ITS rDNA regions (non-coding and variable) and the 5.8S rRNA gene (coding and conserved) are useful and reasonably precise in the rapid identification of fungi species (Peterson 2012).

This study investigated the fungal diversity of 'Hurma' olive fruits that fresh naturally black table olive during the period of between the start of debittering to full ripeness (maturation period) which lasted about 8 weeks between the October-December of 2011 and 2012 harvest years. For the fungi microbiota isolation Potato Dextrose Agar (PDA), Oatmeal Agar (OA), Sabouraud Dextrose Agar (SDA) and Czapek Agar (CZA) culture media were used with incubation at 25°C for 3-5 days. Pure fungal cultures were isolated after subsequent streaks onto potato PDA incubated at 30°C. Then the isolates were grouped based on morphological characteristics as color, texture of the mycelia, fungal cell structures and spore formation. These characteristics were observed under stereo and light microscopic observations of fresh fungal cultures on PDA and CZA agar plates. Identification included comparison of their polymerase chain reaction (PCR) amplicons of the ITS ribosomal DNA region, followed by nucleotide sequence analysis. *Aspergillus* and *Penicillium* were the most frequent genera followed by *Rhizomucor*, *Mucor*, *Rhizopus*, *Geotrichum*, *Alternaria*, *Acremonium*, *Paecilomyces*, *Fusarium*.

Keywords: hurma olive, fungi, molecular, identification, ITS rDNA

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P6.9

Antimicrobial activity of Thai medicinal plants against beverage spoilage microorganisms and *in vitro* evaluation of their phytochemical properties

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In this study, 34 crude methanolic extracts of Thai medicinal plants were tested for their antimicrobial activities against beverage spoilage microorganisms,and their phytochemical properties such as anti-acetylcholinesterase and antioxidant activities, and total phenolic and flavonoid contents were evaluated. The plant extracts of *Acorus calamus* (Mytle grass), *Cassia alata* (Ringworm bush), *Crocus sativus* (Saffron), *Nymphaea lotus* (White lotus) and *Phyllanthus emblica* (Indian gooseberry) showed strong antimicrobial activity against spoilage yeasts, lactic acid bacteria and acetic acid bacteria tested. Two most susceptible yeast strains to the plant extracts were *Rhodotorula glutinis* (inhibited by *N. lotus* extract with the MIC of 0.64 mg/ml) and *Schizosaccharomyces pombe* (inhibited by some plant extracts such as *A. calamus*, *C. alata, Holarrhena curtisii* (Phut Thung), *Kaempferia galangal* (Aromatic ginger) and *N. lotus* with the MIC of 0.64 mg/ml). Extracts of *C. alata* and *A. calamus* strongly inhibited growth of *Lactobacillus casei* and *Lactobacillus plantarum*, respectively (2.56-5.12 mg/ml MIC), while *C. sativus* extract effectively inhibited growth of *Acetobacter aceti* (5.12 mg/ml MIC). The study of anti-acetylcholinesterase activity revealed that the extracts of *Kaempferia parviflora* (Black galingale), *Nelumbo nucifera* (Roseum plenum), *Rauvolfia serpentina* (Serpentine root) and *Centella asiatica* (Asiatic Pennywort) exhibited strong acetylcholinesterase inhibitory activity (>70 % inhibition). The extracts of *Terminalia chebula* (Myrabolan wood), *Cinnamomum bejolghota* (Cinnamon), *Uncaria gambir* (Gambir) and *P. emblica* had the strongest antioxidant activity with the EC50 of 387.23 - 490.47 µg extract/mg DPPH (2, 2-diphenyl-1-picrylhydrazyl) by DPPH radical scavenging activity method and reducing capacity of 774.41 - 656.19 mmol Fe(II)/g by FRAP (ferric reducing antioxidant power) method. The extract of *U. gambir* had the highest total phenolic and total flavonoid contents (771.59 mg gallic acid equivalents (GAE)/g and 2,292.43 mg quercetin eqivalents (QE)/g, respectively), followed by *C. bejolghota* extract (501.25 mgGAE/g and 1,317.61 mgQE/g, respectively).

Keywords: anti-acetylcholinesterase activity; antioxidant activity; total phenolics and flavonoids

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P6.10

Gut microbiota of rats on the consumption of resistant dextrin

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The objective of the present study was to observe whether the diet based on the consumption of resistant dextrin can show differences in the gut microbiota of rats in comparison with microbiota of rats based on a basic diet. Rats were divided into two groups, one on the resistant dextrin diet and one control, each group consisting of five rats. Rats were reared in the Institute of Animal Reproduction and Food Research of Polish Academy of Sciences. All rats were kept in the same, standard conditions, which means the air temperature of around 21-22°C, with relative humidity in the range of 50-70%. The rooms where they were brought up were ventilated intensively, and the light was on for 12 hours daily.
Resistant dextrin, that was added to the forage fed to dextrin group of rats, was obtained during the process of pyroconversion (formation of untypical for starch α-1,2 and α-1,3-glycosidic bonds making the product undigestible for digestive enzymes) and chemical modification of potato starch at Jan Dlugosz Academy in Czestochowa. Pyroconversion was carried out for 120 minutes at the temperature equal 130°C. Chemical modification was done with the use of 40% tartaric acid and 0.1% HCl as a catalyst for the reaction.
The project was performed by observations and calculations of colony forming units: total number of bacteria, *Lactobacillus*, *Bifidobacterium*, Enterobacteriaceae, *Enterococcus*, *Clostridium*, *Escherichia coli*. These bacteria were isolated from the

rats' feaces for four weeks, as well as from the blind gut and colon of each rat.
After obtaining the results, the influence of the uptake of resistant dextrin was observed. The slight increase in beneficial *Bifidobacterium* amount together with significant boost of another favourable bacteria Lactobacillus of magnitude for dextrin group in comparison to control one revealed, that resistant dextrin can be utilised by those bacteria in order to stimulate their growth. On the other hand, the decrease in the rest of bacteria was observed in rats' feaces after the consumption of the diet rich in resistant dextrin. The most significant difference was observed in the amount of *Clostridium*, which turned out to be lower for dextrin-fed rats than control groups by more than one order of magnitude.
The colon matter analysis is concerned, showed also prebiotic consequences of addition of resistant dextrin. Slight increase on *Lactobacillus* and *Bifidobacterium* levels, with simultaneous decrease in unfavourable types of bacteria were observed. Significant changes were observed for four types of bacteria, Enterobacteriaceae family, *Clostridium*, *Escherichia* coli and Bacteroides, revealing the drop of their levels for rats fed with the diet rich in resistant dextrin. The biggest decrease in comparison to control group was observed, similarly to the results from feaces, for *Clostridium*, for which the difference turned out to be higher than one order of magnitude.
From obtained results it can be concluded, that analysed resistant dextrin display prebiotic properties. It increased amount of beneficial bacteria (*Lactobacillus* and *Bifidobacterium*), decreasing simultaneously unfavourable ones (*Clostridium*).
The study was supported by the Ministry of Science and Higher Education, grants No. NN312335339.

Keywords: microbiota; resistant dextrin; rats

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P6.11

Fluorescence in situ hybridization for identification of intestinal bacteria of mice

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One of the methods allowing for detection of microorganisms is a method involving homologous nucleic acid fluorescent in situ hybridization (FISH). It involves combining fluroscencyjnie labeled molecular probe of known sequence with the complementary nucleic acid sequences in the cell.
The aim of the study was a quantitative evaluation was to determine the quantitative composition of bacteria colonizing the intestinal tract of conventional mice and germ-free, who received probiotic preparation.
Experimental studies on animals were carried out in the Academy of Sciences of the Czech Republic, Division of Immunology and Gnotobiology, Novy Hradek.
The study was Primarily mouse-mouse GF sterile (germ free), and mice bred in the laboratory with traditional physiological SPF (specific pathogen free) intestinal microbiota (CONV mice).
The probiotic preparation was administered in the amount of $1x10^9$/ml with intragastric probe. The preparation consisted of three strains: *Lactobacillus casei* LOCK 0900, LOCK 0908 and Lactobacillus paracasei LOCK 0919. They were obtained from the collection of the Institute of Fermentation Technology and Microbiology (LOCK 105), Technical University of Lodz, Poland. The probiotic strains possess full documentation and they are in course of license (no. P-382760, P-382761, P-382762).
For the identification of microorganisms that inhabit the gastrointestinal tract mice used FISH method (fluorescent in situ hybridization). Employ the appropriate molecular probes: Lab 158 (GGTATTAGCA(T/C)CTGTTTCCA- group *Lactobacillus-Enterococcus*); Bif 164 (CAT CCG GCA TTA CCA CCC- *Bifidobacterium* sp.); Eub 338 (GCT GCC TCC CGT AGG AGT- total number of bacteria); Bac 303 (CCA ATG TGG GGG ACC TT- group Becteroides-Prevotella); Erec 484 (GCT TCT TAG TCA GGT ACC G- group *Clostridium*-coccoides); Enter 1432 (CTT TTG CAA CCC ACT- groupEnterobacteriaceae).

Studies have shown that the number of bacteria colonizing the intestinal tract in mice reared under conventional conditions of the order of 10^9 CFU/g. The number of bacteria of the genus Lactobacillus-*Enterococcus* was $5{,}0x10^7$ cfu/g, *Clostridium coccoides* $1{,}4x10^8$ cfu/g *Bifidobacterium* sp $1{,}3x10^8$ cfu/g, Bacteroides-Prevotella $8{,}4x10^7$ cfu/g, and the bacteria present in the number of Enterobacterioceae $3{,}7x10^8$ cfu/g. After the addition of probiotics slightly increased the amount of bacteria in the *Lactobacillus* group and at the same time decreasing the amount of bacteria of the genus *Clostridium coccoides* and Enterobacterioceae.
In mice, germ-free quantitative assessment of the total number of bacteria showed that prior to germ-free mice with a probiotic preparation of the digestive tract was sterile. After administration of the probiotic preparation has been observed their gastrointestinal colonization by bacteria of the genus Lactobacillus. This was due to the fact that the probiotics are living micro-organisms are capable of homing and intestinal epithelium.
This work was supported by grant number 12010110 from National Center for Research and Development, Poland.

Keywords: microbiota; probiotic; mice; germ-free mice

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P6.12

Truffle genomics and post-genomics to understand the symbiotic life-style

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A special interest in an agronomical and food context is bound to the symbiotic mycorrhizal fungi, and among them to truffles (*Tuber* sp.). Thanks to the symbiosis they establish with the roots of woody and shrubby plants, truffles produce fruiting bodies that are prized and highly requested in all the world markets for their organoleptic properties. The Périgord black truffle (*T. melanosporum*) and the Piedmont white truffle (*T. magnatum*) dominate today's truffle market. In 2007, the *T. melanosporum* genome sequencing project has been launched in Turin as a common project by a French-Italian consortium, and the results were published in Nature in 2010 (Martin et al., 2010). The working hypothesis was that identification of processes that condition and trigger fruit body and symbiosis

formation, ultimately leading to a more efficient production, would be facilitated by a thorough analysis of truffle genomic traits. Starting from the sequencing project, several post-genomics activities were then developed, in order to focus on specific gene categories (e.g., cell-wall related genes, environmental response genes, etc.) and, among them specific gene families (Zampieri et al., 2011; Balestrini et al., 2012; Sillo et al., 2013). These results have allowed us to obtain new knowledge on metabolic processes that happen during the complex life cycle of a symbiotic fungus, giving information on the genes/proteins involved in the symbiosis development as well as in the formation of the precious fruiting bodies. On the other hand, a project for the *T. magnatum* sequencing is still in progress, and it will offer the possibility to understand the biological differences between the two species. In the absence of the whole genome sequence, we have produced an inventory of gene expression in *T. magnatum* by sequencing cDNA from fruiting body. This represents the first step towards the understanding of genome functionality and, thanks to the availability of *T. melanosporum* genome sequence, the identification of genes that could be specific for one truffle species and genes that are common to both species. In addition, starting from the first available sequencing data (genomic and transcript sequences), we have performed gene expression on specific genes putative involved in changes during truffle post-harvest storage, considering that, according to the gastronomy rules, *T. magnatum* is usually consumed raw. In parallel, a metabolite profile has been obtained on fruiting bodies conserved as in the previous analyses. Taken together, the results allowed us to highlight some of the molecular events that take place during truffle conservation. The *Tuber melanosporum* genome sequencing project was a collaborative effort involving the Génoscope and the *Tuber* Genome Consortium.

Keywords: *Tuber* sp.; genome and transcript sequencing; symbiotic fungi; fungal cell wall; RT-qPCR

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P6.13

Archaeal intestinal community in healthy children compared with subjects affected by cystic fibrosis: a preliminary study

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The endogenous intestinal microbiota plays an essential role in human health and disease. Archaeal community has recently been described in various human niches, such as intestine; *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* were present in the human gut with variable and low prevalence. Moreover, sequences of 16S rRNA corresponding to uncultured methanogenic clones were described. Methanogenic Archaea were found to represent 12% of anaerobic bacterial population in the distal colon whereas only 0.003% in the proximal colon (Pochart et al., 1993). In intestinal chronic inflammatory diseases an abnormal intestinal microbiota composition has been observed (Scanlan et al., 2012; Duytschaever et al., 2011); intestinal inflammation is a hallmark of cystic fibrosis (CF). We investigated the intestinal Archaeal composition by Denaturing Gradient Gel Electrophoresis (DGGE) and Real time PCR in children aged between 2 and 9 years old affected by cystic fibrosis, comparing their profiles with a control group of the same age. Although different primers have been widely employed to describe Archaeal communities, only a few studies have assessed in parallel the quantitative efficiency of different primer pairs. Thus, initially, a large set of primers was tested and validated on human stool samples, to evaluate their ability to specifically amplify Archaeal bacteria and to produce suitable amplicons for DGGE analyses. The DGGE profiles obtained from healthy controls showed a common core with similar bands detected in all samples, although with slightly different intensity. There was no such common core in CF patients. The biological diversity of CF and healthy Archaeal communities was estimated by the number of bands and their intensity of DGGE profile, whereas the similarity of DGGE profile intra- and inter-group was analysed by similarity coefficient. Real time PCR was performed in order to evaluate the methanogenic Archaea using primers targeting on *mcrA* gene. Preliminary results indicate a reduction of methanogenic Archaea in CF patients but these data needed to be confirmed.

