Use of dairy and non-dairy Lactobacillus plantarum, Lactobacillus paraplantarum *and* Lactobacillus pentosus *strains as adjuncts in cheddar cheese*

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Dairy Science & Technology
Official journal of the Institut National de la Recherche Agronomique (INRA)Formerly 'Le Lait'

ISSN 1958-5586
Volume 93
Number 6

Dairy Sci. & Technol. (2013) 93:623-640
DOI 10.1007/s13594-013-0131-8

Springer

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ORIGINAL PAPER

Use of dairy and non-dairy *Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus pentosus* strains as adjuncts in cheddar cheese

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Received: 25 July 2012 / Revised: 11 April 2013 / Accepted: 22 April 2013 /
Published online: 17 May 2013

Abstract Lactobacilli have been used as adjunct cultures in the manufacture of different cheeses with the objective of accelerating ripening and/or improving cheese quality, but no studies have been conducted with strains from non-dairy origins. A miniature cheddar-type cheese model was used to screen ten dairy and non-dairy *Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus pentosus* strains for their performances as adjuncts in cheese manufacture. All strains were able to grow and survive in the cheese environment and produced only minor, although statistically significant, changes in gross cheese composition. Adjuncts affected secondary proteolysis causing differences in the levels of free amino groups, total free amino acids and reversed-phase HPLC (RP-HPLC) profiles of pH 4.6-soluble extract. Three strains were selected on the basis of differences in proteolysis pattern and used in a pilot-plant production of cheddar cheese, which was ripened for 180 days. The results confirmed that use of *L. plantarum* adjuncts significantly affected secondary proteolysis as measured by free amino acid production with minor impact on gross composition and primary starter performance, but the impact on RP-HPLC profiles of pH 4.6-soluble extracts was not statistically significant. The use of a

Electronic supplementary material The online version of this article (doi:10.1007/s13594-013-0131-8) contains supplementary material, which is available to authorized users.

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strain originally isolated from olive brine fermentation, *L. plantarum* P1.5, resulted in significantly improved preference scores over the control.

Keywords Adjunct cultures · *Lactobacillus plantarum* · Cheddar cheese · Proteolysis

1 Introduction

Microorganisms play a central role in cheese ripening. Mesophilic or thermophilic lactic acid bacteria (LAB) that grow rapidly in milk and curd (*Lactococcus lactis*, *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis*, etc.) producing lactic acid, and which are usually added as starters, are the dominant group in the first days/weeks, but during ripening other microorganisms increase and contribute to several key quality features of cheese (Beresford and Williams 2004). For some cheese varieties, secondary starters (*Penicillium roqueforti*, *P. camemberti*, *Brevibacterium linens* and *Propionibacterium freudenreichii*) are added intentionally (Chamba and Irlinger 2004), but adventitious mesophilic lactic acid bacteria, referred to as non-starter lactic acid bacteria (NSLAB), dominate the microbiota of most ripened cheese varieties, as the environment becomes more selective (low sugar content, decreased a_W, low pH and increasing salt in moisture; Beresford and Williams 2004; Crow et al. 2001). Adventitious NSLAB are known to contribute to flavour development, and for this reason, their growth is desirable, but they may also cause off flavours and defects (Crow et al. 2001; Swearingen et al. 2001).

The dominant microbiota during cheddar cheese making are the starter lactococci: their number rapidly increases (~10^9 cfu.g^{-1}) and then declines as the cheese ripens (Lawrence et al. 2004) while the adventitious NSLAB counts increase from a very low level (~10 to 10^4 cfu.g^{-1} in 1-day-old cheddar cheese manufactured with pasteurised milk) to ~10^7 to 10^8 cfu.g^{-1} within approximately 3 months of ripening, becoming the dominant viable microbiota (Swearingen et al. 2001). The dominant adventitious NSLAB found in cheddar cheese include *Lactobacillus paracasei* and *Lactobacillus plantarum*, while *Lactobacillus curvatus*, *Lactobacillus casei*, *Lactobacillus brevis* and *Lactobacillus rhamnosus* have been found as minor components (Beresford and Williams 2004; Crow et al. 2001; Jordan and Cogan 1993; Swearingen et al. 2001). The heterogeneity of the adventitious NSLAB population in cheddar cheese decreases during ripening; in fact, species encountered in young cheeses (*L. plantarum* and *L. curvatus*) can be replaced at later stages by *L. paracasei* (Fitzsimons et al. 2001). In contrast, Jordan and Cogan (1993) found that the adventitious NSLAB population of 8-week-old Irish cheddar cheese had a smaller proportion of *L. paracasei* and larger proportions of *L. plantarum* and *L. curvatus*. The dynamics of NSLAB population depends on several factors, such as cheese age, the use of raw or pasteurised milk, factory hygiene, starter used and cheese composition (Beresford and Williams 2004).

Improved hygiene and use of pasteurised milk for cheese making has led to an overall reduction in NSLAB numbers, and this may result in slower flavour development in the mature cheese. The use of selected secondary starters (adjuncts) has been suggested to improve development of cheese aroma and flavour intensity (Lynch et al. 1996; Lynch et al. 1999; Milesi et al. 2008b; Williams et al. 2006), to

accelerate ripening and to eliminate defects caused by adventitious NSLAB by inhibiting their growth (Lawrence et al. 2004).

L. plantarum is an industrially important mesophilic LAB that is widespread in the environment and in fermented products of animal and plant origin either as starter (Parente et al. 2010) or as a component of the NSLAB flora (Beresford and Williams 2004; Jordan and Cogan 1993; Swearingen et al. 2001); it is tolerant of low pH and relatively high salt concentration. Although *L. plantarum* has been tested, sometimes successfully, as adjuncts in cheese (Lynch et al. 1996; Lynch et al. 1999; Di Cagno et al. 2006; Milesi et al. 2008b; Ortigosa et al. 2005; Poveda et al. 2003; Williams et al. 2006), to our knowledge, no study using *L. plantarum* strains from non-dairy origins as adjuncts in cheese manufacture has been published. The use of wild LAB strains isolated from dairy and non-dairy environments as adjuncts for the development of new flavours has raised interest since these strains may harbour more amino acid catabolic enzymes than industrial starters (Ayad et al. 2000). The objective of this study was therefore to assess the technological properties of non-starter *L. plantarum, Lactobacillus paraplantarum* and *Lactobacillus pentosus* strains isolated from different sources (cheese, sourdoughs, cassava, olive fermentations and wine). Ten strains, selected in a previous work (Parente et al. 2010), were tested as adjuncts in a screening study in miniature cheddar-type cheese. Three strains which significantly affected secondary proteolysis were then tested in pilot-plant cheddar cheese production, and their ability to affect the composition, proteolysis and sensory scores of cheese was evaluated.

