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ORIGINAL PAPER

Aerobic metabolism and oxidative stress tolerance in the *Lactobacillus plantarum* group

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Abstract Aerobic metabolism and response to oxidative stress and starvation were studied in 11 *Lactobacillus plantarum*, *L. paraplantarum* and *L. pentosus* strains in order to assess the impact of aerobic metabolism on the growth and on the stress response. The strains were grown in aerobiosis without supplementation (AE), with hemin (AEH) or with hemin and menaquinone (AEHM) supplementation and in anaerobiosis (AN) in a complex buffered substrate. Growth rate, biomass yield, glucose and O_2 consumption, production of lactic acid and H_2O_2, catalase activity, oxidative and starvation stress tolerance were evaluated. Aerobic growth increased biomass yield in late stationary phase. Further increase in yield was obtained with both hemin (H) and menaquinone (M) addition. With few exceptions, the increase in biomass correlated with the decrease of lactic acid which, however, decreased in anaerobic conditions as well in some strains. Addition of H or H + M increased growth rate for some strains but reduced the duration of the lag phase. H_2O_2 production was found only for aerobic growth with no supplementation due to catalase production when hemin was supplemented. To our knowledge this is the first study in which the advantages of aerobic growth with H or H + M in improving tolerance of oxidative stress and long-term survival is demonstrated on several strains of the *L. plantarum* group. The results may have significant technological consequences for both starter and probiotic production.

Keywords *Lactobacillus plantarum* · *Lactobacillus paraplantarum* · *Lactobacillus pentosus* · Aerobic growth · Oxidative stress

Introduction

Lactic acid bacteria (LAB) are fermentative organisms which do not need oxygen for growth but they can grow under aerobic conditions and the pyruvate produced from Embden-meyerhof-parnas or phosphoketolase pathway may be metabolized aerobically into acetyl-phosphate and acetate (Hickey et al.1983; Kandler 1983).

Lactic acid bacteria consume molecular oxygen through the action of flavoprotein oxydases, including NADH oxidase, pyruvate oxydase, α-glycerophosphate oxidase, L-amino acid oxidase and lactate oxidase. LAB NADH oxidases catalyse two-or four-electron reduction of molecular oxygen to form H_2O_2 or H_2O respectively and regenerate NAD^+ required for smooth operation of glycolysis. Under aerobic condition, pyruvate may be catabolized in acetyl-phosphate by the enzyme pyruvate oxidase (POX) or by the pyruvate formate lyase/phosphotransacetylase pathway and then it is converted to acetate by the enzyme acetate kinase with accompanying production of ATP (Lopez de Felipe and Gaudu 2009). Accumulation of acetate instead of lactate could have a beneficial effect for the cell ensuring the pH homeostasis and survival during the stationary phase growth (Konings et al. 1997).

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Pyruvate oxidase and flavoprotein oxidases activities results in the production of H_2O_2 and in order to detoxify it, LAB synthesize H_2O_2-degrading enzymes as NADH peroxidase and catalases (Sakamoto and Komagata 1996). Typical catalase has a heme prosthetic group (true or heme-depending catalase) in the active site and forms homotetramer. In contrast, manganese catalase (pseudocatalase) contains a dinuclear manganese active site in place of heme and forms hexameric structure (Barynin et al. 2001; Kono and Fridovich 1983a). Manganase catalase activity has been reported in some strains of *Lactobacillus plantarum* (Kono and Fridovich 1983b) and recent genome analysis of LAB indicated the presence of pseudocatalase homologues in *Pediococcus pentosaceus* and *Enterococcus faecium*.

Enterococcus faecalis, *Lactobacillus sakei*, and *L. plantarum* contains the gene *kat* coding for the true catalase which is produced when an exogenous heme group is provided (Abriouel et al. 2004; An et al. 2010; Frankenberg et al. 2002; Knauf et al. 1992).

Heme addition can also improve growth yield because *Lactococcus lactis*, *E. faecalis*, and other LAB are capable of activating a heme-dependent cytochrome oxidase and of establishing a respiratory chain with positive consequences on ATP yield and on bacterial robustness (Lechardeur et al. 2011). In *L. lactis* the electron transport chain (ETC) includes an electron donor (NADH dehydrogenase, encoded by *noxA* and/or *noxB*), an electron transporter (a menaquinone pool, synthesized in *L. lactis* and in *E. faecalis* by *menFDXBEC* but lacking in other respiration-competent LAB) and a heme-requiring terminal electron acceptor (cytochrome *bd* oxidase, called CydAB) which reduces oxygen to water in the presence of H^+. The *cydABCD* genes, which are needed to produce and for assembly the cytochrome *bd* oxidase are present only in some LAB like *Oenococcus oeni*, *Leuconostoc mesenteroides*, *L. johnsonii*, *L. plantarum*, *E. faecalis, L. casei*, *L. lactis* and *Streptococcus agalactiae* (Brooijmans et al. 2009a).