Keywords: cystic fibrosis; gut microbiota; child; DGGE; real Time PCR

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P6.14

Population dynamics of *Staphylococcus aureus* and *Salmonella typhimurium* in a laboratory medium and rocket extract

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Salmonella and *Staphylococcus aureus* are an important human pathogen capable of causing a diverse array of infections, while international organization (EFSA, FAO/WHO) report that the most related microorganisms for foodborne diseases are these pathogens. In addition, the ability of both species to form biofilm and the increased number of antibiotic-resistant *S. aureus* strains, including strains resistant to methicillin (MRSA) among nosocomial infections stimulates the interest of researchers. On the other hand, the consumption of raw plant tissues, have been associated with the risk of foodborne diseases due to cross contamination. However, the ability of pathogenic strains to survive on the surface of the plant tissues needs to be studied in deep.

In the present study, the growth of *Staphylococcus aureus* and *Salmonella typhimurium* on different growth media was studied. For this purpose, a growth medium (Luria – Bertani broth, LB) and extract from rocket, were inoculated with *Staphylococcus aureus* strain COL (MRSA) and *Salmonella* Typhimurium (CDC 6516-60). After the inoculation, the samples were incubated at 20°C. In the case of *Salmonella typhimurium*, the final population on LB broth was about 1 log cfu/mL higher than rocket extract. On the other hand, the growth of *Staphylococcus aureus* seemed to take an advance on rocket extract at the beginning of storage, while the final population was at the same level in both medium (LB broth and rocket extract).

These results revealed that, the rocket might be a favorable environment for the growth of *Staphylococcus aureus and Salmonella typhimurium*. Further studies are needed to ensure the survival and growth of both pathogens on plants extracts or plant tissues, as well as the study the potential of its pathogenicity during the growth on these media.

Keywords: salmonella; MRSA; rocket

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P6.15

Identification and selection of fluorescent *Pseudomonas* with potential use to improve plant performance

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Pseudomonads are microorganisms present in the soil, water, and colonizing plant roots. Some isolates of *Pseudomonas fluorescens* improve plant performance through several mechanisms: plant protection (antagonistic reactions against plant pathogenic microorganisms, induction of plant defence mechanisms), and plant nutrition and development (enhancing mineral plant assimilation, production of plant-growth regulatory compounds). The mechanistic bases of these plant reactions must be investigated. Pseudomonas has been utilized as a biocontrol agent against pathogen microorganisms (Redondo-Nieto et al., 2013). We are studying Pseudomonas microorganisms in an attempt to develop effective biocontrol systems for the management of leaves and root disease and to improve plant vigor. *P. fluorescens* suppress plant diseases by production of number of secondary metabolites including antibiotics, siderophores and hydrogen cyanide. These cells have the ability to invade the plant vascular system, reach the various organs of the plant and participate as a systemic bio-control agent against various fungal and bacterial diseases. The competitive exclusion of cells from roots, as a result of fast colonization ability of *P. fluorescens* cells may also be an important factor in diseases control. The antagonists bacterial cells were selected, in the first place, based on their ability to promote seed germination and seedlings growth and by their ability to inhibit the growth of pathogenic microorganisms. We perform a series of tests in order to determine the effects of bacterial cell suspensions on the germination of seeds. *Hibiscus sabdariffa*: T16 strain caused inhibition of 79%, while the germination of the seed in the presence of other isolates ranged from 80 to 97%. *Solanum lycopersicum*: Germination in the presence of the bacterial suspensions ranged between 82 and 94%. With the T16 bacterial suspension, germination reached 84%. *Zea mayz*: Germination in the presence of the bacterial suspensions ranged between 90 and 100% but only Pseudomonas fluorescens strain caused an inhibition of 28%.

Keywords: microbial interference; plant performance; disease control

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P6.16

GC-MS-based metabolic profiling of biological samples of nephropatic and autistic individuals: a comparative study

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Metabolomics is a method of high throughput fingerprinting that offers the possibility of measuring metabolic end points that are determined by genetic and environmental factors. On the other hand it can be used to evaluate perturbances of specific metabolic pathways induced both by host and gut bacterial metabolic disturbances leading to altered metabolite profiles in biological samples.
Human diseases such as chronic kidney disease have been shown to have a direct relationship with disorders in the lipids and fatty acids. Autism spectrum disorders (ASD) are a group of biological disorders which have been proved to have strong associations with various metabolic abnormalities, immunological function and gastrointestinal disturbances, although their mechanistic significance is unknown.
In this study a metabolomic strategy based on gaschromatography mass-spectrometry solid phase microextraction (GC-MS/SPME) analysis has been used to investigate the metabolic profiles of biological samples of nephropatic (NP) and autism patients (ASD) compared to healthy subjects (HS). To identify differences between the fecal samples of the 3 groups of individuals, the volatile organic metabolites were elaborated through the Canonical discriminant Analysis of Principal coordinates (CAP). More than 80 volatile organic compounds belonging to different chemical classes including aldehydes, ketones, alcohols, short chain fatty acids, esters, sulphur compounds and phenols were detected by SPME/GC-MS in fecal samples of NP, ASD and HS. The comparison of the metabolic phenotypes of individuals diagnosed with autism and nephropathy and healthy volunteers enabled the identification of perturbations in the presence and levels of specific compounds. In particular the CAP analysis relative to the HS vs ASD evidenced that three groups of molecules were significantly associated with feces of ASD, and namely phenols, methyl esters of short chain fatty acids and indoles. Also NP samples showed high concentrations of phenols (e.g. 4-1,1,3,3-tetramethyl-buthyl-phenol and di-p-terbuthyl-phenol) in addition to 2,6-dimethyl-

heptanone, acetic, propionic and butanoic acids. On the other hand, sulphur compounds such as di-methyl-disulfide and di-methyl-trisulfide were significantly higher in the controls with respect to ASD and NP samples.

Keywords: metabolomics; biological samples; GC-MS/SPME; nephropathy, autism

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P6.17

Food safety significance of potential endophytic bacteria isolated from *Capsicum annuum* var. *grossum*

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Bacteria that live inside plant tissues without being hazardous to the host or gaining benefit other than residency are considered as endophytic microorganisms. Endophytes can enter plant tissues mainly through the roots, nevertheless stems, cotyledons and blossoms can also serve as access for these bacteria. Endophytes can promote the growth and yield of the plant, help to remove contaminants from tissues, and can suppress pathogens, however some enteric human pathogenic bacteria have been isolated as endophytes. Based on these observations the aims of our study were the isolation, identification and characterisation of bacteria from sweet peppers, and determination of interaction between the potential endophytes and *Listeria monocytogenes*.Bacteria were isolated from different tissues of hydro- and soil-cultures of *Capsicum annuum* var. *grossum* cultivars using coliform selective violet red bile lactose (VRBL) agar. The isolates were subjected to phenotypic (morphological, biochemical, physiological) trials and molecular biological analyses (rep-PCR, ARDRA, *Pseudomonas* genus-specific PCR, sequencing), while their antagonistic effect was determined by interaction studies.Altogether 43 bacteria were isolated from primary roots, stem, leaf and fruit tissues, and grouped by STATISTICA 10 software based on phenotypic properties. The isolates showed heterogeneity, but phenotypically identical isolates could also be found between bacteria isolated from different parts of the plants. Using the M13 minisatellite primer for rep-PCR the isolates were clustered into clonally related groups, in which – in contrary to the phenotypic alignment – only isolates from the same tissues and culturing conditions showed clonal identity. The results of restriction analysis of the 16S rRNA amplicons using two restriction

endonucleases showed that this technique had lower discrimination ability compared to rep-PCR, however proved to be suitable for differentiation at genus level. A genus-specific PCR was used for the detection of *Pseudomonas* isolates. Amplicons were generated in case of 15 bacterial isolates, which indicated that approximately 1/3 of the isolates belonged to the *Pseudomonas* genus. Identification of these isolates was done by sequencing the *rpoB* gene, while in case of twenty eight non-*Pseudomonas* isolates the 16S rRNA gene was used as the target in sequence analysis. Results showed that the isolated bacteria represented species of *Achromobacter, Agrobacterium, Comamonas, Delftia, Enterobacter, Erwinia, Leclercia, Mesorhizobium, Pantoea, Pseudomonas, Rhizobium* and *Serratia* genera.Testing the antagonistic effect of the isolates on *Listeria monocytogenes* CCM 4699 by co-culturing inhibition was observed in case of seven isolates. The most significant inhibitory effect was attributed to one of the *Pseudomonas* isolates, which performed the best growth inhibition at 25 °C. Growth curve analysis of *L. monocytogenes* CCM 4699 in the presence of cell-free supernatant of this *Pseudomonas* isolate showed that extracellular substance(s) even in relatively low proportion could inhibit the growth of the pathogen, which indicated the potential biocontrol activity of this isolate. This work was supported by the Hungarian project OTKA 101716, and the work of A. Belak was supported by the European Union and Hungary in frame of TÁMOP 4.2.4.A/1-11-1-2012-0001 project.

Keywords: endophytic bacteria; antagonistic effect; *Listeria monocytogenes*

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P6.18

Antibiotic susceptibility of potential probiotic *Lactobacillus pentosus* strains: screening for resistance genes

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The antibiotic resistances of 15 lactic acid bacteria belonging to the species *Lactobacillus pentosus* isolated from Spanish-style green olive fementations were studied. The strains were previously selected based on their potential probiotic

characteristics. Antimicrobials and range of concentrations used were as follows: ciprofloxacin, 2-512 μg/ml; chloramphenicol, erythromycin, lincomycin, penicillin, and tetracycline, 0.125-32 μg/ml; kanamycin and streptomycin, 32-4,096 μg/ml, and rifampin and ampicillin, 0.125-16 μg/ml. Survival for each strain (as expressed as % of the control without antibiotic) was determined using a Bioscreen C apparatus, incubating the plates at 30 °C for 48 h. Also, MIC50 and MIC90 for each strain and antibiotic were obtained. Furthermore, PCR detection of erythromycin and tetracycline resistance genes in the 15 *L. pentosus* strains and in other 51 additional strains isolated from olive fermentations (brines and olive-surface biofilms) was carried out.

Eleven and three strains were phenotipically resistant to kanamycin or lincomycin, respectively, being three of them phenotipically resistant to both antibiotics. All other strains were susceptible to the other antibiotics tested with MIC breakpoints below those established by EFSA for *L. plantarum*. Genetic determinants for erythromycin-resistance erm(B) were found in 18 out of 76 strains, whereas erm(C) was found in five out of 76 *L. pentosus* strains. From these, only two strains harboured erm(B) and erm(C) simultaneously. Furthermore, tet(M) was found in 10 strains while tet(S) was not detected in any strain. Finally, only two of the 76 strains tested showed simultaneously tet and erm.

The results indicate that most of these strains could be considered suitable for their use as starter cultures in olive fermentations, since they present no risk of transmission of antibiotic resistances.

Keywords: *Lactobacillus pentosus*; olive fermentation; probiotics; antibiotic resistance

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P6.19

Potenial endophytic bacteria of *Capsicum annuum* var. *grossum*

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Endophytic bacteria might have numerous beneficial effects on plant physiology like improved resistance against abiotic and biotic stress factors, phytohormon production and protection against plant and human pathogens, although interactions between endophytic and human pathogenic bacteria may contribute to bacterial penetration into the plant tissues (Teplitski et al., 2009).
In our recent study we aimed to identify and analyse potential endophytic bacteria isolated from different cultivars of *Capsicum annuum* var. *grossum* ("sweet pepper") grown in soil- and hydrocultures and to determine the potential endophytic nature of these bacteria by using the seed germination test.
Isolation of bacteria was performed from seeds, vegetative tissues and fruits of *C. annuum* var. *grossum* (cultivars Ho and KPA) after surface disinfection. Samples from seeds and organs were cultivated on complete, differential chromogenic and coliform-selective agar plates. Altogether 304 bacterial pure cultures were isolated. The isolates were subjected to phenotypic analyses like cell and colony morphology, biochemical and physiological tests, as well as spore forming ability. Based on these characteristics phenograms were constructed by clustering the results with the STATISTICA 10 software. Considering the results it can be concluded that the isolates were mostly heterogenic in phenotypes, but there were some phenotypically identical isolates as well. Concerning the molecular biological analyses, molecular typing was done by rep-PCR method using the M13 minisatellit primer as the first step. Molecular fingerprints were evaluated by the GelCompar II software. It was observed that clonally identical isolates originated from peppers grown under the same conditions (i.e. hydro- or soil cultures) and in most cases they were isolated from the same plant tissue. Isolates belonging to the *Pseudomonas* genus were detected by a genus-specific PCR reaction and they were identified by sequencing the *rpoB* gene. Sequencing of the 16S rRNA gene was used for identification of the non-*Pseudomonas* isolates. Identified isolates belonged to the genera of *Acetobacter*, *Achromobacter*, *Acidovorax*, *Agrobacterium*, *Bacillus*, *Brevibacillus*, *Comamonas*, *Cupriavidus*, *Delftia*, *Enterobacter*, *Erwinia*, *Leclercia*, *Mesorhizobium*, *Nanobacterium*, *Paenibacillus*, *Pantoea*, *Pseudomonas*, *Ralstonia*, *Rhizobium*, *Rothia*, *Serratia*, *Stenotrophomonas*, *Thermoleophilum* and *Xanthomonas*. Considering that endophytic bacteria must not inhibit seed germination, we used the seed germination test as described by Niranjan Raj et al. (2003). The results revealed

that certain species from the genera of *Enterobacter*, *Brevibacillus*, *Delftia* and *Leclercia* had strong inhibitory effect on seed germination, while some species belonging to the genera of *Pseudomonas* and *Paenibacillus* had outstanding stimulatory effect. It can also be concluded that the potential endophytic nature is determined at strain level in certain cases, because different isolates belonging to the same *Pseudomonas* species showed either inhibitory or stimulation effects.
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Keywords: endophytic bacteria; *Capsicum annuum*

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P6.20

Arbuscular mycorrhizal fungi host a so far undetected intracellular microbiome

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The root-associated microbiome greatly influences the nutrition and health of plants, with the fungal component performing pivotal functions (Smith and Read, 2008). Arbuscular mycorrhizal fungi (AMF), an ancient group of soil fungi which belong to the phylum Glomeromycota (Schüßler et al., 2001), are obligate biotrophs that colonize the roots of most land plants, playing a key role in

improving plant growth and resistance to environmental stresses. They also represent a niche for hosting endocellular bacteria in their hyphae and spores, although the functional significance of this association is still unknown. Two types of endobacteria are known in the AMF: the rod-shaped Gram-negative *Candidatus Glomeribacter* gigasporarum (*Ca*Gg) (Bianciotto et al., 2003), which is limited in distribution to members of the order Gigasporales, and the coccoid Mollicutes-related endobacteria (Mre) (Naumann et al., 2010) widely distributed across different lineages of AMF.