2 Materials and methods

2.1 Strains

Lyophilised *Lactococcus lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* (F-DVS pHageControl™ R-600 Culture Series, Chr. Hansen, Hørsholm, Denmark) were added at 0.02% (*v*/*v*) as a starter into cheese-milk at 31 °C. The strains used as adjuncts and their origin are listed in Table 1. The adjuncts were inoculated (1%, *w*/*v*) in MRS broth (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated for 16 h at 30 °C. Cells were harvested by centrifugation (12,000×*g* for 10 min), washed twice with Ringer's solution, re-suspended in milk and added into the milk at level of 10^5 cfu.mL^{-1}.

2.2 Cheese-making trials

2.2.1 Miniature cheddar-type cheese

Miniature cheddar-type cheeses were manufactured in aseptic conditions by the procedure of Shakeel-Ur-Rehman et al. (1998) with some modifications. Raw milk was obtained from a local dairy farm and batch pasteurised at 63 °C for 30 min. The cheeses were prepared in experiments with two treatments, for each of which six cheeses were made; each treatment was replicated on two different days and the combination of treatments for each given experiment was randomised. A total of 11

Table 1 List of bacterial strains used in this study and coding of cheeses in the minicheese experiment

Strain	Cheese	Species	Isolation source	Source
None	A (control)			
P1.5	B	*Lactobacillus plantarum* subsp. *plantarum*	Olives brine, Italy	DOFATA
UBS3	C	*L. plantarum* subsp. *plantarum*	Wine, Italy	DOFATA
5TP	D	*Lactobacillus pentosus*	Olives water fermentation, Italy	DPPMA
38AA	E	*L. plantarum* subsp. *plantarum*	Cassava, Colombia	DBVR
DK022	F	*L. plantarum* subsp. *argentoratensis*	Sour Cassava, Nigeria	DBVR
1069	G	*L. plantarum* subsp. *plantarum*	Pane Carasau sourdough, Italy	PCC
C17	H	*L. plantarum* subsp. *plantarum*	Caciocavallo cheese, Italy	DBPZ
B7N26	I	*Lactobacillus paraplantarum*	Caciocavallo cheese, Italy	DBPZ
MTG30L	J	*L. paraplantarum*	Cornetto di Matera sourdough, Italy	DBPZ
MTD2S	K	*L. plantarum* subsp. *plantarum*	Cornetto di Matera sourdoug, Italy	DBPZ

All strains were identified by multiplex PCR as described in Parente et al. (2010).

DBPZ culture collection of Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali, Università degli Studi della Basilicata, *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, *PCC* Porto Conte Ricerche, Italy

treatments was used (Table 1). Miniature (20 g) cheeses were prepared in a laminar air-flow hood using sterile utensils and salted by immersion in sterile brine (20% NaCl and 0.05% $CaCl_2 \cdot H_2O$ (*w*/*v*) at 30 min at room temperature) and then wiped dry, vacuum packaged and ripened at 8 °C for up to 90 days.

2.2.2 Pilot-plant cheddar cheese production

Three *L. plantarum* subsp. *plantarum* strains (C17, MTD2S and P1.5) were used as single adjunct cultures in cheddar cheese manufacture and compared with the control (no adjunct) using a randomised block design. Four vats, each with a different treatment were made each day, and the experiment was replicated three times. Cheddar cheeses were made in the pilot plant of University College Cork, from whole cow's milk obtained from a local farm according to a standard protocol (Kosikowski and Mistry 1997), utilising open vats filled with approximately 50 L of HTST-pasteurised (73.5 °C, 15 s) milk. Vats were cleaned and sanitised to minimise cross contamination during manufacture of the cheeses. The curd was milled at pH 5.4, dry-salted with 2.5% NaCl (*w*/*w*) of the theoretical curd yield for 20 min, moulded and pressed at 1 bar overnight at room temperature. Cheese blocks were vacuum packed and ripened at 8 °C for up to 6 months.

2.3 Microbial counts

Cheese samples were homogenised in 2% (*w*/*v*) trisodium citrate solution and decimal dilutions were prepared in sterile quarter-strength Ringer's solution. Counts

were performed in duplicate after 7, 14, 30, 60 and 90 days of ripening in the miniature cheddar-type cheese experiment, and at 120 and 180 days in the pilot-plant experiment. The starter was counted on LM17 agar (Merck, Darmstadt, Germany) after incubation at 30 °C for 3 days. In the miniature cheddar cheese experiment, NSLAB were enumerated on LBS agar (Becton Dickinson, Le Pont de Claix, France) and on Rogosa agar (Merck), after incubation at 30 °C for 5 days under anaerobic conditions (anaerobic jar and Anaerocult A gas packs, Merck) while only LBS was used in the pilot-plant experiment. To obtain preliminary evidence that the colonies counted belonged to the *L. plantarum* group, ten colonies (from 60-day cheeses) were randomly selected from a countable plate of both Rogosa and LBS agar for each treatment and purified by streaking on MRS medium containing bromophenol blue (Lee and Lee 2008) using reference cultures (including *L. plantarum* DSM20174 and *L. paracasei* subsp. *paracasei* DSM20020) as a control.

2.4 Gross composition of cheeses

The pH was measured in duplicate by insertion of a combination electrode connected to a pH metre into cheese slurry (1:1 with water). Samples of 90 (miniature cheddar-type cheese) or 14 days old cheeses (cheddar cheese) were analysed in duplicate for moisture (IDF 1982), salt (Fox 1963), fat (IDF 1996) and nitrogen content (IDF 1993).