Respiration metabolism induces positive effects on bacterial robustness, for instance in *L. lactis* increases biomass yield, reduces oxidative and acid stress and improves long-term survival compared to fermentation growth (Duwat et al. 2001; Koebmann et al. 2008; Rezaiki et al. 2004).

Lactobacillus plantarum is an industrially important LAB which can be found in several fermented foods and shares its ecological niche with the closely related species *L. pentosus*, *L. paraplantarum* and with other facultatively heterofermentative members of the genus *Lactobacillus* (Stiles and Holzapfel 1997). In the presence of oxygen and with the exhaustion of the glucose in the substrate the main fermentation end product is acetate produced by the patway LDH (lactate dehydrogenase), POX and ACK (acetate kinase) with ATP generation which improves cells survival in the stationary phase (Goffin et al. 2004; Quatravaux et al. 2006). The POX (coded by *poxB, poxF*), induced by oxygen or H_2O_2, is controlled by carbon catabolite repression (Lorquet et al. 2004; Goffin et al. 2006) and H_2O_2 accumulation in absence of heme, may reduce vitality and activity of stationary phase cells. In the presence of hemin and menaquinone *L. plantarum* can synthesize a limited respiratory chain and in contrast to *L. lactis*, it displays an additional respiratory metabolism with nitrate reductase heme-requirent as terminal electron acceptor (Brooijmans et al. 2009b).

Although a sizable body of knowledge is available on the aerobic metabolism of *L. plantarum* (Brooijmans et al. 2009b; Goffin et al. 2004; Goffin et al. 2006; Lorquet et al. 2004; Murphy and Condon 1984a; Quatravaux et al. 2006; Stevens et al. 2008; Tseng et al. 1991; Watanabe et al. 2012a, b), less is known for the two closely related species *L. pentosus* and *L. paraplantarum*. In addition, most studies have been carried out with a limited number of strains and only recently data on the impact of aerobic growth on the stress tolerance have been published (Zotta et al. 2012b). Therefore, the objective of this work was to compare the aerobic metabolism under different conditions in 11 strains belonging to the species *L. plantarum*, *L. paraplantarum*, *L. pentosus* and to evaluate the resistance to oxidative stress and starvation, in order to provide insight on their ability to grow under aerobic conditions in foods and during production of starter cultures and probiotics.

Materials and methods

Bacterial strains and media

The strains belonging to the species *L. plantarum, L. paraplantarum* and *L. pentosus* used in this study and their sources of isolation are listed in Table 1. All strains were maintained as freeze-dried stocks in the culture collection of the Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali, Università degli Studi della Basilicata, Potenza, Italy, as described previously (Parente et al. 2010) and routinely propagated in MRS broth (Oxoid), for 16 h at 30 °C or 35 °C (optimal for growth).

Fermentation conditions

Batch fermentations were carried out in buffered (pH 6.5) WMB (Zotta et al. 2012b) containing 0.1 M *3*-(N-*morpholino*) propanesulfonic acid (ICN Biomedicals, MOPS). Four different growth conditions were tested: 50 mL buffered WMB without supplements (AE), with 2.5 μg/mL hemin (AEH), with 2.5 μg/mL hemin and 1 μg/mL

Table 1 List of bacterial strains belonging to the *Lactobacillus plantarum* group used in this study

Species and strain	Source	Isolation source
Lactobacillus plantarum subsp. *plantarum*		
1069	PCC	Sourdough (Carasau bread, Italy)
38AA	DBVR	Cassava, Colombia
C17	DBPZ	Caciocavallo cheese, Italy
MTD2S	DBPZ	Sourdough (Cornetto di Matera, Italy)
P1.5	DOFATA	Olives brine
UBS3	DOFATA	Wine
WFCS1	DSANA	Human saliva
Lactobacillus plantarum subsp. argentoratensis		
DKO22^T	DBVR	Sour cassava, Nigeria
Lactobacillus paraplantarum		
B7N26	DBPZ	Caciocavallo cheese, Italy
MTG30L	DBPZ	Sourdough (Cornetto di Matera, Italy)
Lactobacillus pentosus		
5TP	DPPMA	Olive fermentation brine

T type strain. DBPZ our culture collection; DBVR Prof. S. Torriani, Department of Biotecnology, University of Verona, Italy; DOFATA, Prof. C. Caggia, University of Catania; DPPMA, Dr. M. De Angelis, Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi di Bari, Italy; DSANA, Dipartimento di Scienze Ambientali, 2° Università di Napoli, Italy; PCC, Porto Conte Ricerche, Italy

menaquinone (AEHM) in 250 mL shaken flasks, and 25 mL buffered WMB without supplements in screw capped 18 × 180 tubes (AN, anaerobiosis). Shaken flasks and tubes were inoculated (5 % v/v) using an overnight culture in the same medium and incubated at 30 °C or 35 °C (optimal for growth) on an orbital shaker, 150 rpm agitation (Heidolph UNIMAX 2010) (flasks) or in sealed jars (tubes) containing *AnaeroGen* bags (Oxoid) respectively. Two replicate fermentations were carried out for each treatment.