On the basis of previous morphological and molecular analyses, we hypothesized that some AMF could host a double population of endobacteria. Hence, we carried out an extensive investigation of both endosymbionts in thirteen *Gigaspora margarita* spore samples using different approches. We combined molecular PCR-based techniques (molecular phylotyping and quantitative real-time PCR) with microscopical approaches (transmission electron microscopy, fluorescence *in situ* hybridization) to assess the simultaneous presence of the two bacterial populations, to quantify their relative abundance and to observe their morphology and localization. We demonstrate that both the endobacteria are localized in the fungal cytoplasm: *Ca*Gg is compartimented in a fungal vacuole-like structure, whereas Mre are directly embedded in the cytoplasm. Fluorescent *in situ* hybridization experiments confirm their simultaneous presence inside the fungal spore, suggesting a more abundant presence of Mre than *Ca*Gg, as also confirmed by quantitative real-time PCR. Sequences obtained from PCR experiments reveal that, notwithstanding the endobacteria share the same fungal host, they show an important difference in genetic diversity patterns and that Mre seem to be more prone to recombination.

The coexistence of bacterial endosymbionts with diverse genetic features in a single fungal spore reveals that fungi, as other eukaryotic hosts, support a complex microbiome in a single cell.

Keywords: arbuscular mycorrhizal fungi; fungal microbiome; endobacteria

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P6.21

Rhizobacterial communities of *Phragmites australis* grown in hypersaline ponds

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Hypersaline environments are globally distributed on Earth. The effect of salinity on the sediments makes the environment suitable for peculiar microbial communities adapted to these extreme ecosystems. Bacteria are selected to survive at different salinity concentrations and halophyilic bacteria resulted to be the most common group detected in such environments. Few studies have been focused on the differences and effects of roots on microbial communities. Our study was conducted near Wuliangsuhai Lake in the western part of Inner Mongolia (Autonomous Region - China). In June 2011 we collected five samples in duplicates from hypersaline pond: WL70 and WL71, associated to the rizosphere of *Phragmites australis* (Cav.) Trin. ex Steud. and WL 74 of bulk soil. Microbiological analyses have been assessed by Automated Ribosomal Intergenic Spacer Analysis (ARISA) and 454 pyrosequencing.
Cluster analysis based on the ARISA of the bacterial communities showed high similarity of the duplicates within the same sample site. The rhizobacterial communities clustered differently with respect to the samples of bulk soil. In the root-free soil, 454 pyrosequencing data showed that *Cyanobacteria*, *Deferribacteres*, *Opitutae* resulted to be most abundant taxa or to be at least present. On the other hand, *Deinococci* were present only in the rhizosphere samples. The other classes were present both in the bulk soil sample and in the rhizosphere sample. Consequently, here we show that the peculiar rhizosphere environment, rather than saline stress, is a stronger factor determining the overall genetic structure and taxonomic diversity of dwelling bacterial community.

Keywords: rhizobacteria; bacterial community structure; diversity; hypersaline; pyrosequencing

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P6.22

Potential application of nanofilters to recover microbial strains in brewering

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Beer production process often requires a step of pasteurization before commercialization to kill unwanted bacteria and yeast. At the high commercial scale this process may be an energy intensive process, leading to loss of resources. Moreover, fermentative yeasts are permanently lost. A new and promising alternative to the heating process is nanofiltration with nanofibrous membranes prepared by electrospinning.
Electrospinning is a technique which use of a high voltage to eject at high speed a polymer fluid. The working principle is straightforward. A high-voltage is applied between a syringe containing a polymer fluid and a metal foil collector. When the potential reaches a critical value, then a jet of polymer erupts resulting in the formation of a thin fiber, which is collected on the metal foil collector. The resulting membrane has then be used to remove bacterial spoilers from beer. Moreover, the yeast retained by the membrane remain active and can be recovered for later use.
Fouling mechanism and physicochemical characteristics of the brew obtained before and after filtration were studied at different operating conditions, with a dead-end lab-scale filtration set-up. The beer samples were inoculated with different bacterial strains and yeasts. The bacterial strains were selected among a set of strains common in the food processes as well as into the open environment, with some of them known to be closely related to potentially opportunistic pathogens. Bacterial were chosen also to guarantee the wider cell morphology as possible. The beer samples were filtered and the filtrated product was inoculated on Petri dishes in order to see the presence and viability of the cells. The membranes showed a superior performance than commercial membranes regarding removal of bacteria. The result of this investigation will be presented at the congress.

Keywords: bacterial spoilers; yeasts; beer production; nanofiltration; electrospinning

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P6.23

Bacterial profile from hard faeces in growing rabbits fed with *Lythrum salicaria*

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The microbial community of the rabbit's gastrointestinal tract is characterized by its high concentration, wide diversity and complexity of interactions, having a profound influence on immunological, nutritional, physiological and protective processes in the host animal (Fortun-Lamothe et al., 2007). Numerous studies have investigated the effect of dietary supplements with different type and level of fibre to control and prevent the rapid spreading of the diseases that commonly occur in intensive rabbit farms. *Lythrum salicaria* (LS) is an invasive and competitive plant with medical properties such as anti-microbial, anti-fungal, anti-inflammatory and anti-oxidant, as proven by *in vitro* studies (Tunalier et al., 2010). Given the plant's interesting properties, the aim of the present study was to verify its effects on the diversity of bacterial communities from the hard feces of rabbits fed with different amounts of LS. The microbiota were assessed at species level by using Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis of 16S rRNA gene of DNA extracted directly from hard feces (HF) samples. Thirty weaned, 35 days old Hycol rabbits were divided into 3 groups of 10 animals of the same gender. Three different levels of LS powder (0, 0.2 and 0.4%) were added in the common formula diet. HF samples were collected after 0, 3, 5, 7, 14, 21, 35, and 54 days. The dendrograms generated using DGGE profiles of 16S rRNA fragments show that the similarity between rabbit fed with LS was higher if compared with conventional diet. On the other hand the control group fingerprints were different between the older and younger age groups. In conclusion, it appears that only age influenced the dynamic of the microbiota which was observed in this study.

Keywords: PCR-DGGE; rabbit gut microbiota; *Lythrum salicaria*

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P6.24

Survival to simulated gastric juice and simulate gastrointestinal transit in lactic acid bacteria from traditional cheeses

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Cheese has been used as a carrier of probiotic bacteria (Karimi et al., 2011) because it affords better protection to their viability compared to fermented milks and probiotic bacteria in cheese are known to survive simulated gastrointestinal transit (SGT) (Mäkeläinen et al., 2009). On the other hand the secondary microbiota of cheese is dominated by species which include probiotic strains, such as *Lactobacillus plantarum*, *Lb. paracasei* and *Lb. rhamnosus* (Settanni and Moschetti, 2010). Traditional pasta pasta filata cheeses are no exception (Piraino et al., 2005). The objective of this work was to evaluate the secondary microbiota of traditional pasta filata and ewe's milk cheeses produce in Basilicata, Southern Italy, to evaluate its potential to survive to gastro-intestinal transit, and to isolate potential probiotic candidates. Non-starter Lactic Acid Bacteria were enumerated in samples of 20 semi-hard and hard (ripening 1-11 months, median 3 months) cheeses (3 ewe's milk, 17 cow's milk pasta filata cheeses). A selective treatment with simulated gastric juice (SGJ) for 30 min followed by enumeration on a differential medium (mMRS) was used to select for acid resistant strains, and the counts were compared with those obtained from Rogosa agar. In 11 cheeses the survivors to the SGJ treatment exceeded 10^7 cfu/g and were 10-100% of NSLAB enumerated on Rogosa agar. 277 random isolates (from 3 to 20 per cheese, median 14) from mMRS were characterized by using a combination of molecular methods. The majority of isolates were identified as *Lb. paracasei* (35%), *Lb. fermentum* (17%), *Lb. rhamnosus* (13%), *Enterococcus* (10%), or *Pediococcus* (8%). From 2 to 5 species were retrieved from each sample (median=3). Although members of the *Lb. casei* group were almost alwasy present and often dominant, some samples were dominated by heterofermentative species (*Lb. fermentum*, *Lb. buchneri* and *Lb. parabuchneri*) or enterococci. The composition of the microbiota estimated by DGGE-PCR of bulk DNA extracted from Rogosa plates confirmed this pattern. The bile salt tolerance and bile salt hydrolase (BSH) activity were tested on 90 strains. 64% were able to growh with 0.15% bile and 20% with 0.3%; 40% were BSH positive, with little association between bile resistance and BSH. Fifteen strains were tested for their ability to survive a short SGT (5 min simulated saliva,

SS, 30 min simulated gastric juice, SGJ, and 120 min simulated pancreatic juice, SPJ; De Angelis et al. 2006) when suspended in a simulated dairy drink, pH 4.2. Inacivation, as log(N/N0), ranged from -0.15 to -2.93, median -0.64, with no correlation with bile tolerance. The lethality was due mostly to the SPJ stage, while SS and SGJ had little effect. A selective treatment based on simulated gastric juice exposure alone may not provide a correct estimate fo the survivors to GI transit, but allowed to isolate lactobacilli with a high tolerance to SGT, which may be tested as probioti candidates.
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Keywords: cheese; acid stress; simulated gastric transit; *Lb. Paracasei*; *Lb. rhamnosus*

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P6.25

Evaluation of different methods to improve *Bifidobacterium* spp. tolerance to pH and other stress conditions

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The aim of this study is to generate stable resistant Bifidobacterium spp. strains to different and combined stress conditions. *Bifidobacterium* strains are some of the most important probiotics used for human consumption. The high sensitivity of the strains of this genus to pH, water activity, oxygen content and other stress parameters can, however, impair their survival in adverse environments such as,

foods with high concentration of organic acids, phenols, like fruit juices, and, therefore, limits their use as live probiotic cultures. The ability of 31 Bifidobacterium strains to survive to stress conditions as well as the success and limitations of the application of acid stress adaptation treatments to improve their stress tolerance are reported. The strains belonged to different species like, *B. longum subsp. longum, B. longum* subsp. *infantis, B. adolescentis, B. breve, B. pseudocatenulatum, B. catenulatum, B. animalis* subsp. *lactis, B. bifidum*.
Different methods to improve the stability of *Bifidobacterium* spp. in stress conditions were evaluated. One of the methods was daily cultivation of strains in TPY medium pH 3.5 for 2 weeks, and finally isolation of resistant colonies until the end of the 2 weeks treatment. The second method was inoculation of previously isolated acid resistant phenotype of strains in two different cellular concentration, concretely 10^5 and 10^7CFU into 400 ml of a 20% blueberry fruit juice and evaluating their viability every 24 h of incubation. However, this method was not efficient, because the acid resistant phenotypes isolated after 48h of incubation lost from 3 to 5 logarithmic scale of their total cell number and after 62 h of incubation of accelerated shelf life (37ºC) into the fruit juice they totally lost their viability.
A third method was experimented: firstly by screening the strains inoculating them directly into 6 ml of juice fruits and evaluating their viability after 24h at 37ºC. Generally the most resistant strains lost 1-2 logarithmic scale in their viability and belonged to the species *B. animalis* subsp. *lactis*, whereas strains of *B. bifidum* and *B. breve* lost 2-3 logarithmic scale respectively. Strains from *B. longum* subsp. *longum, B. longum* subsp. *infantis, B. adolescentis* showed a very poor resistance failing their survival during this screening procedure. The most promising strains were slowly adapted to survive in a model fruit juice mixture, concretely the strains were inoculated initially in a high cellular concentration (10^8-10^9 CFU) in a mixture formed by TPY broth media and fruit juice, in an increasing volume of the juice every 24 h. The volume of juice added every day was 200 µl substituting the same volume of TPY broth media. The viability of the strains was daily evaluated and it slowly decreased in a strain dependent manner, with *B. animalis* subsp. *lactis* P32 and *B. bifidum* B1958 and *B. breve* B609 as the more resistant strains.
This last method could be successful to gain *Bifidobacterium* strains with improved stability in adverse environments and, probably, contribute to expand the spectra of probiotic species of human origin currently marketed.

Keywords: *Bifidobacterium* spp.; probiotics; pH tolerance; stress condition; blueberry fruit juice

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P6.26

Probiotic activity of riboflavin-overproducing *Lactobacillus* strains

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Probiotic microorganisms are achieving considerable interest because of their health-promoting properties for human. Probiotic attributes include safety as well as antinflammatory, antiallergic, immunomodulatory and intestinal equilibrium-maintaining activities. In addition, some probiotic strains are able to protect from pathogens and synthetize vitamins or fatty acid, thus contributing greatly to ensure a good state of health for the host (FAO/WHO, 2001; Ouwehand et al., 2002). In this context, the aim of this work was to investigate the potential probiotic properties of two Lactobacillus strains, i.e. *Lactobacillus plantarum* B2 and *Lactobacillus fermentum* 11.5, which had been previously selected for their capability to overproduce riboflavin. Firstly, we examined their survival capability through an *in vitro* model which simulates the human gastrointestinal transit. To this aim, three different carrier matrices were used, including saline solution, milk and yogurt. Both of the tested strains showed a high tolerance to the oral, gastric and intestinal stresses, in particular when yogurt was used as a vehicle matrix. Secondly, the strains were characterized for their ability to adhere on human colon adenocarcinoma cells in order to estimate their intestinal colonization potential, which is another desirable feature of probiotic microorganisms. Both strains exhibited a high percentage of adhesion on Caco-2 cells and, in competitive assays, they hindered adhesion by the enteropathogen *Escherichia coli O157:H7*. Furthermore, the tested strains could significantly inhibit the growth of *E. coli O157:H7*, *Listeria monocytogenes* and *Salmonella enteretidis* on agar plates. Finally, Caco-2 monolayers were exposed to Lactobacilli cultures and possible effects on the barrier function of the intestinal epithelium were evaluated by trans-epithelial electric resistance measurements and by assessing transcriptional variations of genes involved in modulating the integrity of epithelial cell barrier. Lack of both hemolytic activity and antibiotic resistance was verified and confirmed in the tested Lactobacilli.

In conclusion, the strains analyzed in the present study seem to comply with most of the pre-requisites of probiosis, which are routinely considered during *in vitro* selection of potentially beneficial microorganisms. In addition, their ability to over-produce, virtually in situ, an essential vitamin as is the riboflavin, makes such strains even more attractive in view of potential therapeutic applications.