2.5 Assessment of proteolysis

Proteolysis was monitored by measuring the percentage of N soluble at pH 4.6 (pH 4.6-SN) using the method of Kuchroo and Fox (1982), the *o*-phthaldialdehyde assay (OPA; Church et al. 1983), and the trinitrobenzene-sulphonic acid method (TNBS; Adler-Nissen 1979). In the latter case, a calibration curve was prepared using leucine (Sigma) as standard (range, 0.0–1.0 $mmol.L^{-1}$ of Leu), and results were expressed as milligrammes Leu per gramme of cheese.

Peptide profiles of the pH 4.6-soluble extracts were determined by reversed-phase HPLC (RP-HPLC) as described in Milesi et al. (2008a).

Casein degradation was assessed by electrophoresis of pH 4.6-insoluble extracts in polyacrylamide gels (urea-PAGE; 12.5% T and 4% C at pH 8.9) on 14- and 90-day-old cheeses as described before (Milesi et al. 2008a).

2.6 Sensory evaluation

Consumer preference was evaluated on 6-month-old cheeses by an untrained consumers panel (n=216; 114 females and 102 males) in individual booths illuminated with white lighting. The panel was further divided in classes on the basis of age (18–28, 70%; 29–38, 18%; and 39–58, 12%, all with approximately equal number of males and females) and on the basis of frequency of consumption of cheddar cheese (often, 44%; very Often, 56%). The samples were assigned with three-digit random numbers and served under identical conditions. Cheeses were randomly assigned to days in a balanced block design, whereas order of tasting within each day was balanced to account for first-order and carry-over effects (MacFie et al. 1989). Consumers evaluated the four cheeses using a hedonic scale ranging from 1 ("dislike

extremely") to 9 ("like extremely") (Drake et al. 2008), and then ranked them in their order of preference. Each consumer evaluated each cheese for overall acceptability.

2.7 Statistical analyses

Data from gross composition, chemical analysis, and microbiological counts for all experiments (miniature and pilot-plant production) were analysed by ANOVA with Tukey's HSD as a post hoc test. A complete random design was used for the miniature cheese experiment while a randomised block design was used for the pilot-plant experiment. A paired *t* test and χ^2 were used to test the hypothesis that LBS medium was more selective than Rogosa agar for the enumeration of *L. plantarum*. Data processing of the RP-HPLC chromatograms of the pH 4.6-soluble extracts of cheeses was carried out as described by Piraino et al. (2004) using 61 classes from 4 to 70 min elution time with flat range and membership in the flat range values of 30 and 90, respectively, for the miniature cheddar cheese experiment, while for the pilot cheese experiment, 61 classes were used from 4 to 95 min retention time. Repeatability was evaluated on three randomly selected samples using the procedures described in Parente et al. (2012). Covariance and presence of outliers in RP-HPLC data were initially assessed using principal component analysis (PCA). Chromatographic data were then transformed to *Z*-scores and used in partial least squares discriminant analysis (PLS-DA), a supervised data analysis technique that has the ability to cope with multicollinearity among variables and uses group information to maximise the separation between groups of observations. The PLS-DA linear model was:

$$Y = XB + R$$

Y Matrix of dependent variables
X Matrix of independent variables
B Matrix of regression coefficients
R Matrix of model residuals

In addition, RP-HPLC pseudo-chromatograms from pilot cheese experiment were analysed using a single mean linear mixed effect model (LME) where all factors are considered as random with the intent of performing variance decomposition:

$$y_{ij} = \mu + \beta_i + \tau_j + \varepsilon_{ij}$$

y_{ij} The RP-HPLC class measured in the *i*th cheese, at *j*th time.
μ Overall mean
β_i $\sim N(0, \sigma^2{}_\beta)$=random deviation due to *i*th cheese from overall mean
τ_j $\sim N(0, \sigma^2{}_\tau)$=random deviation due to *j*th time from overall mean
ε_{ij} $\sim N(0, \sigma^2)$=random residual error

Sensory data were evaluated using the Friedman test (O'Mahony 1985). All statistical and graphic analyses were performed using Systat 13.0 for Windows (SPSS, Chicago, IL) except PLS-DA which was performed using "pls" and "lme4" packages of R (http://lme4.r-forge.r-project.org/; Pinheiro and Bates 2000) of R (R Development Core Team 2010) as described in Parente et al. (2012).

3 Results

3.1 Miniature cheddar cheese experiment

3.1.1 Gross composition and pH

Compositional data for 90-day-old miniature cheddar cheeses made with or without adjuncts are shown in Table 2. One-way ANOVA showed significant differences ($p<0.01$) among treatments in pH, moisture, salt in moisture (S/M%) and protein content. Differences between cheeses made with adjuncts and the control in pH, moisture and S/M% values, however, were small (2–3% of the mean of the control). The pH changed with 0.2–0.3 units throughout ripening, with some exceptions (Fig. 1 in the Electronic supplementary material (ESM)). At the end of ripening, cheeses C, E, F, and G had slightly higher pH values (5.19 compared with 5.07–5.12 for the other cheeses).

3.1.2 Microbiological analyses

In both control and experimental cheeses, the starter population grew to similar numbers in the first stages of ripening (~10^9 cfu.g^{-1}) (Fig. 1 in the ESM) suggesting that the addition of adjuncts did not influence the growth and survival of the starter. Viable counts on M17 decreased almost 1.5 log cycles over the course of ripening, with a similar trend in all the cheeses. NSLAB were enumerated on LBS and Rogosa agar. Counts on Rogosa, with a few exceptions were 0.4-0.8 log units higher ($p<0.001$, paired t test) than counts on LBS. For the cheeses made with adjuncts out of 200 isolates obtained on modified MRS medium (Lee and Lee 2008), 182 (91%) had a colony morphology identical to *L. plantarum*, which was clearly different from other LAB species commonly found in cheddar cheese. The proportion of non-*L. plantarum* colonies was significantly higher on Rogosa agar (13%) compared with LBS (5%, $p<0.05$, χ^2 test).