Growth, chemical, and biochemical analyses

Growth was monitored by measuring the absorbance of the culture at 650 nm (OD_{650nm}) until the end of fermentation (24 h, $OD_{650nm} = 3.0$). Growth was modelled using the dynamic model of Baranyi and Roberts (1994) with DMFit v. 2.0 (Baranyi and Le Marc 1996).

Spectrophotometric methods were used for the measurement of glucose (Miller, 1959), H_2O_2 (Quatravaux et al. 2006), DL-lactic acid concentration (Figenschou and Marais 1991) on the exponential (E, $DO_{650} = 1$) and/or early (ES, 6 h) and late stationary phase (LS, 16 h) culture supernatants. Lactic acid ($Y_{P/S}$) yield was calculated by dividing the net acid production by the substrate (glucose) consumed. Catalase activity was measured on whole cells using the method described by Risse et al. (1992).

O_2 uptake was measured on all strains ES whole cells and on 4 selected strains LS whole cells grown in aerobiosis (AE, AEH, AEHM) from 5.5 mM glucose and 9 mM Na-lactate. ES and LS cells were harvested by centrifugation at 12,000 × g for 10 min, washed twice with 20 mM sterile phosphate buffer, pH 7, and re-suspended in the same buffer containing 5.5 mM glucose and 9 mM Na-lactate. Oxygen consumption was measured by polarographic electrodes (Applisens Applikon) previously polarized and calibrated. The test, was conducted at 35 °C for 15 min in stirred bottles put in a thermostatic bath, and was monitored using BioXpert 2. Dissolved oxygen concentration (DO, %) was transformed into mM O_2 using the Henry's law.

Stress tolerance

The tolerance to oxidative and starvation/cold stress was evaluated on ES and E/ES cells respectively. The cells were recovered by centrifugation (10,000 × g, 5 min at 4 °C), washed twice in 20 mM potassium phosphate buffer pH 7 (PB7) and re-suspended in PB7 to obtain a final A650 = 1. Oxidative stress tolerance was tested by spotting 5 µL of a 0.75 % H_2O_2 on the top layer (7 mL) of WMA (containing 0.6 % w/v agar bacteriological) inoculated with 100 µL of standardized cell suspension, on a 10 mL bottom layer of sterile WMA. Two replicate plates were used for each suspension with two replicate spots. The area of the inhibition zone was measured with a calliper after incubation at 30 °C, 48 h.

Tolerance to starvation and cold storage was evaluated by storing cell suspensions in PB7 at 4 °C for 30 days. The proportion of metabolically active cells was evaluated by epifluorescence microscopy using Propidium iodide (PI) and 5-(6)-carboxyfluorescein diacetate (cFDA) after 15 and 30 days (Zotta et al. 2012a).

Statistical analysis

Statistical analysis (ANOVA and multiple mean comparisions for all results and Partial Least Square Discriminant Analysis for a subset of variables) and graphing was carried out using Systat 13 (Systat Inc.).

Reagents, culture media and ingredients

Unless otherwise stated all reagents were obtained from Sigma-Aldrich (Milan, Italy) while culture media and ingredients were obtained from Oxoid Ltd. (Basingstoke, Hampshire, UK).

Results

Growth parameters and metabolite production

The growth in aerobiosis (AE, AEH, AEHM) and anaerobiosis (AN) of 7 *L. plantarum* subsp. *plantarum*, 1 *L. plantarum* subsp. *argentoratensis*, 2 *L. paraplantarum* and 1 *L. pentosus* strains (Tab. 1) was compared in batch fermentations in a complex buffered medium at controlled temperature (30 °C or 35 °C). Growth parameters and metabolite concentrations in aerobiosis and anaerobiosis are shown in supplementary Table 1 while an example of different kinetics of growth, modeled using the D-model, is shown in Fig. 1.

The highest maximum specific growth rate value (μ_{max}) was found for *L. plantarum* subsp. *plantarum* WCFS1 in all growth conditions (from 0.90 ± 0.03 to 0.81 ± 0.02 h^{-1}). *L. plantarum* subsp. *plantarum* MTD2S, UBS3 and *L. plantarum* subsp. *argentoratensis* DKO22 also showed high μ_{max} values (0.70–0.82 h^{-1}) in all growth conditions. For some strains which showed slow growth at 35 °C, incubation was carried out at 30 °C and the specific growth rate was distinctly lower. However, strains behaved differently under the aerobic and anaerobic growth conditions: *L. plantarum* subsp. *plantarum* 38AA, UBS3, P1.5, WCFS1 and *L. pentosus* 5TP, grew better in aerobiosis while for the other strains μ_{max} was significantly higher in anaerobiosis. Within aerobic growth conditions supplementation with hemin and with hemin and menaquinone conferred an advantage only for some strains and generally reduced the duration of the lag phase (Fig. 1). High concentrations of hemin (10 μg/mL) caused only a slight toxicity (data not shown).