Keywords: *Lactobacillus fermentum*; *Lactobacillus plantarum*; riboflavin; probiotic activity

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P6.27

Exploring the colonization ability of probiotic lactic acid bacteria strains in zebrafish: an *in vivo* model

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Probiotics are viable microorganisms that are able to reach the intestine in an active state and thereby exert positive health effects (De Vrese and Schrezenmeir, 2008). A number of Lactobacillus species have been proposed as probiotic strains and commercialized as live microorganisms for food supplemention (Bove et al., 2013). Although the microbial potential to colonize the human gut lumen has been investigated in in vitro models, there is still much to discover about their *in vivo* behaviour. Zebrafish (*Danio rerio*) is a vertebrate model that is being widely used to study various biological processes shared with humans. Particular advantages of

using Zebrafish are that i) the embryos are transparent and the internal organs can be easily seen, ii) the costs are relatively low, iii) it is preferred from an ethical point of view to the use of mammalian models and iv) its genome is known thus allowing functional genomics assays. In this work we report the first use of the zebrafish model to investigate the *in vivo* colonization ability of probiotic strains belonging to the genus Lactobacillus, monitoring the expression of a recombinant fluorescent protein.

Germ-free zebrafish larvae were obtained by disinfecting the embryos with a combination of antibiotics (Pham et al, 2008) and a pulsed light treatment to obtain completely axenic organisms. Recombinant strains of *Lactobacillus fermentum*, *Lactobacillus plantarum* Lp90 and *L. plantarum* Lp8 were constructed using the new pRCR12 vector (unpublished results), containing a synthetic mrfp, designed for optimal mCherry expression in lactic acid bacteria, under control of Px promoter (García-Cayuela et al. 2012). These were used to infect germ-free (gnotobiotic) and conventional (non–axenic) larvae (5 dpf) by immersion at 27 °C and agitation (120 rpm) at a concentration of 107 cfu ml-1 of probiotic cells. After 15 h, the larvae were washed in order to eliminate the microorganisms from the medium and the colonization ability of the strains was qualitatively monitored at 5 and 24 h respectively by using a fluorescence stereomicroscope.

The observation of the fluorescence at 5 h indicated differing adhesion capabilities, with the proximal and distal intestinal tract mainly colonized by the *L. plantarum* strains and *L. fermentum*, respectively. In addition, the intensity of the fluorescence emitted by the larvae infected with L. plantarum strains appeared to be greater than with L. fermentum. 24 h after removal of the probiotics by washing no fluorescence was detected in larvae infected with *L. fermentum*, while approximately 50% of the gnotobiotic fish infected with *L. plantarum* continued to fluorescence. Interestingly, at this time the microbial colonization seems to move to the distal part of the intestine of the host. No significant differences were detected between gnotobiotic and conventional larvae.

In conclusion, our results suggest that zebrafish represent an alternative animal model that could be applied as a powerful tool to investigate, *in vivo*, the activity of probiotic microorganisms.

Keywords: probiotic; lactic acid bacteria; zebrafish; colonization; gnotobiotic

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P6.28

Bioactivity of *Lactobacillus plantarum* and *Bifidobacterium animalis* subsp. *lactis* combined with citrus extract towards *Zygosaccharomyces bailii* in a model fruit juice

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A new challenge for juice producers is the preparation of functional beverages, inoculated with different kinds of probiotic microorganisms (lactobacilli or bifidobacteria); moreover, there is an increasing trend towards some alternative approaches for the preservation of juices (both physical methods or natural antimicrobial compounds) to avoid the loss of nutrients and the browning of juices caused by the thermal treatments.
Nowadays the thermal treatments are designed to control and/or inactivate the growth of some spoiling microorganisms (yeasts belonging to the genera *Saccharomyces*, *Zygosaccharomyces*, *Pichia*, and *Candida*, moulds or bacilli); therefore, in a functional juice an additional trait required to the probiotic is the ability to compete and inhibit the growth of the spoiling microflora. The aim of this research was the evaluation of the bioactivity of two probiotic strains (*Lactobacillus plantarum* c19 and *Bifidobacterium animalis* subsp. *lactis* DSM 10140) in a model system, simulating a fruit juice and containing a natural citrus extract.
A commercial beverage (red fruit juice), diluted with water (1:1), was inoculated to 6-7 log cfu/ml with either *L. plantarum* and *B. animalis*; thereafter, the samples were inoculated with *Z. bailii* (2-4 log cfu/ml), added with a citrus extract and stored at 15°C for 24-48 h (thermal abuse) and then at 4°C. The level of the yeast (0-2-4 log cfu/ml), as well as the concentration of citrus extract (0-20-40 ppm) and the duration of the thermal abuse (0-24-48 h) were combined through a factorial design. Samples of diluted juice, with and without the citrus extract and inoculated with the yeast but not containing the probiotics, were used as controls.
The samples were stored for 18 days and the viable count of both the probiotics and the yeast was assessed throughout the running time; the results were used to build a polynomial equation through the theory of the Design of Experiments and

the black-box approach.
The viability of *Z. bailii* in the juice was affected by both the amount of the citrus extract and the initial level of inoculation; an interesting trait was that *B. animalis* and *L. plantarum* were able to control the growth of spoiling yeast and avoid the increase of its cell count throughout the time. Moreover, the combination of *L. plantarum* with 40 ppm of citrus extract exerted a strong bioactivity towards *Z. bailii* and reduced its level by more than 2 log cfu/ml.

Keywords: juices; probiotics; spoiling yeasts; interactions; citrus extract

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P6.29

Effect of whole grain consumption on gut microbiota of overweight and obese subjects

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Epidemiological studies associate whole grain (WG) consumption to a reduced risk of cardiovascular disease, diabetes, obesity and some types of cancer. Recent evidence suggests that intestinal microbiome may play a role in the risk and progression of several non-communicable chronic diseases. (Martìnez et al., 2013). In the lower gut, WG represents a natural source of complex carbohydrates, some key vitamins, minerals and polyphenols. In addition, intestinal bacteria can ferment WG polysaccharide moiety releasing phenolic acids (FA), which can trigger microbiota modification and even drive systemic metabolic benefits once absorbed through the colon (Vitaglione et al., 2008).
The aim of this study was to evaluate gut microbiome variation in healthy overweight and obese subjects including in habitual diet 70 g of Shredded Wheat (100% whole grain) (WGG, n=31) or some refined cereal foods (CTG, n=17) for 2 months in substitution of iso-nutrient/energy portions of habitual refined cereal foods. Total DNA from fecal homogenates was extracted by using Power Soil DNA Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). PCR amplicons of the V4 region of the 16S rRNA gene were obtained and sequenced. Sequences were analyzed by using the QIIME software version 1.5.0. Unweighted UniFrac clustering of the samples showed a high level of similarity between WGG and CTG subjects. The distribution of OTUs with an incidence greater than 3% showed a complex microbiota consisting of up to 229 OTUs. Sequence data processing was performed by comparing the OTUs of subjects with the treatment group, age and

sex as independent variables. Data showed that gut microbiome was different in male vs female individuals ($p<0.05$), while no significant variation was found in relation to dietary treatments or age. However, some interesting trends of variation upon the study period in relation to diet and gender were noticed for some groups of OTUs. *Prevotella* sp. increased in WGG subjects particularly in male individuals (ca. 42%). *Dialister* sp. decreased in WGG male subjects while it increased in CTG regardless of the gender. *Roseburia* sp. increased in subjects independently of the diet but particularly in the males; *Bifidobacterium* sp., *Blautia* sp. and *Collinsella* sp. reduced while *E. coli* increased independently of the diet and gender. The correlation of these data with several biochemical data monitored upon the study will allow us to clarify mechanisms underlying health benefits exerted by WG consumption in overweight/obese population.

Keywords: gut microbiota; metabolic disorder; whole grains

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P6.30

Effect of probiotic *Lactobacillus reuteri* on gastrointestinal microbiota modulation and on clinical symptoms in patients with SNAS allergy

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Systemic Nickel Allergy Syndrome (SNAS) is a pathological condition related to the ingestion of Ni with food and it is characterized by cutaneous reactions and/or gastrointestinal (GI) symptoms which shows after ingestion of Ni-containing foods (Falagiani et al., 2008; Gangemi et al., 2009) Clinical symptoms are typically: eczema, urticaria, itch, dyspeptic symptoms, such as nausea and/or vomiting, heartburn, bloating, chronic abdominal pain, diarrhoea and constipation (Falagiani et al., 2008).

The purpose of this study was evaluate the effect of the probiotic *Lactobacillus reuteri* DSM 17938 strain (NOOS S.r.l., Italy), on the intestinal microbial population of SNAS patients, inducing the reduction of severity and frequency of gastrointestinal symptomatology as well as allergic cutaneous symptoms rather than a low Ni diet alone. For these reasons, a prospective double-blind randomized placebo-controlled study was planned. Sixteen patients, all females, aged from 18 to 65 years, with clinical symptoms correlated to SNAS were enrolled. The clinical severity of both allergic cutaneous and gastroenterological symptoms was evaluated. All patients followed a specific diet, in order to reduce the daily intake of nickel, and they were randomly assigned to two groups (experimental and placebo). The duration of the whole intervention was of 6 weeks. Faecal samples from patients were collected before, during and after the intervention, stored at -80°C and analysed throughout a culture-independent approach. In order to characterize the bacterial diversity in human fecal samples, ribosomal DNA (rDNA) was extracted from fecal samples, subjected to PCR amplification, by using *Lactobacillus* specific primers for the V1 to V3 regions (Walter et al., 2001) and universal primers for V6 to V8 regions of 16S rDNA (Randazzo et al., 2002) and analyzed by PCR-DGGE (Muyzer et al., 1993). Moreover, the impact of symptoms on the quality of life of each patient, by a Visual Analogic Scale, the frequency of gastrointestinal symptoms, consistency of stools, according to the Bristol Stool scale, side-effects bowel habits and also stool frequency were investigated. Significant changes in the severity scoring index were recorded in patients who received the probiotic supplementation compared with patients who received placebo as well as a significant reduction in drug consumption (antihistamines, antisecretory) and a significant increase in well days. Moreover, SNAS patients, which received the probiotic supplementation, showed a significant improvement in cutaneous and GI symptoms. DGGE fingerprinting obtained using universal primers revealed quite stable profiles during the treatment while results obtained with *Lactobacillus* specific primers highlighted significant shift during the treatment with an increase in LAB population in samples of patients which received probiotic supplementation. Hence, our findings confirm that the *L. reuteri* supplementation could be proposed as a valid tool for the SNAS pathology treatment.

Keywords: probiotic; PCR-DGGE; Nickel allergy; double-blind randomized placebo-controlled study

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P6.31

Microbial enrichment of compost from olive waste with biological control agents to enhance plant health and growth at farm level

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It has been estimated that over 20 thousand tons of olive husks and more than 15 thousand m3 of olive wastewater are generated per year in Molise Region. Furthermore, a large undefined amount of olive prunings are produced each year. So far, the widely used methods for disposal of olive waste (OW) has been field spreading and combustion (Alfano et al., 2009). The composting process proved to be one of the most reliable method for bioremediation and valorization of OW, and it allows to avoid the harmful effect on the soil that could be caused by the spreading of untreated wastes, combining the recovery of valuable resources with the environmental protection (Roig et al., 2006; Alfano et al., 2011a, 2011b).
Composting proved to be an effective way to transform OWs into valuable, high-quality agricultural amendments and fertilizers particularly suitable for organic farming systems. Moreover, it has been reported that OW compost can actively contribute to the biological control of several plant pathogens.Compost varies considerably in chemical, physical and biotic composition, and, consequently, in ability to suppress or cause soil-borne diseases (Termorshuizen et al. 2006; Alfano et al., 2011b).
In this context the BioCompost project (POR-FESR 2007-2013) was born as a response to the double need of some olive oil producer to sustainably and economically dispose of OW and of some farm/nursery to reduce fertilizers and pesticides costs.
Aims of the project are: (i) to createan olive mill waste transformation and reuse chain, linking several different regional farms and companies including olive mills, farms, nurseries, biotech companies; (ii) to improve biological productions through the use of olive waste compost; to improve plant growth and health through the use of compost inoculated with biological control agents and plant growth promotion rhizobacteria.
The use of OW composts (inoculated or not) significantly improved health, growth and production of several horticultural and ornamental plants including tomato, grape, olive, peach, holm oak, polygala, both in open field and nursery cultivation

systems.

Keywords: olive waste; compost; organic fertilizer; bio-pesticide; biocontrol agents

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P6.32

Human gut microbiota of children with autism and pervasive developmental disorder not otherwise specified

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Autism spectrum disorders (ASD) are a complex neurodevelopmental disorders characterized by impairments in social interaction and communication. ASD (0.6-1% of prevalence) include autism (AD), Asperger's Syndrome, and Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS). ASD patients often suffer from gastrointestinal (GI) problems (Adams et al., 2011). Recently, a gut-brain-microbiota axis is coined to describe interactions between the three systems (Forsythe and Kunze, 2013). It was hypothesized qualitative and quantitative differences of the microbiota in AD patients compared to healthy control (HC) (Finegold et al., 2010). This study aimed at comparing the differences of the fecal microbiota between AD, PDD-NOS and HC children. Fecal microbiota was characterized through an integrate approach of culture-dependent and -independent methods. Bacterial tag-encoded FLX-titanium amplicon pyrosequencing (bTEFAP) and Biochrom 30 series Amino Acid Analyser were carried out for genomic and phenotypic analyses. Each group (AD, PDD-NOS or HC) was composed by ten children. Based on 16S DNA analisys, the main bacterial phyla

(Firmicutes, Bacteroidetes, Fusobacteria and Verrucomicrobia) significantly ($P<0.05$) changed between the fecal microbiota of PDD-NOS, AD and HC children. As estimated by rarefaction, Chao and Shannon diversity index, highest microbial diversity was found in AD children. Since the level of total RNA in a bacterial community is proportional to its metabolic activity (Mills et al., 2004) to highlight the metabolic role of the microbiota on AD and PDD-NOS disorders, metabolic active microbiota was analyzed by partial sequencing the 16S rRNA genes.