3.1.3 Proteolysis

Assessment of primary proteolysis by urea-PAGE of the pH 4.6-insoluble extracts (not shown) showed no differences between the control and the experimental cheeses at 14 days and 3 months of ripening, suggesting that the adjuncts did not provide a significant contribution to casein breakdown. Experimental cheeses B (strain P1.5), E (38AA), F (DKO22) and G (1069) yielded values of absorbance for OPA significantly higher than the control cheese, indicating a higher level of proteolysis. In general, levels of proteolysis estimated by the OPA assay were in good agreement ($r=0.84$) with estimates obtained by measuring pH 4.6-SN by Kjeldhal (Table 2).

At 1 and 2 weeks ripening, the concentrations of free amino acids (FAA) measured by TNBS assay were similar in all cheeses except for E (38AA) and F (DKO22), which had significantly higher values (data not shown). Differences emerged over the ripening: at 90 days, cheeses B (P1.5), E (38AA) and F (DKO22) had significantly higher levels of FAA compared with the control while cheeses C (UBS3), G (1069), H (C17), I (B7N26) and J (MTG30L) had levels of FAA significantly lower than the control (Table 2).

A bar plot of RP-HPLC profiles of pH 4.6-soluble extracts for the control cheese and cheeses B, is shown as an example in Fig. 2 in the ESM. PCA was performed on the

Table 2 Microbial counts and gross composition of miniature cheddar-type cheeses produced without (A) or with addition of *Lactobacillus plantarum*, *Lactobacillus paraplantarum* or *Lactobacillus pentosus* adjunct cultures (see Table 1)

Cheese	LM17 (log cfu.g^{-1})	Rogosa (log cfu.g^{-1})	LBS (log cfu.g^{-1})	pH	Moisture (%)	Protein (%)	S/M (%)	pH 4.6-SN (%TN)	OPA (abs.340 nm)	FAA (mg Leu.g^{-1})
A	7.63±0.04^{a}	7.73±0.04^{a}	6.74±0.03^{a}	5.10±0.01^{ab}	42.5±0.2^{c}	21.0±0.2^{c}	4.16±0.02^{d}	20.2±0.1^{f}	0.47±0.01^{e}	0.86±0.02^{b}
B	8.02±0.03^{ab}	8.14±0.03^{ab}	7.99±0.03^{d}	5.12±0.02^{b}	43.2±0.3^{e}	21.3±0.2^{d}	4.05±0.02^{a}	21.0±0.4^{g}	0.52±0.01^{g}	1.13±0.01^{d}
C	7.37±0.65^{a}	7.87±0.04^{a}	7.59±0.03^{c}	5.19±0.02^{c}	43.9±0.5^{c}	20.5±0.2^{c}	4.13±0.02^{bc}	17.4±0.2^{d}	0.44±0.01^{c}	0.84±0.01^{b}
D	8.13±0.02^{ab}	8.33±0.04^{bc}	8.23±0.01^{e}	5.10±0.02^{ab}	42.4±0.2^{b}	20.7±0.2^{b}	4.23±0.02^{e}	16.7±0.2^{cd}	0.45±0.01^{d}	0.94±0.09^{c}
E	7.87±0.03^{ab}	7.65±0.05^{a}	7.47±0.03^{b}	5.19±0.02^{c}	41.5±0.1^{b}	21.5±0.2^{c}	4.25±0.02^{ef}	20.3±0.2^{fg}	0.50±0.01^{f}	1.14±0.01^{d}
F	7.86±0.03^{ab}	7.65±0.05^{a}	7.49±0.03^{b}	5.19±0.02^{c}	41.5±0.2^{b}	22.4±0.2^{e}	4.26±0.02^{f}	17.1±0.4^{cd}	0.49±0.01^{f}	1.19±0.01^{e}
G	8.16±0.02^{ab}	7.92±0.05^{a}	7.65±0.08^{c}	5.19±0.01^{c}	37.5±0.4^{a}	20.5±0.3^{a}	4.36±0.02^{g}	20.6±0.3^{fg}	0.48±0.01^{f}	0.75±0.02^{a}
H	7.97±0.03^{ab}	8.45±0.81^{bc}	8.05±0.03^{d}	5.10±0.02^{ab}	43.7±0.3^{c}	22.1±0.1^{f}	4.12±0.02^{b}	18.5±0.7^{e}	0.44±0.01^{c}	0.87±0.01^{b}
I	8.03±0.03^{ab}	8.37±0.03^{bc}	8.19±0.02^{e}	5.13±0.03^{b}	42.5±0.4^{c}	21.0±0.1^{c}	4.17±0.02^{d}	15.4±0.7^{b}	0.41±0.01^{a}	0.82±0.01^{b}
J	8.57±0.03^{b}	8.70±0.04^{c}	8.35±0.02^{f}	5.07±0.02^{a}	43.3±0.4^{c}	23.0±0.1^{g}	4.16±0.02^{cd}	14.4±0.2^{a}	0.41±0.01^{a}	0.83±0.01^{b}
K	8.24±0.04^{ab}	8.56±0.05^{bc}	8.00±0.04^{d}	5.12±0.03^{b}	42.7±0.4^{c}	21.0±0.1^{c}	4.18±0.02^{d}	16.3±0.2^{c}	0.43±0.01^{b}	0.94±0.01^{c}

All values (mean±standard deviation) refer to 90-day-old cheeses. Means of duplicate analyses followed by the same superscript are not significantly different (Tukey's HSD, α=0.05)