Aerobic growth conditions increased biomass yield in early stationary phase in most strains with the exception of *L. plantarum* subsp. *plantarum* 38AA, C17, UBS3, P1.5 and *L. plantarum* subsp. *argentoratensis* DKO22. However, for strains 38AA, C17, UBS3 a greater biomass concentration was obtained with hemin or hemin and menaquinone additions in stationary phase. After 16 h of growth, hemin and menaquinone additions resulted in a slight decrease in biomass yield only in *L. plantarum* subsp. *plantarum* WCFS1, MTD2S, *L. paraplantarum* B7N26 and *L. plantarum* subsp. *argentoratensis* DKO22. The increase of biomass, except for a few strains, correlated with a decrease of lactic acid and, possibly due to lactate consumption, pH increased in aerobiosis. Some strains, however, showed a decrease of lactate even in anaerobiosis upon prolonged incubation.

Catalase activity and H_2O_2 production are shown in supplementary Table 2. During the exponential phase H_2O_2 production was always very low (≤0.2 mM) in all conditions, while it slightly increased (<0.4 mM) for few strains in the conditions of aerobiosis without supplementations after 6 h of growth and significantly increased (3–8 mM) in the same growth condition in stationary phase in all strains except for *L. paraplantarum* MTG30L, *L. pentosus* 5TP, and *L. plantarum* subsp. *plantarum* 1069 and UBS3. H_2O_2 production correlated with the decrease of lactate while catalase activity in absence of hemin was always below the sensitivity of the method used: higher activities were found in aerobiosis with hemin supplemented after 6 and 16 h of growth (from 14 to 29 mkatal/g and from 17 to 31 mkatal/g, respectively).

O_2 uptake

Oxygen consumption in the presence of 5.5 mM glucose or 9 mM Na-lactate was measured in early stationary phase cells of all the strains in study and in stationary phase cells of the selected strains *L. plantarum* subsp. *plantarum* 38AA, C17, UBS3 and WCFS1. Oxygen consumption of cells grown in anaerobiosis was very low (not shown) and only cells grown in aerobiosis (AE, AEH, AEHM) were used for the assay. In early stationary phase cells, oxygen

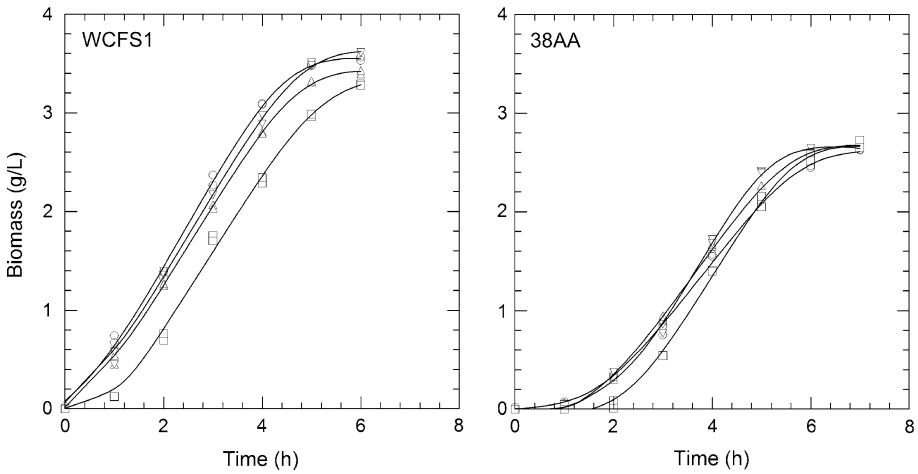

Fig. 1 Kinetics of growth of *L. plantarum subsp. plantarum* WCFS1 and *L. plantarum subsp. plantarum* 38AA in batch fermentations in a complex buffered medium at 35 °C (optimal for growth of these strains). Circle, AE; Triangle, AEH; Inverted triangle, AEHM; Square, AN

uptake from Na-lactate was not detected with the exception of *L. plantarum* subsp. *plantarum* C17 and UBS3 in presence and in absence of supplementations, respectively. In the presence of glucose, with the only exception of *L. plantarum* subsp. *plantarum* 38AA in AE, all strains showed a O_2 consumption, varying from 0.43 ± 0.02 to 2.45 ± 0.04 μmol min/g/biomass while *L. plantarum* subsp. *plantarum* WCFS1 showed an unusual respiratory capacity with values from 5.32 ± 0.79 μmol min/g/biomass in AEH to 6.97 ± 0.19 μmol min/g/biomass in AE. Most strains showed a lower O_2 consumption in the presence of hemin and menaquinone; however, oxygen consumption was significantly higher in the presence of supplementation for *L. plantarum* subsp. *plantarum* 38AA, C17, P1.5 and UBS3, (supplementary Table 3).

Stress tolerance

The tolerance to oxidative and starvation/cold stress was assayed on ES and E/ES cells respectively. The diameters of the zone of inhibition caused by 5 μL of a 0.75 % H_2O_2 solution for ES cells were significantly different from strain to strain and within the four different growth conditions. In general cells grown in aerobiosis with hemin and menaquinone were significantly more resistant than cells grown in aerobiosis with only hemin supplemented and than cells grown in the presence of oxygen without supplementation, while cells grown in anaerobiosis were significantly more sensitive to oxidative stress, (supplementary Table 2). *L. plantarum* subsp. *plantarum* WCFS1 and C17 showed in AEHM the best tolerance to oxidative stress with zone of inhibition 0.45 ± 0.07 and 0.52 ± 0.02, respectively.