Faecalibacterium and *Ruminococcus*, the main genera of Ruminococcaceae, were present at highest level in PDD-NOS and HC children. With a few exceptions (e.g., *Clostridium barlettii*), the identified Clostridiaceae species belonging to *Caloramator*, *Sarcina* and *Clostridium* genera, were highest in AD children. Clostridiaceae are the principal bacteria group that produces some metabolic products that are potentially toxic (e.g., phenols, p-cresol, certain indole derivatives). Compared to HC, the composition of Lachnospiraceae family (*Roseburia*, *Dorea*, *Coprococcus* genera) also differed in PDD-NOS and, especially, in AD. The composition of lactic acid bacteria species belonging to *Enterococcus*, *Lactobacillus* and *Streptococcus* genera differed between AD and HC children. The level of Bacteroidetes genera (*Bacteroides*, *Barnesiella*, and *Parabacteroides*) and some *Prevotella* species were almost highest in AD children. This study also showed that almost all identified Sutterellaceae (*Parasutterella* genus) and Enterobacteriaceae (e.g., *Proteus*, *Shigella*) were higher in AD children compared to PDD-NOS and HC. Compared to HC children, *Bifidobacterium* species decreased in AD. According to the level of dominant proteolytic genus (e.g., *Clostridium* and *Bacteroides*), the free amino acids were highest in PDD-NOS and, especially, in AD fecal samples. It was hypothesized that glutamatergic neurotransmission plays a role in the pathophysiology of AD (Shimmura et al., 2011). In agreement, Glu was found at the highest level in the fecal samples of AD children. If the main microbial differences in AD and PDD-NOS compared to HC are found to be a causative or consequent factor in these types of autism, they may have implications for treatment and prevention.

Keywords: autism; human intestinal microbiota; pyrosequencing

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P6.33

Arbuscular mycorrhizal fungi of Laguna Salada: an arid environment at Baja California, Mexico

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Arbuscular Mycorrhizal Fungi (AMF) are important component in the terrestrial ecosystems (Bever et al., 2001). These microorganisms are considered to be critical for the survival of many plants in arid environments (Titus et al., 2002). AMF contribute to drought tolerance and the uptake of phosphorous and nitrogen. This study was conducted to characterize the AMF species associated to the predominant plant species of Laguna Salada, and to determine the level of AMF colonization to the roots of the plants in the area. Laguna Salada is a vast dry lake surrounded by scarce vegetation thus considered as an extremely arid zone. It is located in the northwest of Mexico, 30 km southwest from Mexicali city (32°21´56´´ N, 115°40´20´´ W; elevation 7 masl). Annual precipitation varies from 54.9 to 127.4 mm and the temperature can reach up to 52°C during the summer. The basin of the lake is not capable to support vegetation due to its high level of salinity. Sampling was carried out along the west edge of Laguna Salada. Roots and soil samples from nine plant species were collected (n=5 for each species). Soil samples have sandy loam texture, pH 8.1 to 9.7, EC 9.78 dS/m, 0.18% of organic matter and 12 kg ha-1 of nitrogen. Percentage of mycorrhizal colonization was determined in the roots of *Allenrolfea occidentalis*, *Tamarix ramosissima*, *Suaeda divaricata*, *Pluchea sericea*, *Larrea tridentata*, *Haplopappus sp*., *Atriplex canescens*, *Prosopis sp*., and *Lycium andersonii*, including the number of spores and sporocarps from the soil samples of these plants. *Allenrolfea occidentalis*, *Suaeda divaricata*, *Atriplex canescens* and *Tamarix ramosissima* were nonmycorrhizal. *Pluchea sericea*, *Larrea tridentata*, *Haplopappus sp*., *Prosopis sp*., and *Lycium andersonii* were highly AM colonized (96–98%). Spores and sporocarps of AMF were present in soil samples from all plant species studied, even in those nonmycorrhizal plants. The number of spores of AMF present in the

soil varied depending on the plant species. The number of spores and sporocarps was lower (p>0.05) in nonmycorrhizal plants (42-97 spores; 46-172 sporocarps/50 g dry soil) than those mycorrhizal plants (160-248 spores; 242-377 sporocarps/50 g dry soil). In general, sporocarps were more abundant than spores. Six spores and two sporocarps morphotypes were observed, predominantly of *Glomus* genus. This study reveals the abundance and diversity of AMF in an environment that is highly saline and poor in organic matter and nitrogen. Most plants of Laguna Salada survive in symbiosis with AMF. Although some plant species were nonmycorrhizal, their rhizosphere were found to be abundant with AMF hyphae and spores. High colonization of the roots by other fungi was also observed which could play important roles for the survival of these plants.

Keywords: arbuscular mycorrhizae; Laguna Salada; arid environment

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P6.34

Survey of endophytic fungi living in pepper (*Capsicum annuum* L.)

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Endophytic organisms play a fundamental role in plant life and are important for breeding, agrotechnology and food safety. The presence of endophytes is a risk factor during *in vitro* production of dihaploid breeding material. Several endophyte species were shown to be beneficial, while others may behave as opportunistic pathogens or human pathogens. Their enrichment in soil or in growth media in greenhouse may influence plant production and give rise to safety problems.
In this study, we examined the frequency and species composition of filamentous endophytic fungi in different organs of two F1 hybrid cultivars of *Capsicum annuum* var. *grossum* (Hó and Kárpia), grown either in greenhouse or under field conditions.
Sampling was performed four times during the vegetation period from roots, shoot, leaves, pedicles and differentially developed pericarp as well as from seeds. Surface sterilized organ slices were put on PDA medium supplemented with

chloramphenicol to prevent bacterial growth. After morphotyping the colonies of putative endomycota, individual strains were isolated and then monosporated or – the non-sporulating ones – monohyphated. The isolates were divided into morphological groups, then from each group representative samples were chosen for DNA-based identification. In the selected strains we used PCR to amplify the ITS1+5.8S RNA+ITS2 region of the rDNA with the ITS1 and ITS4 primers. Fungal strains were identified on the basis of the nucleotide sequences of PCR-products.

Our results indicate that greenhouse-grown and field-grown peppers harbour different endomycota. We also observed differences between individual plant parts and seasonal difference in the samples of the same plant. Plant parts of the cultivar 'Hó' usually showed higher colonisation rate by endophytic fungi than those of 'Kárpia'. As expected, colonization rates were higher in field-grown plants and in older organs. Highest colonisation frequency was found in old leaves and in stalks of fruits. Several strains belonging to the *Alternaria* genus were detected; members of this genus occurred in every plant part. From the root we also isolated *Plectosphaerella, Colletotrichum, Paecilomyces, Penicillium* and *Fusarium* strains, while in the shoot *Cladosporium, Acremonium, Chaetomium* and *Lewia* strains were identified.

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Keywords: *Capsicum*; endophytic fungi; cultivation conditions

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P6.35

Variability of cryptic viruses in *Beta* and *Capsicum* species and cultivars

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Viruses are now considered as important players of host's evolution, and it is widely acknowledged that a significant number of viruses is not only non-pathogenic, but mutualistic, i.e. its presence is advantageous to the hosts. Plant cryptoviruses (*Partitiviridae, Alphacryptovirus* genus) are widely distributed in the plant kingdom. According to our current knowledge cryptoviruses are only dispersed by seed and pollen, and remain associated with their host plants during its whole life cycle. Their genomes consist of 2-3 monocistronic dsRNA segments ranging from 1-3 kbp in size. One of them always encodes the replicase (RdRp),

the other (two) dsRNA the capsid protein(s) (CP) of isometric virions. They do not induce any symptoms in the host, but may enhance stress tolerance of the host (Nakatsukasa-Akune et al., 2005).

We investigated the variability of cryptic viruses in *Beta* and in *Capsicum* species and cultivars to find out whether the continuous association with the host results in co-evolution and segregation of host-specific sequences. In the *Beta* genus three alphacryptoviruses have been described: *Beet cryptic virus* 1, -2 and -3 (BCV1, -2, -3). In sugarbeet (*Beta vulgaris* convar. *crassa* provar. *altissima*) mainly BCV2 and BCV1 are present, while for chard (*Beta vulgaris* L. convar. *vulgaris)* the presence of BCV1 and BCV3, for beetroot (*Beta vulgaris convar. crassa provar. conditiva)* the occurrence of BCV2 are characteristic. We determined the nucleotide sequences of BCV1 occurring in the chard cultivar 'Lucullus' and those of BCV2 occurring in the beetroot cultivar 'Bíborgömb', and then compared them to the corresponding sugar beet virus sequences. In BCV1 RdRp we observed 89 nucleotide exchanges, but only 6 resulted in amino acid changes. BCV2 sequences are also highly conserved. In BCV2 RdRp 5 nucleotides were mutated in comparison to sugar beet, from these three led to amino acid change. The mutations didn't affect the RdRp motifs of the Partitiviridae family. We conclude that BCV1 and BCV2 cryptovirus sequences are strongly conserved at the amino acid level suggesting a strong selection pressure acting in the host plant.

Furthermore, we investigated dsRNA viruses occurring in different varieties of *Capsicum annuum, C. chinense, C. frutescens, C. baccatum, C. praetermissum, C. pubescens, C. chacoense* and *C. eximium* by dsRNA-specific immunoblotting and by PCR. We found that *C. annuum, C. chinense, C. chacoense* and *C. frutescens* contain both endorna- and cryptoviral sequences, but in *C. baccatum* only the endornaviruses were present. In *C. chinense* we identified and partially cloned a new cryptic virus, differing in its RdRp sequence from both of the known pepper cryptic viruses (PCV1 and PCV2).

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Keywords: *Capsicum*; *Beta vulgaris*; cryptoviruses

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P6.36

Endophytic fungi in cherry trees and their effect on in vitro growth of some cherry pathogens

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Endophytic fungi colonize healthy tissues of plants, at least for part of their life cycle, and inhabit asymptomatically different organs of the host plant. Host-endophyte interaction is generally considered as a kind of symbiotic association which is either neutral or beneficial for both organisms. Although some endophytes can behave as opportunistic pathogens, there is a growing body of evidence regarding the antagonistic effect of fungal endophytes against pathogens in angiosperm hosts (Arnold et al., 2003).
In this study we investigated the species composition and frequency of endomycota in cherry trees grafted on different rootstocks and the effect of selected strains on *in vitro* growth of two cherry pathogens, *Monilinia laxa* and *Agrobacterium tumefaciens*.
To identify putative endomycota surface sterilized organ slices were put on PDA medium and the outgrowing fungal isoltes were visually classified after 1 and 2 weeks. Monosporation and monohyphation technique was applied to generate single colonies of each individual fungus. The isolates were divided into morphological groups, and from each group representative samples were chosen for DNA-based identification using ITS-specific primers. A total of 5161 putative endomycota isolates were identified in root (1353 colonies), twigs (2854 colonies) and leaves (954 colonies), they belonged to 13 orders. A strain of *Alternaria* (*Alternaria sp. 1*) was found as the most frequent endophitic fungus in all tissue samples with 518, 725 and 423 colonies isolated from root, twig and leaf specimens, respectively. A genetically different strain, *Alternaria sp.2* was highly frequent in twigs (1131 colonies), much lower represented in leaves (109 colonies) and missing in the root samples. *Fusarium* showed the highest species diversity: *F. solani* was isolated only from twigs, *F. oxysporum* only from root samples. Two further species, *Fusarium sp.*1 and *Fusarium sp.2* were found in root as well as in twigs, an additional species, *Fusarium sp.3* occurred in all ivestigated organs.
27 strains of putative endophytic fungi were screened whether they show antagonistic effects against *M. laxa* and *A. tumefaciens in vitro*, and the 7 best-growing taxa were selected for more detailed analysis. Out of these, *Botrytis cinerea* showed the strongest groth inhibition against *M. laxa*, while

Agrobacterium was most strongly inhibited by *Ceratobasidium sp.1*, *Embellisia sp.* and by *Alternaria sp.1*.
This work was supported by Hungarian grant TÁMOP 4.2.1./B-09/01/KMR/2010-0005.

Keywords: cherry; endomycota; antagonism

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P6.37

Interaction between potential probiotic lactic acid bacteria and *Listeria monocytogenes* in functional human gut models

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Listeria (*L.*) *monocytogenes* is one of the most important foodborne pathogen causing severe systemic infections posing a significant health risk for pregnant women, newborns and other immunocompromised individuals. In order to maintain intracellular lifestyle this pathogen has evolved a number of mechanisms to exploit host process to grow and spread cell to cell without damaging the host cell. The traditional vaccination is not affordable for the treatment and control of listeriosis, therefore an intake of probiotic bacteria could be considerate as a novel and effective strategies to prevent it in susceptible people. In relation to the pathogenesis of *L. monocytogenes* the inhibition of its penetration into the epithelial human cells could be considered as the most desirable feature. In this contest, *in vitro* gut models are fundamental tools to investigate the potential inhibitory action of probiotic bacteria versus *L. monocytogenes* infection mechanism.
Therefore, the aim of this study was the identification of lactic acid bacteria (LAB) strains capable of reducing *L. monocytogenes* invasion on *in vitro* human gut model.

With this purpose, 17 strains of *Lactobacillus* (*Lb.*) *plantarum*, *Lb. pentosus* and *Leuconostoc mesenteroides* isolated from olives fermentations and resistance to a simulation of human digestion were tested in 2D and 3D models. *In vitro* 3D models were built using foetal human epithelial cells grown in microporous inserts and human macrophages placed under the epithelial layer, called H4 and TLT respectively. The 2D model was prepared using H4 cells only in the undifferentiated status. After a preliminary assay of adhesion in 3D model, all strains were tested in a simulation of *L. monocytogenes* infection performed in the 2D model. Inhibition of pathogen adhesion and invasion was assessed and the strains with the greater inhibition potentiality were selected and tried in 3D models. Invasion, translocation assays and the evaluation of macrophage viability were performed on 3D model, both treated or not with the selected strains. In order to enhance the knowledge on their *L. monocytogenes* inhibition action, the macrophages secretion of interleukin (IL)-10 and IL-12 was quantified by sandwich ELISA.
Concerning the results, the *Lb. plantarum* S11T3E, together with *Lb. plantarum* S2T10D and *Lb. pentosus* S3T60C showed the ability to inhibit the *L. monocytogenes* invasion in 2D epithelial model ($P<0.05$). The *L. pentosus* strain was also able to reduce the pathogen adhesion ($P<0.05$). However, the three strains did not confirm in a significant way ($P>0.05$) the reduction of pathogen invasion when tested in 3D model, despite the S11T3E decreased the number of recovered *L. monocytogenes* by more than 50%. Moreover this strain reduced the stress conditions in the macrophages infected with *L. monocytogenes* but, concerning the modulation activity on the human cells, neither IL-12 nor IL-10 were detected.
In conclusion, the strain *Lb. plantarum* S11T3E has shown the ability to significantly inhibit the invasion of *L. monocytogenes* in 2D model, but the mechanisms of inhibition did not seem related to its immunomodulation activity.