Cheese A control cheese, *B–K* cheeses made with adjuncts (see Table 1), *S/M* percent salt in moisture, *pH 4.6-SN* pH 4.6-soluble nitrogen as per cent total of nitrogen, *OPA* o-phthaldialdehyde assay, *FAA* free amino acids

covariance matrix of the RP-HPLC data. A model with five components explained 49.7% of the *X* variance, and it was relatively ineffective in discriminating between cheeses and ripening times, probably because of variability intrinsic to the miniature cheese model (variability within and between replicate treatments made on different days) and, to a lesser extent, technical factors (extraction and injection) (see below). Therefore, in order to obtain a model which had a better ability to discriminate the cheeses, classes of retention time were used as *X* vectors for PLS-DA, and cheeses and time as *Y* vectors. The model had a relatively low predictive ability (cross-validated R^2 was 0.72 for ripening time and 0.35–0.67 for cheeses), but all treatments were clearly discriminated on the *X*-score plots for the first two components shown in Fig. 1. Larger hulls for cheeses D, C, J and B at 60 days, and for cheeses C, G, H and A at 90 days, reflect a larger variability which was due in part to biological variation (different cheeses made in different days) and in part to technical variability (due to extraction or chromatographic runs). However, the control cheese (A) was clearly separated from all cheeses with adjuncts at 60 days of ripening and from most adjuncts cheeses at 90 days. The cheeses which showed the largest differences from the control were those made with adjuncts B, D, E G and J at 60 days and all those made with adjuncts with the exception of G and F at 90 days. Almost all peak classes contributed to differences due to ripening time, use of adjuncts, or both, as judged by PLS regression coefficients (Fig. 2) with the largest contribution from peak classes at 5.1<RT<8.4, 13.9<RT<15.0, 32.6<RT<38.1, 44.7<RT<45.8, 5.1<RT<8.4, 50.2<RT<51.3 and 56.8 min. Differences due to adjuncts

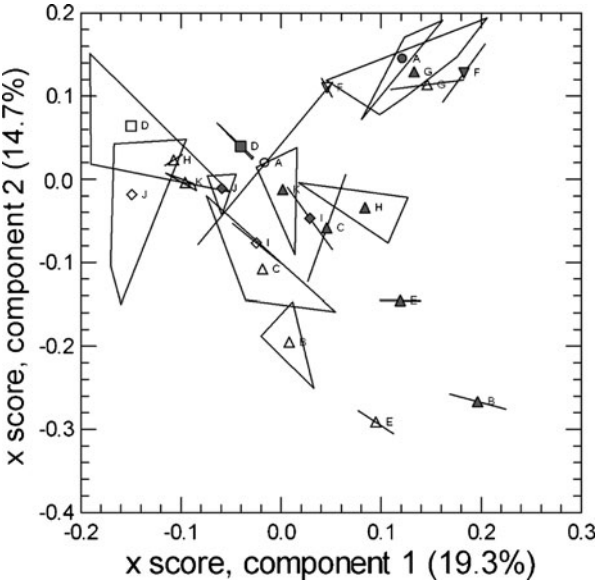

Fig. 1 *X*-score plots for PLS components 1 and 2 for PLS-DA analysis for RP-HPLC profiles of pH 4.6-soluble extract from miniature cheddar-type cheeses made without (*A*) or with (*B–K*, Table 1) *Lactobacillus* adjuncts. *Empty circles*, control cheese; *empty triangles*, *L. plantarum* subsp. *plantarum*; *empty inverted triangles*, *L. plantarum* subsp. *argentoratensis*; *empty squares*, *L. pentosus*; *empty diamonds*, *L. paraplantarum*. Ripening: *empty symbols*, 60 days; *closed symbols*, 90 days. The *symbols* mark the average score while the *convex hulls* mark the area of the graph in which the individual points for technical and biological replicates fall

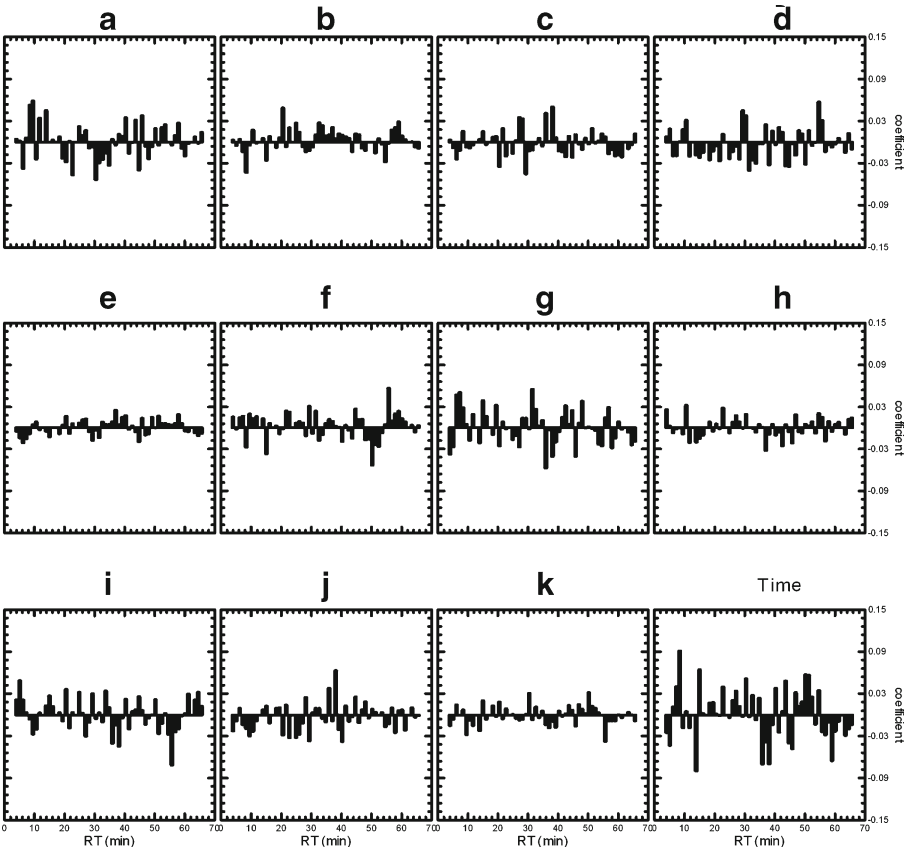

Fig. 2 PLS coefficients plots for PLS-DA analysis for RP-HPLC profiles of pH 4.6-soluble extract from miniature cheddar-type cheeses made without (**a**) or with (**b**–**k**, Table 1) *Lactobacillus* adjuncts

were more complex and varied with time, and the largest differences were found at RT<10 min, 22<RT<38 min, and RT>54 min (Fig. 2).