The survival of both exponential and early stationary phase cells after cold storage in a nutrient depleted buffer (PB7) was evaluated after 15 and 30 days by direct counts in epifluorescence microscopy images stained with cFDA/PI. The double staining cFDA-PI was not effective for *L. plantarum* subsp. *plantarum* C17 which was excluded from the study.

Exponential phase cells were significantly more sensitive than early stationary phase cells and strains grown in aerobic and anaerobic growth conditions behaved differently.

After 15 days of storage, early stationary phase cells were significantly ($p < 0.01$) more tolerant of cold and starvation stresses than exponential phase cells for almost all strains (with the exception of *L. plantarum* subsp. *plantarum* P1.5 grown in anaerobiosis) and the difference in survival for aerobic grown cells was negligible; for the strains *L. plantarum* subsp. *plantarum* P1.5 and *L. pentosus* 5TP the resistance in anaerobiosis was the lowest (reduction of viability of 40 and 50 %), Fig. 2. For expoential phase cells different, strain dependent response patterns were found. For some strains (38AA, MTD2S and 5TP) no significant difference was found among treatments. For others (1069, P1.5, B7N26) showed significantly ($p < 0.05$) incresed survival, while increased survival in aerobiosis without supplementation (AE) was observed with WCFS1 and DKO22 only. Addition of hemin improved survival only for strain UBS3 while menaquinone supplementation usually decreased survival.

After 30 days, the survival of cells grown in aerobiosis were significantly ($p < 0.01$) better than for cells grown under anaerobic conditions with the sole exception of *L. pentosus* 5TP, at least for early stationary phase cells (data not shown).

Multivariate analysis of variables involved in aerobic metabolism

To evaluate the relationships between factors affecting the aerobic metabolism in *L. plantarum* subsp. *plantarum*, *L. plantarum* subsp. *argentoratensis*, *L. paraplantarum* and *L. pentosus*, a PLS Discriminant Analysis (PLS-DA, Fig. 3a, b) was carried out on standardized (mean centered and scaled by dividing by the standard deviation for each variable) data. A model with 5 components explained 80 % of the x-variance, but had a relatively poor predictive ability, probably due to the high number of Y variables (strains and culture conditions). However, the first two components explained 55.5 % of the total variance and allowed to obtain a good graphical representation of the effect of strains and culture conditions. Figure 3 shows the X-scores and loadings plots, (panels a and b, respectively). Most of the variance (33.5 %) was associated with the first component, which, in turn, was strongly related (Fig. 3b) with the catalase activity in early stationary phase (KATS) and stationary phase (KATS16) cells, with the maximum specific rate of O_2 uptake from glucose in early stationary phase (O2GLUS), pH in stationary phase (PHS16), biomass yield in stationary phase (YXSS16), while oxidative tolerance (OX075S) and lactic acid yield in early stationary phase (YPSS) and stationary phase (YPSS16) were inversely correlated. Additionally, the first component was weakly related to maximum specific growth rate (MUMAXRATIO) and H_2O_2 production in early stationary phase (H2O2S) and in stationary phase cells (H2O2S16). Variation over the second component axis (22.0 % of the total variance) was associated with H2O2S, H2O2S16, MUMAXRATIO, O2GLUS, PHS16 while KATS, KATS16, OX075S and YPSS, YPSS16 were negatively associated with this component.

Strains/culture condition combinations were divided into two groups because of differences resulting from the growth conditions (aerobiosis/anaerobiosis and supplementation): strains grown in anaerobiosis and aerobiosis