Keywords: human gut model; probiotics; *Listeria monocytogenes*; pathogen inhibition; listeriosis

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P6.38

Analysis of bacterial communities involved in the parasite symbiosis between *Apis mellifera* and *Varroa destructor*

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The interpretation of host-parasite interaction is one of the most intriguing themes in biological studies. In a symbiotic relationship the partners reciprocally influence their physiology and, more in general, their evolution. In the recent years, the identification of the microbiome, thought as all the microscopic living beings found in a symbiotic relationship in different host body compounds, ranging from the gut to the skin, is considered pivotal in the understanding of the hosts physiology The influence on the hosts is so strong that the microbiome could be considered as a "hidden organ".
We decided to test these opportunities on the biological model honey bee (*Apis mellifera*) and its parasitic mite varroa (*Varroa destructor*), implementing the use of NGS techniques on microbiomes. We focused our study on the bacterial communities involved in the symbiosis. It is reasonable to expect that the analysis of the microbiome of both varroa and honey bee larvae, could open new perspectives to shed light on the role of the parasite on colonies health.
The characterization of microbial communities involved in *A. mellifera* biology has proven to be a good indicator of its state of health. Nevertheless, the ecological dynamics of this parasitic symbiosis are still largely unknown.
Our hypothesis is that varroa mites play a fundamental role in the alteration of bacterial community composition of honey bee larvae, not only being a vector, but also acting as an open "door" through which exogenous bacteria alter the mechanisms of primary succession in honey bee microbiome.
We studied varroa and honey bee bacterial communities through selected marker amplicon pyrosequencing methods, taking advantage of the of hight-throughput sequencing technologies and the opportunity to detect uncultured and uncultivable bacteria allowed by such techniques. In summary, we characterized the honey bee-associated microbiota in the larval stage and the parasiteassociated microbiota, and explored the possible patterns of interaction between the two microbiomes.

Keywords: symbiosis; microbiome; parasite; honey bee; Varroa; NGS

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P6.39

Impact of pre- and probiotic on Indian gut microbiota – an in vitro evaluation

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With increasing demand of food additives in the country, the study aims to address the existing research gap with respect to effect of probiotics and prebiotics on indigenous human gut microflora. To meet this aim, gut microflora was simulated in the form of a chemostat with inoculum derived from a healthy individual. Cultivation-dependent and independent analysis was performed to assess the population of introduced probiotic strain (*Lactobacillus* sp.), with and without selected prebiotics (inulin). Colony counts and the technique of denaturing gradient gel electrophoresis, were employed to resolve microbial community in the simulated gut. The results revealed better build-up of the probiotic strain in the presence of prebiotics. Inulin was found to decrease the doubling time of *Lactobacillus* sp. significantly. The study not only established the sensitivity of the methodology employed to detect changes in microbial communities with time, but also demonstrates for the first time the stimulatory effect of prebiotic on probiotic population.

Keywords: probiotic, prebiotic; gut microflora; DGGE; chemostat

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PSS1.1

Safe food microbes

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The overwhelming use of antibiotics for about six decades has been determining an increasing development of antibiotic resistance bacteria to conventional antibiotics. In particular, the food chain has been considered the main route for the introduction of antibiotic resistant (AR) bacteria in the human gastrointestinal tract where AR genes may be transferred to pathogen and opportunistic bacteria. In this context, the European Food Safety Authority (EFSA) demands the absence of acquired antibiotic resistance in microorganisms used in food and feed and establishes in FEEDAP technical guidance (EFSA 2012) the criteria for assessing the safety of AR bacteria. Lactic acid bacteria (LAB), because of their large use in fermented food, may represent a potential concern since they may act as AR gene reservoir. In this study, the Minimum Inhibitory Concentrations (MICs) of a collection of 189 LAB strains (69 *Streptococcus thermophilus*, 49 *Lactococcus lactis*, 63 and 8 *Lactobacillus* and *Bifidum* strains, respectively), used as added starter cultures and/or probiotics in foods, were determined and compared to the epidemiological cut-off values established by EFSA. Approximately 40% of strains showed a resistant phenotype to at least one out of the nine antibiotics under study. As far as streptococci, a high proportion of strains (72%) showed MIC values for kanamycin equal or higher than the cut-off value. Similarly, the 49% of lactococci showed MIC values for streptomycin equal or higher than the breakpoint value, suggesting in these cases a possible revision of the established cut-off values. In addition, the MIC distributions of tetracycline in streptococci and clindamycin in lactococci were bimodal suggesting the potential presence of acquired AR genes in some strains, though in these species the overall incidence of AR strains was low. The distribution of antibiotic resistance was different among different *Lactobacillus* species and the number of strains evaluated for each species was insufficient to propose cut-off values. However, remarkable AR phenotypes were observed for kanamycin, tetracycline and clindamycin. Furthermore, in order to clarify the genetic basis of the acquired resistances, whole genome sequencing has been carried out. First results obtained from genome sequencing of ten

microbial strains indicated generally a discrete correlation between phenotype and genotype, with the exception of kanamycin and streptomycin probably due to the occurrence of genomic mutations in indigenous genes. On the whole, the data obtained can give a contribution to the knowledge of antibiotic resistance of LAB and can be used to revise and improve the established cut-off values.

Keywords: antibiotic resistance; lactic acid bacteria; minimum inhibitory concentration; safety assessment

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PSS1.2

Bacterial diversity of crude oil polluted sites in Niger Delta, Nigeria using molecular techniques.

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The research was carried out to study the diversity of bacteria responsible for oil degradation in polluted areas of Niger Delta, Nigeria. Oil-degrading bacteria were isolated from environmental samples (soil and water) collected from different habitats in Niger Delta, Nigeria using selective enrichment techniques. The isolates obtained were cultivated in Bushnell-Haas broth (bacteria) supplemented with 2% oil (kerosene, diesel, petrol and crude oil) as sole source of carbon and energy. The microbial growth of the strains were determined using spectrophotometer. The isolates were identified by conventional microbiological methods. The identities of the isolates were confirmed by subjecting their total genomic DNA to 16S rRNA gene analysis in a polymerase chain reaction (PCR) using the primers: E9F and U1510R. The PCR products were purified and sequenced using Sanger method. Molecular assessment of the bacterial community of oil-degraders was monitored with the aid of denaturing gradient gel electrophoresis (DGGE). The screening for oil-degraders revealed 20 distinct bacteria with *Bacillus subtilis* having the highest percentage of occurrence of 58.33%. The results obtained from the conserved sequence of the 16S rRNA coupled with the nucleotide sequence, revealed that the isolated bacteria have 96% similarities to *Citrobacter freundii* and 95% to uncultured bacterium clone aab17f10 and *Klebsiella oxytoca*. Others show between 72–94% similarities to *Pseudomonas pseudoalcaligenes*, uncultured *Citrobacter*

F4 Jan. 7, uncultured bacterium clone aab20d12, uncultured bacterium clone aab17f05, *Enterobacter spp.*, *Ewingella americana*, and *Bacillus megaterium*. The molecular assessment of bacterial community revealed that the degradation of oil was done in succession.

Keywords: bacterial diversity; crude oil; 16S rRNA; DGGE; oil-degrading bacteria

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PSS1.3

Integrating soil characteristics, land management and soil microbial communities

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Soil ecosystems are highly complex and are considered to be one of the most diverse microbial systems on earth. Soil microbiology plays an important role in numerous ecosystem functions, including, nutrient cycling, organic matter decomposition and energy fluxes. To truly understand the role that microbiology has in these different ecosystem functions, it is essential to quantify the baseline range of microbial communities and their activities across a spectrum of soils and agricultural land-use types. Agriculture is the dominant land use in Ireland, accounting for approximately 65% of the land area. Therefore anthropogenic influences are considered to play an important role in shaping microbial populations in Irish soils.
The Irish Soil Information System project (ISIS) is a national survey of soil types to provide a soil map at a scale of 1:250,000. This national survey incorporates sampling of 250 sites for full soil pedological and microbiological assessment, sampled across the soil horizons down to a depth of 1m. This has been completed over a 2yr period, with sampling being carried out from March to October of each year. Soil parameters analysed include soil type (Irish classification system and WRB), land-use, vergetation, texture, pH, Organic C, C:N, cation exchange capacity (CEC), base saturation (BS) and Fe/Al.
The microbial assessment of these soils includes phenotypic, genotypic and functional analysis.
Phenotypic includes: Phospholipid Fatty Acid Analysis
Genotypic includes: T-RFLP, ARISA and quantitative PCR

Functional includes: microresp, microbial biomass and nitrification potential.
For soil surface horizons total microbial biomass will be related to substrate induced respiration data (Microresp) as well as microbial community structures and abundances, made visible by PLFA and DNA fingerprinting through T-RFLP and ARISA. Physicochemical properties throughout the soil profile will also be related to PLFA, T-RFPL and ARISA results, to determine community changes on the vertical level and identify driving forces of change. Furthermore, the study aims to develop distinctive microbial fingerprints for varying soil parameters.
Nitrification is an important process in providing mobile nitrogen for grassland and crop uptake. This study will assess the abundances of the bacterial and archaeal amoA genes in Irish top soils. The amoA gene has been used as a marker gene for studies of ammonia oxidising bacteria and archaea and was related to potential nitrification rates. We therefore seek to uncover which soils have the highest gene abundance and relate these to driving physicochemical factors.
This study will use a range of multivariate analysis to generate an extensive knowledge on the role soil properties and land management play in determining soil microbial diversity, abundance and community structure in Ireland. Furthermore it will provide a baseline for future research, resulting in the identification of possible indicators of soil types and soil quality with the overall aim to sustainably manage and protect soil as a vital resource in the future.

Keywords: national soil survey; soil characteristics; microbial diversity; PLFA; DNA Fingerprinting; ecosystem functioning

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PSS1.4

Meat Tips: Improving the fermented meat products sector via training on innovation in products, processes and safety management

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Leonardo da Vinci projects are funded by the European Commission and focus on vocational education and training. Meat tips (www.meattips.gr) is a Leonardo da Vinci project (LdV 2010-GR1-LE005-03952) aiming at the production of training material on various innovative technological developments, as well as a suitable accompanying methodology, for the fermented meat products sector of the food

industry. The project consortium consists of 7 partners from 4 EU counties, namely Greece, Spain, Italy and Hungary. The training elements developed cover (i) the use of lactic acid bacteria as means of improving product quality and safety, (ii) the elaboration of efficient safety management systems, (iii) the presentation of major innovative developments on processing and packaging of fermented sausages.
These topics are of high relevance to the needs of the sector, and although they can contribute to competitiveness and consumer trust, they often remain unexplored by the industry. Meat tips aims at training food safety professionals and meat industry trainers through developing training material and corresponding training methodology, including audio-visual aids and interaction scripts that may be adapted to suit characteristics and needs of the local audiences. An E-learning platform module is also available to allow for a wider dissemination of the project's results. The training material is available in the 4 languages of the countries participating in the project and in English and has been evaluated by external experts, in each of the 4 countries. Furthermore, in each of the participating countries, training seminars were organized, with industry trainers as main target audience group, where the material developed was implemented.

Keywords: Meat tips; training; fermented sausages

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PSS1.5

Different molecular approaches reveal how arbuscular mycorrhizal fungal communities thrive in a winter-wheat rotation management system

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In the frame of EU project PURE (Pesticide Use-and-risk Reduction in European farming systems with Integrated Pest Management) our research group is involved with EU researchers in the topic "Pathogen suppression by soil microbial communities" (Task 10.1), which objective is the exploration of soil microbial communities' response to existing management actions, and considers their role in suppressing pathogens.
The first aim of the research task is the investigation of soil microbiome associated with a rotation based management system typical of arable farming. In particular, a wheat based rotation provides the experimental platform where soil and winter wheat, grown under different management systems (including crop rotation, tillage

and fertilization), are sampled twice in the winter wheat cropping seasons.In order to study and design new strategies to promote microbial mediated soil suppressiveness toward soil-borne pathogens and measure changes is soil biota due to different management strategies, some important groups of soil organisms have been selected (fungi, Arbuscular Mycorrhizal Fungi (AMF), bacteria, *Lysobacter* spp., nematodes).

Among the microbial targets that could provide complementary measures of soil quality as well as indication on general and specific diseases suppressive capacity of soil, our research group analyzes Arbuscular Mycorrhizal Fungi (AMF), obligatory mutualistic symbionts that supply plants with inorganic nutrients and protect them against diverse abiotic and biotic stresses.

The complexity of AMF community was estimated by morphological and molecular analysis in five different experimental sub-plots characterized by different crop rotations and managements (ploughed, or no-tilled).

Since recent development of molecular tools and Next-Generation Sequencing (NGS) techniques have revolutionized the study of microbial ecology, providing insights into complex systems, we applied either Sanger sequencing or tag-encoded 454 pyrosequencing to investigate AM fungal communities (Davison et al., 2012; Lumini et al., 2010).

Raw sequencing data plus metadata describing the samples were processed using the Quantitative Insights Into Microbial Ecology (QIIME) software pipeline (Caporaso et al., 2010). In brief, sequences below quality score of 30 and 200 bp in length were trimmed, and then assigned to different samples based on unique 5-bp barcodes. Sequences were clustered into OTUs using a 97% identity threshold. Taxonomy was assigned to representative sequence for each OTU. AM Fungal OTUs were identified using a customized reference repository, derived from the web-based MaarjAM database (Opik et al., 2010), and specially developed to overcome the limitations and biases of currently used fungal AMF databases. These approaches allowed us to describe wheat based rotation field AMF biodiversity and to highlight the differences between AMF communities associated to the different management systems.