3.2 Pilot-plant cheddar cheese trials

Three *L. plantarum* strains were chosen for further study in pilot-plant experiments. Strain P1.5 was one of the most proteolytic strains (as judged by OPA and FAA, Table 2; Fig. 2 in the ESM) and significantly affected the RP-HPLC pattern of pH 4.6-SN. Although neither C17 nor MTD2S were able to increase secondary proteolysis compared with the control (Table 2), they both produced RP-HPLC profiles which were clearly different. Finally, both strains C17 and P1.5 have potentially probiotic traits (acid and bile tolerance, surface hydrophobicity, adherence to fibronectin and biofilm formation; data not shown).

3.2.1 Gross composition

Gross composition for 14-day-old cheeses is shown in Table 3. Differences for salt in moisture and protein were not significant, while differences for pH and moisture, although

Table 3 Microbial counts and gross composition of pilot cheddar cheeses produced without (control) or with addition of *Lactobacillus plantarum*, adjunct cultures (see Table 1)

Cheese	LM17^{ns} (log cfu.g^{-1})	LBS^{s} (log cfu.g^{-1})	pH^{s}	Moisture^{s} (%)	Protein^{ns} (%)	Fat^{s} (%)	S/M^{ns} (%)	OPA^{s} (abs.340 nm)	FAA^{s} (mg Leu.g^{-1})
Control	8.90±0.02	5.61±0.06^{a}	5.40±0.01^{a}	39.1±0.2^{a}	26.0±0.3	28.8±0.4^{a}	3.55±0.02	0.61±0.01^{a}	0.24±0.01^{a}
C17	8.86±0.01	6.93±0.08^{b}	5.41±0.01^{a}	39.8±0.1^{b}	26.3±0.1	29.9±1.0^{a}	3.54±0.01	0.64±0.01^{b}	0.31±0.01^{b}
MTD2S	8.90±0.01	6.89±0.09^{b}	5.44±0.01^{b}	40.4±0.2^{c}	26.6±0.3	29.7±1.1^{a}	3.52±0.04	0.89±0.01^{c}	0.33±0.01^{c}
P1.5	8.95 ± 0.08	6.94±0.09^{b}	5.42±0.01^{ab}	40.1±0.1^{b}	26.6±0.2	32.5±0.1^{b}	3.57±0.01	0.98±0.01^{d}	0.35±0.01^{c}

All values (mean±standard deviation) refer to 14-day-old cheeses. Values followed by the same superscript are not significantly different (Tukey's HSD, α=0.05)

S/M per cent salt in moisture, *ns* no significant difference among treatments, *s* significant differences among treatments (p<0.001)

small (1–2%) were significant; larger differences (10–13%) were found in fat content for P1.5. All values were within the range of those found for cheddar cheese.

3.2.2 Microbiological analyses

Starter counts were not significantly different at 14 days, while differences in NSLAB counts on LBS agar were highly significant. The time course for viable counts on LM17 and LBS and pH is shown in Fig. 3 in the ESM. While viable counts for lactococci on LM17 showed a similar trend for all treatments, with a decrease from 60 days, the counts of NSLAB never increased over 6.6 log (in colony-forming units per gramme) for the control while exceeded 8 log (in colony-forming units per gramme) for cheeses made with adjuncts and then decreased to 7–8 log (in colony-forming units per gramme) at the end of ripening (180 days). The ratio between

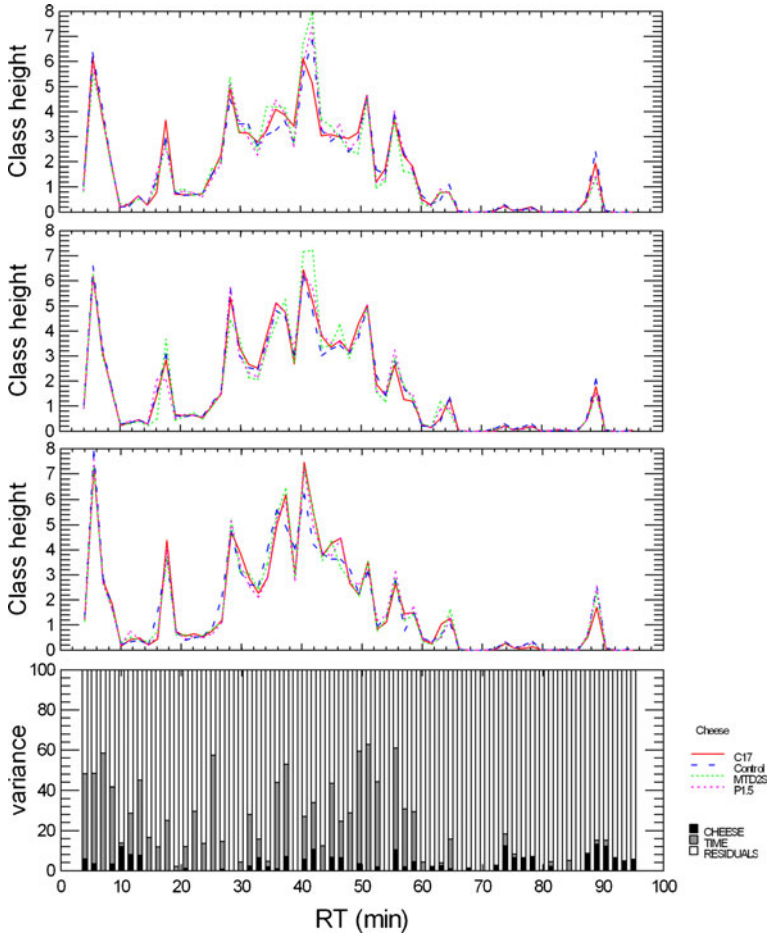

Fig. 3 Variance components and mean profiles for a single mean random effects model carried out on pre-processed RP-HPLC chromatograms of pH 4.6-soluble extract of cheddar cheese made without (control) or with *L. plantarum* adjuncts (C17, MTD2S and P1.5). *Bottom*, the proportion of variance explained by each factor, for each of the variables (height of retention time classes extracted from the raw chromatograms); *top line plots*, the mean profile of each cheese at 60, 120 and 180 days of ripening

presumptive *L. plantarum* and other NSLAB was checked as described for the miniature cheddar-type cheeses. Fraction of non-*L. plantarum* colonies was 70% and 20% for the control at 14 and 180 days while was <5% for the cheeses made with adjuncts (with the exception of cheese made with strain C17 at 14 days, 30%).