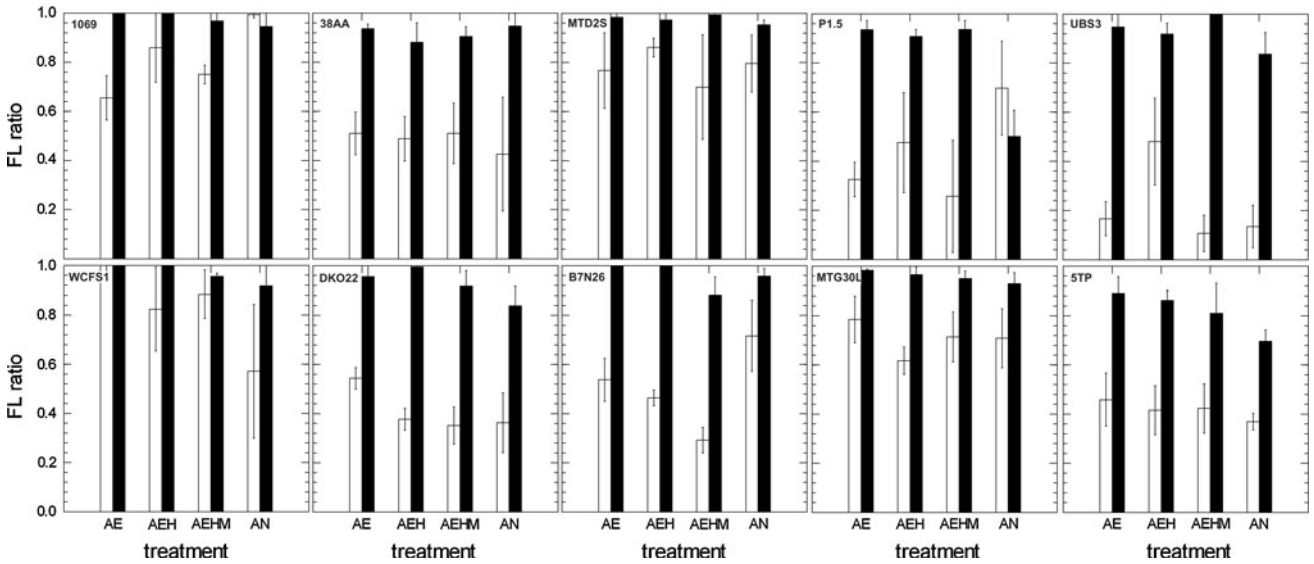

Fig. 2 Bar plot showing the fraction of survivors (*FL*) after exposure to cold and starvation stress (4 °C in 20 mM potassium phosphate buffer, 15 days) of exponential (*E, empty bars*) and early stationary phase (*ES, black bars*) cells of 6 *L. plantarum* subsp. *plantarum*, 1 *L. plantarum* subsp. *argentoratensis*, 2 *L. paraplantarum* and 1 *L. pentosus* strains in batch fermentations in a complex buffered medium at controlled temperature (30 °C or 35 °C) as determined by direct epifluorescence microscopy counts. Mean results with standard error are shown

without supplements were clearly separated from the same strains grown in aerobiosis with menaquinone and/or hemin supplemented. All strains grown aerobically in the presence of hemin or hemin and menaquinone are grouped in the lower part of the graph. The latter were characterized by high catalase activity, resulting in low or absent H_2O_2 concentration and higher resistance to oxidative stress; on the contrary, all strains grown anaerobically or aerobically without supplementations are found in the upper part of the graph, to the left, with no catalase activity and higher H_2O_2 concentrations and low tolerance to oxidative stress.

In aerobiosis with supplementations, *L. paraplantarum* B7N26, MTG30L and the strain *L. pentosus* 5TP were slightly separated from *L. plantarum* strains because of a lower specific growth rate, lower pH values and higher lactic acid yield values, probably due to a lower conversion of lactate to acetate in these two species.

Lactobacillus plantarum subsp. *plantarum* WCFS1 showed in AE a different behavior compared to the other strains in the same growth condition because of its unusual ability to use oxygen which in turn caused a greater H_2O_2 accumulation in the absence of heme dependent catalase.

Discussion

Lactobacillus plantarum has been known for several years to be capable of aerobic metabolism (Murphy and Condon 1984a; Yousten et al. 1975). In the presence of oxygen and when carbon catabolite repression is relieved (Lorquet et al. 2004; Goffin et al. 2006), lactate is oxidized to acetate with oxygen as final electron acceptor and extra ATP biosynthesis. As shown previously for *L. lactis* (Gaudu et al. 2002), in *L. plantarum* group, respiration, which requires supplementation with hemin and menaquinone (Brooijmans et al. 2009a, b) enhances biomass formation without progressive acidification, increases oxygen consumption rate and resistance to H_2O_2 (Watanabe et al. 2012a) but has a negative impact on the survival of stationary-phase cultures at low pHs (Watanabe et al. 2012b). Recently we have shown that in *L. plantarum* WCFS1 aerobic growth under non-respiratory conditions results in enhanced heat and oxidative stress tolerance (Zotta et al. 2012b).

In *L. plantarum* aerobic metabolism generates extra ATP compared to anaerobiosis (Tseng et al. 1991), providing additional energy needed for survival in the stationary phase, but pyruvate oxidase expression and H_2O_2 accumulation may impair survival upon prolonged incubation (Murphy and Condon 1984b; Sedewitz et al. 1984; Tseng et al. 1991). In our study, the addition of Na-MOPS as a buffer and the low initial sugar concentration prevented pH drop below 4.88. *L. plantarum* is a aciduric microorganism and can grow and produce acid even at pH < 4 (Passos et al. 1994) and growth was unlikely to be affected by variations in tolerance to low pH. In addition, a low concentration of carbohydrate source was needed because aerobic metabolism is known to be controlled by catabolite repression in *L. plantarum* (Lorquet et al. 2004). This is the first study in which significant strain to strain variation is found in aerobic metabolism: only some strains