Keywords: Arbuscular Mycorrhizal Fungal Communities; winter wheat rotation; soil managements; 454 metabarcoding; PURE FP7 Project

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PSS1.6

Multi-strain indigenous yeast & bacterial starters for 'wild-ferment' wine production - WILDWINE

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Aforetime, wines were produced by the resident grape/winery microbiota. Currently commercial *Saccharomyces cerevisiae* and *Oenococcus oeni* starter cultures are widely used to ensure a manageable process. Despite advantages, this may lead to sensory resemblance of wines from diverse origins, whereas exotic starters may fail to take over fermentation. Nowadays, the competitive nature of global wine market urges for the production of premium wines with regional character. Consumers also call for allergen-free wines made according to natural and organic procedures. To this end, the use of indigenous S. cerevisiae or non-Saccharomyces (wild) yeasts is a tool to create wine complexity and authenticity, while selected lactic acid bacteria (LAB) may effectively control malolactic fermentation and thereby eliminate biogenic amines (BA). The innovative scope of this project is to combine native *S. cerevisiae* with wild species and native *O. oeni* with other LAB in the development of peculiar yeast and bacterial blend starters, respectively. These formulations will be carefully designed to fulfill all the essential and desirable winemaking properties to serve as starters in induced wild fermentations for the production of specialty organic or conventional wines. For this purpose, the biodiversity of key EU viticultural areas will be thoroughly screened to identify strains of enological importance as per their phenotypic characters and genetic traits. Their eligibility will be validated in plant-scale fermentations and wines will be evaluated by sensory analysis and consumer acceptance testing. Outcomes will enable (a) 'wild ferment' technology in winemaking, (b) production of innovative, safe terroir wines and (c) meeting rules on organic wine production and BA content. By these means, the project will assist the SME-AGs from leading wine producing EU countries (France, Greece, Italy, Spain) to enhance marketing abilities towards a more competitive and sustainable wine industry. The project is funded by the EU FP7 under Grant Agreement 315065 - WILDWINE (www.wildwine.eu)

Keywords: wine; wild ferment; *Saccharomyces cerevisiae*; *Oenococcus oeni*

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PSS1.7

Biodiversity and VBNC state in *Brettanomyces bruxellensis* isolated from Apulian wineries

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Some yeast species belonging to the *Brettanomyces* genus (e.g *B. bruxellensis*, *B. anomalus*) are the main microbial causes for wine spoilage, entailing significant economic losses. *Brettanomyces* yeasts are well adapted to survive during winemaking process, largely due to their ethanol tolerance and relative resistance to the normal concentrations of sulphur dioxide found in wine (Licker et al., 1998). Although a large number of culture-based techniques are currently available for assessing the presence of this undesirable spoilage yeast during winemaking processes, in several cases *Brettanomyces* is not detected, a dilemma which could potentially be explained by the ability of *Brettanomyces* to enter in a viable but non cultivable (VBNC) state (Agnolucci et al., 2010). The VBNC phenotype is characterized by an inability of cells to divide on bacteriological media, even though they are still alive and maintain metabolic or cellular activity. Very little is known about the *Brettanomyces* biodiversity during winemaking correlated with the different VBNC behaviours.
In this study 170 putative strains of *Brettanomyces* were isolated from Apulian wines and identified. The identification was performed amplifying using the primers ITS1/ITS4, with a successive restriction analysis (Hae III, Hinf I e Cfo I). In addition, another specific PCR (primers DB90/DB394), that form amplification products only with the species *B. bruxellensis* and *B. anomalus* was used and the differences between these two species was assessed after restriction enzyme analysis (DdeI) of the amplified products. The strains identified as *B. bruxellensis* (50) were characterized genotypically through the technique of SAU-PCR, a

method previously used to characterize lactic acid bacteria populations (Corich et al., 2005), to define the intra-species diversity of *Saccharomyces cerevisiae* isolates obtained from former and modern wineries in Italy (Cocolin et al., 2004b), and to study the biodiversity of *B. bruxellensis* in north-western wine (Campolongo et al., 2010). The SAU-PCR fingerprinting was used to investigate *B. bruxellensis* diversity at the strain level, highlighting differences related to geographical origin and to sample type. With this method, genomic DNA is first subjected to enzymatic cleavage using restriction enzyme Sau3AI and then amplified using a specific primer constructed on the basis of the restriction site of the enzyme, so that the obtained profiles reflect the presence or the absence of Sau3AI restriction site within the genome of the strain under investigation.
One representative strain from each of the 7 different clusters (SAU-PCR) were subjected to analysis of volatile phenol production in synthetic media and in wine with chemical analysis by GC-MS. The strains were also tested to assess their VBNC behaviour using different concentration of SO_2 as recently reported (Serpaggi et al., 2012). The VBNC condition is a survival strategy adopted by many microorganisms when exposed to harsh environmental stresses. The VBNC is a reversible state, indeed under appropriate conditions the yeasts can resuscitate to culturable state. The comprehension of strain behaviour in relationship of the VBNC state is crucial, giving that VBNC state of *Brettanomyces* can be responsible of significant spoilage risks.

Keywords: *Brettanomyces bruxellensis*; SAU-PCR; biodiversity; VBNC

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PSS1.8

Variability of riboflavin overproduction in *Lactobacillus plantarum*

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The genetic informations for riboflavin (vitamin B2) biosynthesis are species and/or strain-specific traits in Lactic Acid Bacteria (LAB) (Burgess et al., 2009; Capozzi et al., 2012). Regulation of riboflavin biosynthesis and transport in LAB is usually achieved by means of a conserved regulatory region, the RFN element, located upstream of both the rib operon, which encodes the riboflavin biosynthetic genes, and the riboflavin transporter genes. Mutations in the conserved RFN regulatory region, usually cause riboflavin overproduction. In bacteria this trait can be achieved by exposure to purine analogues and/or the toxic riboflavin analogue roseoflavine (Burgess et al., 2009). In this work we present several strains of *Lactobacillus plantarum* able to overproduce riboflavin following roseoflavine treatments. In all the strains analysed, the RFN regulatory elements were sequenced and analysed. The results reported suggest a clear correlation between riboflavin overproduction and single changes in the RFN regulatory element. The distribution of the mutations observed in the RFN element allowed us to divide the overproducing *L. plantarum* strains in three different subgroups: low, medium and higher vitamin B2 overproducers. One strain of each group was used for the preparation of cereals-based products. The applied approaches resulted in a considerable increase of vitamin B2 content. The final concentration of riboflavine was strongly correlated to the *L. plantarum* strain used in the fermentation process.

Keywords: *Lactobacillus plantarum*; riboflavin overproduction; cereals fermented products; RFN

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PSS1.9

Ropy phenotype of *Lactobacillus plantarum* confers higher tolerance to acidic and bile stress

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Lactic Acid Bacteria (LAB) are able to secrete long-chains of homo- or hetero-polysaccharides, consisting of branched, repeating units of sugars or sugar derivatives (Ruas-Madiedo et al., 2002). Such exopolysaccharides (EPS) can be either highly adherent or loosely bound to the microbial cell surface and are thus distinguished into capsular or secreted forms.
In nature, microbial EPS are thought to provide protection from adverse environmental conditions as well as to contribute to biofilm formation and to cell adhesion/recognition mechanisms (Ruas-Madiedo et al., 2002; Broadbent et al., 2003). A *Lactobacillus plantarum* strain, previously isolated from fermented beverages, exhibited a characteristic ropy phenotype which was presumably associated to its ability to produce EPS. Therefore, genotypic and phenotypic analyses were performed in order to identify the genetic basis of ropy phenotype in *L. plantarum* and the physiological roles of the EPS produced, respectively.
Transmission electron microscopy revealed the presence of a thick matrix of extracellular material which appears to partially cover the cell and to be spread around to form a large reticulate structure. This typical aspect, which was not observed in the WCFS1 strain of *L. plantarum*, was ascribed to the specific ability to produce EPS by the isolated *L. plantarum* strain. EPS were extracted and quantified; furthermore, their composition was determined by thermodegradation and size exclusion chromatography. Different saccharide subunits were detected, indicating a hetero-polysaccharide, complex structure. In order to investigate the genetic background underlying the ability to produce EPS, selected genomic regions of the EPS-producing *L. plantarum* strain, were sequenced and compared with corresponding loci of the fully sequenced genome of the non-ropy WCFS1

strain. The physiological roles of EPS in relation to stress tolerance was analyzed by growth curves, CFU count data and culture spotting on agar plates.
Overall, the results presented suggest that EPS production in *L. plantarum* contributes to significantly enhance resistance to abiotic stress, including those deriving from acid and bile exposure. It is therefore plausible that the EPS-producing feature in *L. plantarum*, connected to higher stress tolerance, might have evolved in response to the particularly harsh and competitive environment characterizing food fermented ecosystems.

Keywords: EPS; *Lactobacillus plantarum*; stress tolerance

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PSS1.10

Biodiversity of *Oenococcus oeni* strains isolated from wine produced in Apulian region

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The lactic acid bacterium *Oenococcus oeni*, naturally present on grapes, is the main species involved in malolactic fermentation (MLF) during winemaking process. MFL consists in a decarboxylation of L-malic acid to L-lactic acid and carbon dioxide and leads to i) an increase in wine pH, from 0.2 to 0.5 units, which translates into a decrease in wine sourness, ii) an increase of microbial stability, thanks to the removal of potential carbon sources which can be used by spoilage yeast and bacteria, and iii) a bacterial production of various secondary metabolites, which can improve the organoleptic properties of wine. MLF is improved by

inoculating selected *O. oeni* strains. Among *O. oeni* strains there is a large degree of phenotypic heterogeneity and the strains have been proven to be diversely adapted and efficient in terms of totally degrading malic acid (Claisse et al., 2012). *O. oeni* possesses metabolic pathways and enzymes involved in the generation of volatile secondary compounds at concentrations well above of their detection threshold (e.g. ethyl and acetate esters, higher alcohols, carbonyls, volatile fatty acids and sulphur compounds), with clear strain specific variations.
In this work, we analyse the biodiversity of *O. oeni* strains isolated from wine produced in Apulia region and undergoing spontaneous malolactic fermentations. Our attention was focused on ther genetic biodiversity of *O. oeni* strains and on their different efficiency in degrading malic acid. For genotypic characterization we utilized variable number tandem repeat (VNTR), a techniques successful used in literature for molecular typing of *O. oeni* strains. VNTR approach is based on the presence of a variable number of tandem repeats (TR) at a specific locus in the genome of a microorganism. Analyzing 50 strains isolated from different wines, VNTR identified 31 profiles, of which 11 unique profiles. Variations in the TR regions directly induce changes at the genetic level in the DNA molecule itself. These changes impact the genetic repertoire of a given microorganism and may play a role in the evolution of the species. This study confirmed the worldwide presence of two *O. oeni* phylogenetic subpopulations (A and B). All the strains have been screened for their capacity of degrading malic acid, bacteria were inoculated with two different times of inoculum, either together with yeast (co-inoculum) or after the completion of alcoholic fermentation (sequential inoculum).
Our aim is to study the natural biological diversity of *O. oeni* of specific a terroir to select strains suitable for of autochthonous malolactic starter cultures design.

Keywords: *Oenococcus oeni*; MLF; biodiversity; VNTR; autochthonous

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PSS1.11

Non-starter lactic acid bacteria strategy to grow during cheese ripening

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Lactic acid bacteria (LAB) are the most important groups of microorganisms involved in the production of different fermented products as fermented milks, cheese, sourdough, sausages, fermented vegetables etc.
In fermented foods LAB can be defined as starter microorganisms, which are added specifically to allow the fermentation process and/or non-starter microorganisms, that can be already present in the raw material or derived from environmental contamination. The dynamics of growth, survival and biochemical activity of all these microorganisms in fermented foods are the result of microbial interactions and their stress reactions in response to the changing of the physical and chemical conditions into food micro-environment.
In long ripened cheese, a traditional fermented food, starter lactic acid bacteria (SLAB) and non-starter lactic acid bacteria (NSLAB) dynamically develop according to modifications of environmental conditions during the long aging time. A previous study reports that in a long ripened Italian cheese, SLAB are the dominant microflora at the maximum of curd acidification (De Dea Lindner et al., 2008); when ripening begins, SLAB start to undergo autolysis and the number of NSLAB increases (Gatti et al., 2008). This demonstrates the NSLAB ability to grow in ripened cheese, that is a low sugar content environment due to the almost complete utilization of lactose by SLAB.
This leads to hypothesize that NSLAB can grow using only carbon sources available in the ripened cheese matrix, therefore also macromolecules arising from SLAB lysed cell (i.e. carbohydrates, amino acids and peptides, fatty acids and nucleic acids)(Fox and McSweeney, 2004).
To focus on this aspect, the aim of this study was to evaluate whether *Lactobacillus casei*, NSLAB strain isolated from ripened Parmigiano Reggiano (Neviani et al., 2009), was able to grow using only *Lactobacillus helveticus* cell lysate products as a nutrient source. The results showed that NSLAB can grow using only SLAB cell lysis products. Chemical analyzes were carried out to observe changes in the composition of SLAB cell lysis product after NSLAB growth.
The experiment was carried out formulating a culture medium containing only the SLAB cell lysis products and developing an appropriate co-cultural method.

Keywords: Non-starter lactic acid bacteria; cheese ripening; bacterial lysis

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PSS1.12

Effect of reuse of agro-industrial wastewaters on the agricultural soil microbial community, product safety and product yield in tomato cropping

PON 2007-2013 cod. 01_01480 In.Te.R.R.A. INnovazioni TEcnologiche e di processo per il Riutilizzo irriguo delle acque Reflue urbane e Agro-ndustriali ai fini della gestione sostenibile delle risorse idriche (Technology and process innovations for irrigation reuse of treated municipal and agro-industrial wastewaters in order to achieve sustainable water resources management.). Funded by Italian Ministry of University and Research (MIUR)

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Sustainable management water resources in agriculture is of increasing global concern. Irrigation of agricultural lands with wastewaters, after different treatments, is increasing around the world (Barkle et al., 2000). Utilization of agro-industrial wastewaters as irrigation water source is a potentially sustainable management practice, and could also be of significant benefit in water-poor regions of the world (Mossea et al., 2012). There are, however, concerns regarding the contaminants that are present in raw wastewater, as well as those that may persist in the treated wastewaters with respect to their effects on soils, crop growth, and consumers of the crop (Toze, 2006). Moreover, it is worthy of investigation the determination of factors that influence microbial community composition in the field, since it can have significant impact on understanding how management practices affect crop quality, disease ecology, and biogeochemical cycling (Buyer

et al., 2010).
In the frame of PON In.Te.R.R.A. project, we evaluated the effect of two different irrigation water (conventional well water, CW vs. agro-industrial wastewater, IW) on the microbial community of irrigated soil during tomato (*Solanum lycopersicum*) production. Triplicate soil samples were collected at 6 time points, during 4 months interval that comprises pre-culture phase and the day before harvest. Bacterial populations were analyzed by Automated Ribosomal Intergenic Spacer Analysis (ARISA). The principal foodborne pathogenic bacteria (*Salmonella* spp. *Campylobacter jejuni*, *Listeria Monocytogenes* and STEC) where monitored in water, soil and final product. Canonical Correspondence Analysis (CCA) showed that ARISA clustered the samples according to temporal variation and to different water used for irrigation. Moreover, molecular fingerprinting analysis showed good correlation with different geochemical parameters, with positive correlation for nitrogen, phosphorous and conductivity and negative correlation with pH, TOC and SOM. Interestingly crop yield was found to be lower (-8,6 ±3,2 %) for the fields irrigated with IW. Both water types, so as tomato plants and berry were found to be pathogen-free, whereas *L. monocytogenes* and *Salmonella* spp. were detected in soil, independently from the water source used for irrigation. Basing upon described results, microbial community dynamic is strongly influenced by different water used for irrigation, and even if microbiological quality of agro-industrial wastewater was satisfactory, the chemical composition of IW (particularly high salinity) may have negatively influenced the product final yield.