3.2.3 Proteolysis

No significant differences were found in the urea-PAGE patterns of pH 4.6-insoluble extracts (results not shown). Amino groups measured with OPA and FAA measured with TNBS were significantly higher in adjunct cheese, with the highest values for P1.5) at 14 days (Table 3). At 180 days the FAA (in milligrammes Leu per gramme) values were 0.243±0.03, 1.422±0.009, 1.452±0.008 and 1.474±0.003 mg Leu.g^{-1} for control, C17, MTD2S and P1.5, respectively. Differences between control cheese and cheese made with adjuncts were significant, with highest values for cheese made with P1.5.

The PLS-DA model of the processed RP-HPLC profiles explained 71% of *X* variance and 65% of *Y* variance in the first eight factors. After cross-validation, although the model had an excellent predictive ability for time (predictive R^2, Q^2=0.91), predictive ability for type of adjunct was low (Q^2<0.50). LME was used to evaluate the proportion of variance which could be attributed to adjunct or ripening time on RP-HPLC profiles of pH 4.6-soluble extracts. Variance components of each chromatographic class and mean profiles for each cheese at 60, 120 and 180 days are shown in Fig. 3. Only a few retention time classes (7.03, 25.2, 37.4, 49.5, 51.0 and 55.6 min) were significantly affected by ripening time. Some of these corresponded to classes which were affected by time in the minicheese experiment (7.3, 25.2 and 51.0). Pseudochromatograms of the RP-HPLC profiles of pH 4.6-soluble extracts are shown in Fig. 4 in the ESM.

3.2.4 Sensory evaluation

Raw preference results are shown as box plots in Fig. 4. The preference scores were significantly different (p<0.0001) and, overall, the preference ranking was P1.5>C17≥MTD2S≥control. The group which consumed cheddar very often had a significantly higher preference for the cheese produced with strain P1.5.

4 Discussion

The objective of this study was to compare ten strains belonging to the *L. plantarum* group and isolated from dairy and non-dairy sources as potential cheese adjuncts using a combination of a screening in a miniature cheddar-type cheese model and of cheese making on pilot scale.

The gross composition of the miniature and pilot-plant cheeses was close to the range typical for miniature and pilot cheddar-type cheese, as reported by other authors (Lynch et al. 1996; Lynch et al. 1999; Milesi et al. 2008a, 2008b; Shakeel-Ur-Rehman et al. 1998). The type of adjunct resulted in small, but significant, differences in cheese composition and final pH. Other authors (Hynes et al. 2001) found similar results, with significant differences only in the dry matter content of miniature washed-curd cheeses made with different strains of starter LAB and a *L.*

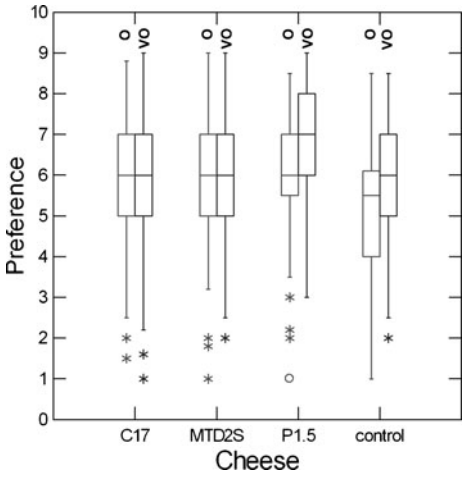

Fig. 4 *Boxes* and *whiskers* plot for raw preference results (from 1, "dislike extremely" to 9 "like extremely) for 180-day-old cheddar cheese samples produced without (*control*) or with the addition of *L. plantarum* subsp. *plantarum* adjunct cultures (*C17*, *MTD2S* and *P1.5*). Box plots for consumers who declared to consume cheddar cheese often (*O*) or very often (*VO*) are shown separately. Outside values (falling outside ±1.5×IR where IR is the interquartile range) are plotted as *asterisks*; extreme values (falling outside ±3×IR) are plotted as *circles*

plantarum adjunct culture, while decrease in pH of cheeses produced with adjuncts has been attributed to the production of acetic acid (Ong et al. 2006; Ong et al. 2007), although this compound was not measured in this study. It is not clear why the use of some strains significantly affected the protein or fat content of the cheeses, but potential measurement errors were discounted. However, it is unlikely that this differences significantly affected proteolysis.

NSLAB did not affect starter growth, which was similar to the control. A decrease in counts of lactococci was observed in both experiments starting from 14 to 30 days: this might have been due to lysis or loss of cultivability of the starter. In our study, the control cheeses without added cultures were virtually free of lactobacilli for the first weeks and the lactobacilli counts in the experimental cheeses were almost 3 log cycles higher than the control, even toward the end of the ripening. The high level of NSLAB counts in the cheeses with adjuncts at the beginning of ripening, the increase in counts to $>10^7$ cfu.g^{-1} within the first month (when NSLAB counts in the control were still lower than 10^5 cfu.g^{-1}), and the high proportion of *L. plantarum* colonies in cheese with adjuncts compared with the control strongly support the hypothesis that the adjuncts grew well in cheese, although molecular assays may be needed to obtain conclusive evidence.

Similar starter and NSLAB counts have been found in both semi-hard Argentinean cheese manufactured using *L. plantarum* adjuncts (Hynes et al. 2003) in Cremoso and cheddar cheese (Milesi et al. 2008a, b) and in cheddar cheese (Lynch et al. 1996; Lynch et al. 1999). The dynamics of the lactobacilli throughout the ripening in the experimental cheeses examined were also similar to those reported in commercial Irish cheddar cheeses made from pasteurised milk after ripening at 8 °C (Fitzsimons et al. 2001).