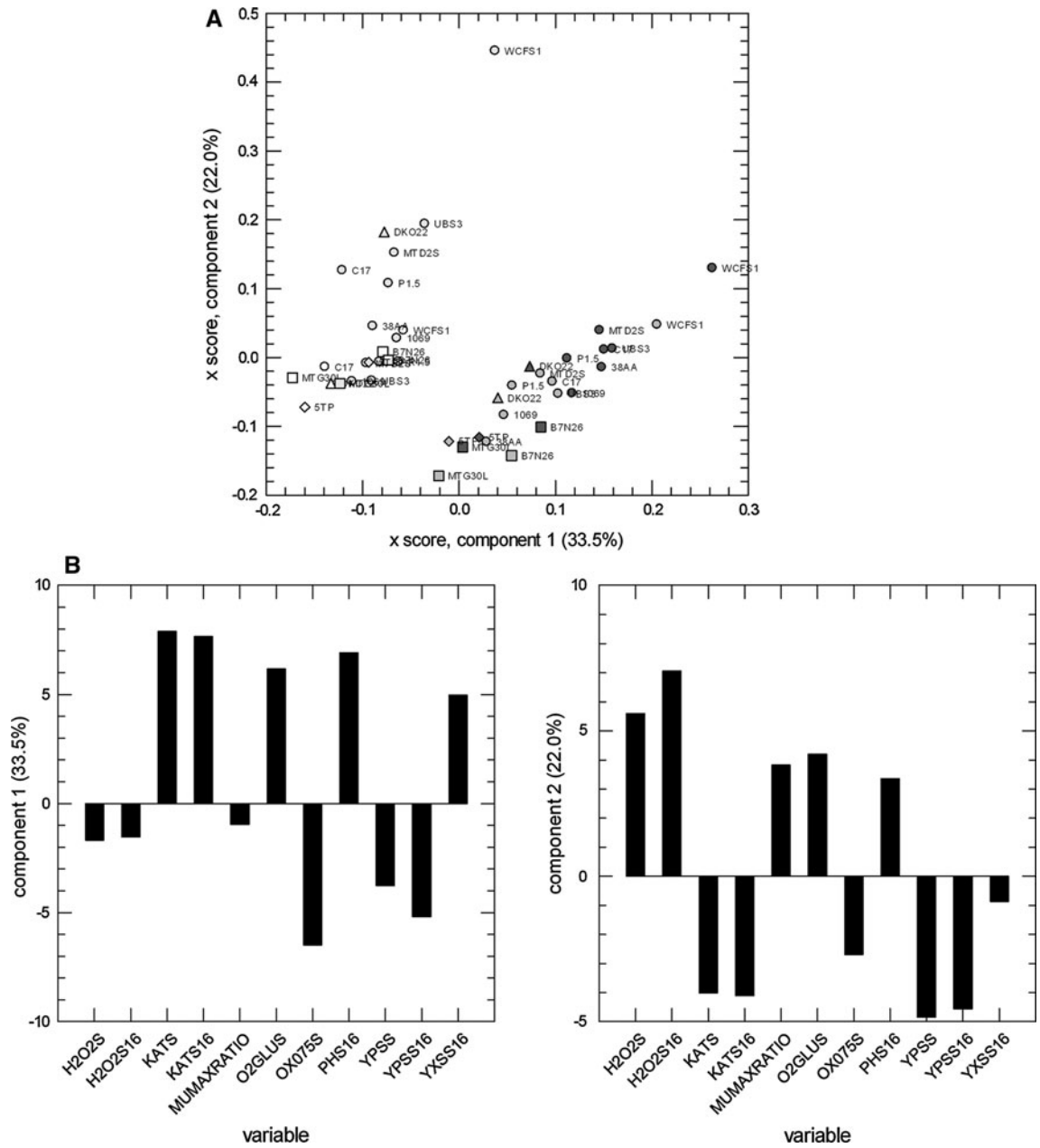

Fig. 3 **a** X-score plots of the first two PLS Discriminant Analysis (PLS-DA) factors. O, *L. plantarum subsp.plantarum*; Triangle, *L. plantarum subsp.argentoratensis*; Diamond, *L. pentosus*; Square, *L. paraplantarum*. Empty symbols, AN; light gray symbols, AE; dark gray symbols, AEH; closed symbols, AEHM. **b** X -Loading plot of the first two PCA factors

showed an increased biomass yield and/or growth rate in stationary phase in aerobiosis without supplementation compared to anaerobiosis.

H_2O_2 production was found at 16 h but was very low at 6 h stationary phase and correlated with the decrease in lactate concentration; *L. paraplantarum*, *L. pentosus* and one *L. plantarum* strain (1069) did not produce H_2O_2. Although some strains of *L. plantarum* can produce a Mn-dependent pseudocatalase (Kono and Fridovich 1983a,b; Yousten et al. 1975) none of our strains produced catalase in the absence of hemin; however, other enzymes (like NADH peroxidase, Götz et al. 1980) may be involved in H_2O_2 detoxification, but were not assayed in this study. We found that H_2O_2 concentration ranged from 3 to 8 mM in stationary phase cultures with the highest values in strain WCFS1. This is in agreement with what has been found by Quatravaux et al. (2006). Murphy and Condon (1984a) reported that a concentration of 3.5 mmol L^{-1} H_2O_2 was needed to completely inhibit growth of *L. plantarum* P5 but we detected a concentration of H_2O_2 until 8 mM in the

stationary phase of *L. plantarum* subsp. *plantarum* WCFS1 in the absence of heme, thus confirming that some strain may show a very high H_2O_2 tolerance.