Keywords: wastewater reuse; soil microbial communities; irrigation; waterborne pathogens

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PSS1.13

Interaction *Trichoderma-Stenotrophomonas* and its effect on plant growth and health

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The use of beneficial microorganisms to suppress plant diseases has become a reliable alternative to reduce the application of pesticides (Alfano et al., 2007, 2011). There is a strong growing market for microbial inoculants worldwide with an annual growth rate of approximately 10%. The use of 'Omic technologies leads to products with more predictable and consistent effect. The future success of biological control will depend on interdisciplinary researches about the ecological interactions taking place in soil and root environment (Berg, 2009; Ryan et al., 2009).
Aim of the present research is to gain insight on the interaction between biological control agents (BCAs) and to assess its potential synergistic effect on suppression of the fungal pathogen *Verticillium dahliae* and on plant growth and health. The protagonists of the research are one of the most important beneficial fungus for agriculture, *Trichoderma velutinum* G1/8 and one of the most promising candidate for application as a BCA and plant growth promotion, the bacterium *Stenotrophomonas rhizophila* DSM14405T. Character of absolute innovation is the assessment of the role played by secondary metabolites and volatile organic compounds (VOCs) in the interaction between the BCAs, between the BCAs and the pathogen and between the BCAs and the plant.
Controlled lab-based and environmental microcosms will be developed to carry out the molecular ecological interactions. DNA-based methodologies in combination with proteomic and confocal laser scanning microscopy will be used to assess the dynamics of interaction, biocontrol activity and expression of each other's primary biocontrol genes.
This research effort will result in new knowledge of the mode of the interaction between BCAs, BCAs and the pathogen, and BCAs and the plant. This will constitute the basis for the development of innovative biotechnological applications.
The research leading to these results has received funding from the European Union Seventh Framework Programme FP7/2007-2013under grant agreement n° 302228.

Keywords: interaction; bioControl; plant growth promotion; *Trichoderma* spp.; *Stenotrophomonas* spp.

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PSS1.14

Evolution of archaea population in piglet stools at different age

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Several methanogens are common inhabitants of the gastrointestinal tract ecosystems and have been isolated from feces of different animal species (Miller et al., 1986; Lin et al., 1998); however, in literature only few studies on pig feces were reported, showing that the most predominant methanogen bacteria belong to the genus of *Methanobrevibacter*. The main aim of this study was to identify and quantify the methanogenic archaeal species from piglet feces and, for this purpose, fecal samples were collected at two different ages (28 and 63 dd). DNA extraction was performend using the Maxwell® (Promega, Madison, WI, USA). The V6-V8 region of the 16S rRNA gene was amplified and analysed by Denaturing Gradient Gel Electrophoresis (DGGE). The archaea-specific primers used to obtain the amplification products for 6% (w/v) polyacrylamide DGGE gels (40–60% v/v gradient), were GC-915af and 1386r, as described by Jeyanathan et al., 2011. To evaluated the amount of archaea population a real time PCR has been used. Several sets of primers, previously described in literature, were used and, to obtain the best result, were choosen a couple of primers target on *mcrA gene*.
The DGGE fingerprints of the methanogenic archaeal species showed, at 28 days of life, a single band identified as *Methanobrevibacter smithii*. Instead the same animals analyzed at 63 days of life exhibited multiple DGGE bands identified as *Methanobrevibacter smithii*, *Methanobrevibacter gottschalkii* and *Methanobrevibacter wolinii*. The latter was the band common to all individuals.
The results obtain with real time PCR technique showed that the methanogenic Archaea population increased in animals analyzed at 63 days of life.

Keywords: Archaea; *Methanobrevibacter*; piglet; DGGE; real Time PCR

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PSS1.15

Development of prebiotics for livestock use

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Weaning imposes tremendous stress on piglets and the period following is characterized by a high incidence of intestinal disturbances with diarrhea and depression of growth performance in piglets. For the last several decades, antimicrobial compounds have been used to promote piglet growth at weaning through the prevention of subclinical and clinical disease.
At the same time in recent years, there is an ongoing interest to minimize the use of antibiotics and to find alternatives for antimicrobial growth promoters and new strategies that have been used to enhance gastrointestinal function.
Aim of this study was to develop a prebiotic food for piglets characterized by bifidogenic activity, capable of improving the health gut of the animals.
Thirty strains of *Propionibacterium freudenreichii subsp. shermanii* (Cousin *et al.* 2011) were isolated from dairy products and were tested for their ability to produce bifidogenic growth stimulator (BGS), a prebiotic preparation that selectively stimulates the growth of Bifidobacteria through the action of its component 1,4-dihydroxy-2- naphthoic acid.
The BGS activity was determined by an agar diffusion plate assay as reported by Mori *et al.* , 1997. Bifidogenic activity has been tested on several species of Bifidobacteria previously isolated from piglets stool samples: *Bifidobacterium tsurumiense, B. pseudolongum subsp. pseudolongum, B. choerinum, B. thermophilum B. thermacidophilum, B. indicum, B. boum*. The two strains of *Propionibacterium freudenreichii subsp. shermanii*, which showed greatest BGS activity, were reproduced in a pilot plant. The surnatant was submitted to a spray treatment in order to obtain a powder product mixable with the feed. The results showed that BGS activity was unaffected by spray treatment and remained stable in the 12 months after production.
The oral BGS administration with the feed could be a possible way to improve the

well-being of the weaned animals by modulating intestinal microflora and reduce the incidence of diarrhea.
These encouraging preliminary results lead us to propose a randomized, controlled trial of BGS in piglets to demonstrate its efficacy *in vivo*.

Keywords: BGS; Propionibacteria; Bifidobacteria; piglets

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Cousin F.J. et al. (2011) Dairy Sci Technol 91: 1-26.
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PSS1.16

Isolation of lactic acid bacteria from ecological grapes and wines of DOQ Priorat in South Catalonia

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The malolactic fermentation (MLF) usually occurs after alcoholic fermentation (AF), especially in red wines. MLF is the decarboxylation of L-malic acid into L-lactic acid and CO_2, which contributes to an organoleptic improvement of wine and to its microbiological stability. This process is carried out by lactic acid bacteria (LAB), particularly by *Oenococcus oeni*.
The trend of consumer preferences in recent years to the ecological wines represents an opportunity for traditional and peculiar terroirs. In this sense, the use of autochthonous LAB as inoculum can offer great potential. To achieve this goal requires a good collection of isolated and well typified strains, and an accurate assessment of their potential in winemaking.
In relation with that, this work is part of a project whose main objective is to identify indigenous microbial diversity of vineyards and wines of the DOQ (*Denominació d'Origen Qualificada*, Qualified Appellation of Origin) Priorat (South Catalonia), and from other European wine regions. We collected samples of Grenache and Carignan grapes from vine properties with minimized pesticide treatment. With these samples, laboratory fermentations were carried out without inoculation and there were obtained 40 LAB isolates. All these were lactobacilli, which were identified by the technique of 16S-ARDRA followed by digestion with MseI. Nine different profiles were obtained and 13 isolates were consistent with

the profile of *Lactobacillus plantarum*. Other profiles fit other *Lactobacillus* species (*L. sakei* and *L. brevis*), and some profiles do not match to those of known species.
Samples were taken also from 28 wines, many of them performing MLF, from cellars of the same DOQ which had not used commercial strains of LAB. These wines had high alcohol content (13.5 to 16%), and came from different varietals, mostly Grenache and Carignan. Most of isolates obtained from these samples (more than 900 isolates, 90% of total) have been identified as *Oenococcus oeni* by multiplex PCR and RAPD PCR with primer Coc. The remaining isolates of these wines (10%) were lactobacilli found along AF and MLF in some of experimental wines.
In conclusion, in this work we have isolated several *Lactobacillus* from grapes and during AF and the beginning of MLF, and a significant number of strains of *O. oeni* isolated from the MLF, which are an important source of study for the selection of indigenous potential starters form the DOQ Priorat.
We thank the European project funding Wildwine FP7-SME-2012-315065, involving the Council of the DOQ Priorat and Ferrer-Bobet winery. We also acknowledge the assistance of the other wineries and vine properties from which samples were obtained. Judit Franquès enjoys a predoctoral fellowship of the URV.

Keywords: wine; vine; lactic acid bacteria; *Oenococcus*; *Lactobacillus*

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PSS1.17

Use of antiseptics and disinfectants in public clinical hospital

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This research is directed towards the prevention of intra - hospital infections, the way that achieves this and the used methods. Intra-hospital infections are major issue in modern medicine, especially where they do not comply with the basic rules and methods. Disinfection and sterilization as two basic methods that contribute to the prevention of intra-hospital infections are described in detail and are presented the most commonly used antiseptics and disinfectants. Attention is given to the description of the control of sterilization, due to frequent lapses in sterilization, and occurrence of contaminated instruments in the operating room. In operating rooms, sterility must always be at the highest level for the most direct route of entry of microbes in wounds, instruments and so on. The control of

intrahospital infections is performed by Commission for intra-hospital infections which is responsible for taking swabs and sediments air for proving non / presence of bacteria, as well as taking measures if contamination occurs and timely detection of intra-hospital infections. The presence or absence of bacteria in the taken materials is presented with graphs and percentages, making it easy to determine what is the situation in the public clinical hospital Stip, R.Macedonia in the period from 2007 to 2011.

Keywords: Infection; control; swabs; departments

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PSS1.18

Diversity of *Oenococcus oeni* strains in five wine-producing regions and selection for the production of indigenous malolactic starters

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Oenococcus oeni is the main species of lactic acid bacteria responsible for malolactic fermentation (MLF) in wine. This stage of the winemaking process occurs after alcoholic fermentation and results in wine deacidification (transformation of L-malic acid to L-lactic acid), sensory changes and microbiological stability. MLF can be performed spontaneously by indigenous strains originating from the grape, or by adding commercial *O. oeni* starters to control its progress and avoid alterations due to some spoiling strains. However, with the development of organic agriculture, many winemakers choose to carry out the MLF spontaneously.

Our aim is to study the diversity of indigenous *O. oeni* strains in several wine-producing regions and to select candidates, according to genetic and phenotypic criteria, to produce malolactic starters specific for wine-producing regions. The sampling was carried out on different geographical areas: Lebanon, three French regions (Aquitaine, Burgundy and Languedoc-Roussillon) and cider-producing regions. In the beginning, 224 wine samples were collected during spontaneous MLF, from which we isolated nearly 3000 clones of *O. oeni*. The identification and typing of *O. oeni* strains were made by the VNTR method (Claisse et al., 2012). The results show a wide diversity of indigenous strains. For each of the studied regions, 50 to 100 different profiles of strains have been identified. Furthermore, even the samples from the same region, posses only some strains in common. However, quantitative analysis shows that some strains are predominant in each

region. The strains were genetically typed by MLST (Bilhère et al., 2009) to determine their phylogenetic position in the species *O. oeni*. We checked that the strains do not cause any alterations in wine (production of beta glucan and biogenic amines), and then we tested their survival to an industrial production process, and their ability to perform MLF.
This study reveals a high diversity of *O. oeni* strains and opens the possibility to select and produce local malolactic starters, specific for each wine-making region.

Keywords: *Oenococcus oeni*; indigenous; diversity; MLF; VNTR

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Bilhère E. et al. (2009). Appl Environm Microbiol 75: 1291-1300.
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PSS1.19

Biocidal actvities, phytochemical constituents and free radical-scavenging capacities of *Adansonia digitata* stem bark extracts

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Background: Food preservation is a major concern in food industry. There is a gradual departure from the use of chemical preservatives in foods due to its adverse effects.
Objectives: This work investigated the antimicrobial and antioxidant properties of extracts from *Adansonia digitata* stem bark.
Methods: Fresh *Adansonia digitata* stem bark was collected from Ilaju village, Oyo State, Nigeria and identified at the Forestry Research Institute of Nigeria (FRIN), Ibadan . Extracts from *Adansonia digitata* stem bark were obtained using different solvents (water, ethanol and petroleum ether). The screening of the extracts for antimicrobial activities involved the use of some food borne pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Shigella dysenteriae*, *Bacillus cereus*, *Candida valida* and *Aspergillus flavus*) at 0.500, 0.250, and 0.125 g/ml concentration respectively to detect the inhibitory properties of the extracts using agar-well diffusion method. Phytochemical and free radical-scavenging screening was also evaluated.
Results: All the three extracts of the stem bark consist of phenols; tannins, cardiac glycosides, phlobatannins; while saponins and flavonoids were present in water

extract only. The antimicrobial screening of the extracts of the stem bark water, ethanol and petroleum ether showed zone of inhibition range of 10.00 – 19.75 mm; 13.50 - 26.00 mm; and 18.75 – 19.75 mm at 0.500 g/ml, 0.250 g/ml and 0.125 g/ml for all the test organisms. The free radical-scavenging screening (DPPH test) of the extracts showed antioxidant capacities of 18.1-87.1% at 1.0 mg/ml and 16.2-76.5% at 0.5 mg/ml.
Conclusions: The extracts of the stem bark of *Adansonia digitata* contained antimicrobial and antioxidant properties that can be used as natural preservatives and antioxidant in foods.

Keywords: *Adansonia digitata*; antimicrobial; antioxidant; phytochemical constituents

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Complex microbial ecosystems, characterized by a high level of microbial biodiversity, are fascinating subjects of investigation. Members of different microbial domains live together and interact in order to reach a specific equilibrium. Microbial ecosystems are often exploited in agricultural, food and environmental microbiology. This volume collects the abstracts of keynote, plenary, selected oral and poster presentations at the Microbial Diversity (MD) Conference, held in Torino, Italy, October 23-25, 2013. This is the second edition of the event organized by the **Italian Society of Agricultural, Food and Environmental Microbiology (SIMTREA)**. More than 200 participants, coming from all over the world, attend the conference creating an important opportunity for discussion over the main themes considered by the conference. Six subjects are taken into consideration: **biofilms in complex microbial ecosystems; the potential of microorganisms in reclamation and remediation; competition, dominance and evenness: how microorganisms manifest their supremacy; communication among microorganisms; fight between microbes: antibiosis and other strategies; symbiosis of microbes with humans, animals and plants.** Moreover, two sessions are dedicated to the presentation of European and National Projects and to Young Researchers.

This book represents an up-to-date, state-of-the art overview of the phenomena at the basis of the organization, functioning and interactions in complex microbial ecosystems.

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