The adjuncts had no significant effect on primary proteolysis, but several strains significantly affected secondary proteolysis, as measured by the OPA and TNBS

assays, with values higher or lower than the control. Other authors also reported that *Lactobacillus* adjuncts do not contribute to hydrolysis of casein during cheese ripening (Di Cagno et al. 2006; Poveda et al. 2003) and our results are in agreement with those obtained in other studies (Lynch et al. 1996; Lynch et al. 1999; Milesi et al. 2008b) in which no differences in primary proteolysis were found by urea-PAGE of cheddar cheeses made with and without adjuncts.

Some of the adjuncts (P1.5, 38AA and DK022) caused a significant increase of pH 4.6-SN, free amino groups and in a higher concentrations of FAA in miniature cheddar-type cheeses, while for others secondary proteolysis was lower than the control at the end of ripening (90 days). However, even the two strains with the lowest proteolytic activity (C17 and MTD2S) in the miniature cheese experiment, resulted in levels of FAA significantly higher than the control in the pilot-plant experiment. Notably, the three strains with the highest proteolytic activity (38AA, DK022 and P1.5) were originally isolated from non-dairy fermentations. Higher secondary proteolysis in cheddar cheese containing adjunct lactobacilli has been noted by other authors (Lynch et al. 1996; Lynch et al. 1999; Ong et al. 2006; Ong et al. 2007) who attributed this increase to higher peptidase activity in cheeses made with adjuncts.

Ripening time and adjuncts significantly affected the peptide profiles of pH 4.6-soluble extracts for the miniature cheddar-type cheeses, and, to a lesser extent, for the pilot-plant cheeses. At 2 and 3 months, almost all cheeses with adjuncts had an RP-HPLC profile which was different from that of the control cheese, as judged by PLS-DA. In the pilot-plant experiment variability due to cheese-making and sampling obscured in part the effect of adjuncts which, however, was highly significant. The largest differences among adjuncts and control in the miniature cheddar cheese experiment were found in areas of the chromatogram that correspond to small water-soluble peptides (Hynes et al. 2003). They are produced by the action of the lactococcal CEP, or perhaps endopeptidases, from products of chymosin or plasmin action. NSLAB supplement the peptidolytic activity of the starter, especially in the production of amino acids (Upadhyay et al. 2004). Interactions with the starter, differences in peptidase patterns and in autolysis and differences in cheese moisture are all factors that may affect secondary proteolysis in cheeses made with adjuncts (Di Cagno et al. 2006; Hynes et al. 2003; Hynes et al. 2001; Lynch et al. 1999; Poveda et al. 2003). In addition to differences related to treatment and ripening time, some variability was observed among replicates for a single treatment. This is not uncommon and can be easily noticed when replicates are shown in PCA score plots for RP-HPLC profiles (Poveda et al. 2003; Parente et al. 2012). Variability in profiles is probably not due to technical factors related to the preparation of extracts or to the chromatographic separation, since technical replicates of the same extract showed very low coefficients of variations (data not shown). All the trials were performed over a very short time using milk from the same dairy, but milk variability may also be a contributing factor, although this was not explicitly assessed in this experiment. Nonlinear and/or non-monotonic trends in the evolutions over time of chromatographic profiles, difficulties in accurately controlling some important parameters (especially moisture and salt) in the miniature cheese model, lack of homogeneity in cheese blocks in the pilot-plant experiment may all contribute to the difficulties of identifying areas of RP-HPLC chromatograms which were significantly affected by adjunct addition. A mixed linear model, in which cheese and time were considered as fixed factors (with time nested within the adjunct effect) and

replicate cheese making trials was considered as a random effect, was also tested on the pilot-plant cheese data (results not shown). Although this model did improve the ability to detect significant differences due to ripening time and, sometimes, to adjuncts, it required the estimation of a very large number of parameters compared with the simple variance decomposition model whose results are shown in Fig. 3. In addition, the occurrence of heteroscedasticity in the distribution of residuals suggested the possibility of violation of some of the assumptions underlying the model. PLS-DA and, to a lesser extent, a completely random linear model, even if riddled by limitations (inability to cope with nonlinear patterns and need to use a relatively large number of components for PLS-DA, potential occurrence of type II errors in the linear model) provided a more parsimonious solution and still confirmed that differences due to ripening time and to adjunct occurred in some regions of the chromatogram.

Although differences in proteolysis measured by the RP-HPLC pattern of pH 4.6-soluble extracts of cheddar cheese manufactured in pilot-plant were very small, the 180-day-old cheeses produced with strains P1.5 (from olive fermentation) and C17 (from cheese) obtained significantly higher preference scores compared with the control, while the cheese produced with strain MTD2S (from sourdough) was not significantly different. Adjuncts made of mesophilic lactobacilli, including *L. plantarum* have been shown to significantly improve sensory properties of cheese (Briggiler-Marcò et al. 2007; Lynch et al. 1996; Lynch et al. 1999; Swearingen et al. 2001; Williams et al. 2006).

5 Conclusions

Our results suggest that even adjunct strains isolated from very different environments (wine, sourdough or starchy products, olives) are able to survive and proliferate in the cheese, possibly because of the ability to use residual sugars and amino acids and their high salt tolerance, which help them to adapt to the environmental conditions of the cheese during ripening.

The ability of some strains to increase secondary proteolysis and to affect RP-HPLC profiles of pH 4.6-soluble extracts may significantly contribute to cheese quality enhancement. *L. plantarum* subsp. *plantarum* MTD2S from sourdough, C17 from cheese, and P1.5 from olives were selected for further study in cheddar cheese manufacture and strain P1.5 resulted in significantly improved sensory preferences score. This confirms that strains from non-dairy foods can be a potential source of novel starter and adjunct culture for cheese making.

Acknowledgements This work was partly funded by Ministero dell'Istruzione, dell'Università e della Ricerca, Rome, Italy, PRIN n.20088SZB9B and by a grant from the Food Industrial Research Measure administered by the Irish Department of Agriculture, Fisheries and Food. The support of Regione Basilicata and ENI, which funded Dr. Felicia Ciocia Ph.D. scholarship, is acknowledged.

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