When exogenous heme was provided in the substrate all strains showed catalase activity after 6 h, with maximum activity after 16 h of growth. Synthesis of heme dependent catalase is known to be specifically dependent on induced oxygen influx (Watanabe et al. 2012a; Zotta et al. 2012b), pH, concentration of salt (Abrioul et al. 2004), and nitrite salt (Mares et al.1994). Although the heme dependent catalase activity has been reported previously for *L. pentosus* (Wolf et al. 1991), to our knowledge this is the first report in which it is demonstrated for *L. paraplantarum* strains. Compared with anaerobiosis and aerobiosis growth conditions, heme supplementation resulted in an increased biomass yield in more than 50 % of the strains, but only one strain showed a μ_{max} higher than anaerobiosis under this condition.

Cell death and lysis in *L. plantarum* may be regulated by acetate (Goffin et al. 2006) when the conversion of lactate to acetate stops in the stationary phase with the resulting dissipation of the proton motive force (PMF) and the subsequent autolysis. On the other hand menaquinone addition in presence of heme is able to activate the (ETC) than can couple the oxidation of NADH and the reduction of oxygen to form PMF that ATPase complex can use to generate ATP with in increasing in biomass production and pH (Brooijmans et al. 2007). At the end of growth a slight (from 0.02 to 0.15) pH increase was observed for some strains. Even if the increase of the pH was relatively low, possibly due to the use of a buffered substrate, a small but statistically significant increase in pH in early and late stationary phase was observed in all the strains with heme and menaquinone supplementation.

Oxygen consumption was measured in stationary phase cells for selected strains. Only a few strains were able to consume oxygen in the presence of 9 mM Na-lactate. The limited use of lactate may be due to the inability to transport lactic acid which at pH 6.5 is predominantly dissociated (pKa = 3.86). Heme dependent catalase activity (with production of O_2 from H_2O_2 produced by POX) in AEH and AEHM may be responsible of lower net O_2 consumption compared to AE cells, but it is not clear why consumption of O_2 both from Na-lactate that from glucose was in some cases lower in AEHM cells (which should show respiratory activity) compared to AEH cells.

In comparison with the others *L. plantarum* strains, *L. plantarum* subsp. *plantarum* WCFS1 showed an unusual ability to use oxygen, at least in early stationary phase from glucose. Our results for *L. plantarum* subsp. *plantarum* WCFS1 are not in agreement with Brooijmans et al. (2009b) who measured a much higher O_2 uptake for the same strain with heme and menaquinone addition compared to aerobiosis without supplementations; this may be due to use of different media and different duration of incubation (16 h in our study, compared to 48 h).

It is known that intracellular oxygen levels cause oxidative damage. The increased resistance to H_2O_2 of AEHM cultures might be related to the consumption of oxygen by the active ETC, which reduces oxygen into water. These results are in agreement with Watanabe et al. (2012a) who studied the survival of exponential-phase and stationary-phase *L. plantarum* WCFS1 cells grown under fermentative, aerobic, aerobic with heme and respiratory conditions after exposure to 0.2 or 0.5 % H_2O_2.

This is the first study in which the survival to cold and starvation stress of *L. plantarum* grown under different aerobic conditions is measured. Even if after 15 days of storage the difference in survival for aerobic grown cells was negligible, after 30 days, the survival of cells grown in aerobiosis was better than for cells grown under anaerobic conditions with early stationary phase cells more resistant than exponential phase cell: this is probably due to a generalized stress response which increases their stress tolerance as previously found for 63 strains of *Lactobacillus plantarum* subsp. *plantarum*, *L. plantarum* subsp. *argentoratensis, L. paraplantarum* and *L. pentosus* (Parente et al. 2010). Our data are show that aerobic growth with supplementation may provide in *L. plantarum* advantages similar to those found for *L. lactis* (Gaudu et al. 2002; Rezaiki et al. 2004).

The results for *L. plantarum* WCFS1 confirm our previous findings (Zotta et al. 2012b) with higher survival of aerobically grown exponential phase cells related to higher oxidative stress tolerance. Menaquinone supplementation did not usually increase survival.

Conclusions

To our knowledge this is the first study in which the aerobic growth with and without supplements (hemin and/or menaquinone) is evaluated in batch fermentations for several strains of the *L. plantarum* group, compared with anaerobic growth and in which tolerance of stress factors relevant for starter and probiotic cultures growth and preservation is measured in all growth conditions.

We demonstrated a high variability among strains, but aerobic growth with heme and menaquinone supplementation determined, except for some cases, benefits comparable to the single supplementation of heme. The preparation of starter culture and probiotics under respiratory conditions may be of great practical significance in terms of improvement of the survival of strains in treatments such as cold storage or spray-drying (which causes oxidative stress).

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