Contribution of autochthonous non-starter lactobacilli to proteolysis in Caciocavallo Pugliese cheese

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Abstract

Caciocavallo Pugliese cheese was manufactured according to a traditional protocol and with added autochthonous *Lactobacillus paracasei* subsp. *paracasei* and *L. parabuchneri* strains, isolated from a well-flavoured Caciocavallo Pugliese cheese, to evaluate their contribution to the cheese biochemical characteristics. Using a "two step REP-PCR" protocol, *L. paracasei* subsp. *paracasei* strains were shown to sustain high viability during ripening, while the *L. parabuchneri* strains were not recovered. The inoculated cheese showed higher levels of free amino acids, as well as differences in the profiles of individual free amino acids in comparison with the control cheese. The addition of autochthonous *Lactobacillus* strains with interesting technological properties in Caciocavallo Pugliese cheese manufacturing could make it feasible to improve cheese processing, while still maintaining the sensory characteristics of this typical pasta-filata cheese.
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1. Introduction

Caciocavallo, one of the most typical pasta-filata cheeses obtained from natural microflora, is manufactured in three different areas—the Balkans, Russia, and Italy. Various types of Caciocavallo are produced in the south of Italy under names such as Caciocavallo Silano, Molisano, Pugliese, and Corleonese. Caciocavallo Silano, made from cows' milk, is the most important Italian Caciocavallo variety with protected denomination of origin (DOP). Most Caciocavallo cheeses produced in Italy use a natural whey starter, corresponding to a backslopping from a previously successful cheese batch.

Natural whey starters are preferred as they contribute to the typical flavour and aroma of the final cheese, qualities which are attributed to the complex microflora, and to the resistance to phage attack due to the multi-strain culture.

For an exhaustive review on this subject, refer to Parente and Cogan (2004).

In recent years, autochthonous cultures have also been used in Tetilla cheese (Menéndez, Godìnez, Hermida, Centeno, & Rodríguez-Otero, 2004), Proosdij-type cheese (Ayad, Verheul, Bruinenberg, Wouters, & Smit, 2003), Reggianito Argentino cheese (Candioti et al., 2002), Turkish White Pickled Cheese (Karakus & Alperden, 1995), Cheddar cheese (Lynch, McSweeney, Fox, Cogan, & Drinan, 1996) and New Zealand Cheddar cheese (Crow, Curry, & Hayes, 2001) in order to study their influence on the ripening process and improve their sensory characteristics.

In a previous paper (Gobbetti et al., 2002), mesophilic lactobacilli such as *Lactobacillus paracasei* subsp. *paracasei* and *L. parabuchneri* were found to make up much of the lactic acid microflora in Caciocavallo Pugliese, after 42 and 60 days of ripening, respectively. Non-starter lactic acid bacteria (NSLAB) have been shown to contribute to flavour development in some varieties of cheeses and could

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therefore be considered as a desirable contaminant of either the milk supply or the subsequent cheese (El Soda, Madkor, & Tong, 2000).

To the best of our knowledge, no studies have been conducted on the contribution of autochthonous non-starter lactobacilli to the biochemical properties of different types of Caciocavallo cheese.

In the last few years, various molecular typing methods such as restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis, ribotyping, amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD) and other PCR-derived techniques have been used to distinguish the strains present in complex microflora isolated from many sources. Unlike RAPD protocols, in which the patterns are obtained by using a short non-specific primer, repetitive element sequence-based PCR (REP-PCR) protocols are based on the amplification of short repetitive sequence elements, dispersed throughout the chromosome of diverse Gram-negative species (Versalovic, Koeuth, & Lupski, 1991).

In this work, several bacterial strains previously isolated from Caciocavallo Pugliese cheese (Gobbetti et al., 2002) were characterized for their peptidase activities. Four of them, belonging to *L. paracasei* subsp. *paracasei* and *L. parabuchneri* species, were added to natural whey and their growth kinetics in cheese were followed by using a new typing protocol named "two step REP-PCR". The contribution of autochthonous NSLAB added to the microflora present in natural whey to proteolysis of cheese was evaluated in order to standardize Caciocavallo Pugliese cheese processing, safeguarding the peculiar traits of this typical southern Italian dairy product.

2. Materials and methods

2.1. Bacterial strains and peptidase activities

The lactic acid bacterial strains belonging to *L. delbrueckii* subsp. *bulgaricus* (strain B15Z), *L. helveticus* (B26W), *L. gasseri* (B24W), *L. paracasei* subsp. *paracasei* (B44f3t and B25f3), *L. parabuchneri* (B10f5, B23f3, B14f3, B48f3, B51f5, B9f5) and *Pediococcus pentosaceus* (C9f5), used in this study, had been isolated previously from Caciocavallo Pugliese cheese (Gobbetti et al., 2002). All strains were routinely cultured in MRS broth (Difco Laboratories, Detroit, MI, USA) at 37 °C, frozen at −80 °C and stored in the Institute of Sciences of Food Production (ISPA, Bari, Italy) bacterial collection.

Peptidase activity was determined on cells from 12-h-old cultures collected by centrifugation (8200 × g, 5 min), washed twice with 50 mM phosphate buffer pH 7.0 and suspended in the same buffer to give a ten-fold concentrated cellular suspension. Synthetic substrates (Ala-pNA, Leu-pNA, Lys-pNA and Pro-pNA; Sigma Chemical Co., St. Louis, MO, USA) were dissolved (20 mM) in absolute ethanol and used for in vitro assaying the aminopeptidase and iminopeptidase activities of non-starter lactic acid

bacteria (Gobbetti, Corsetti, Smacchi, De Angelis, & Rossi, 1997). Twenty microlitres of each substrate were incubated with 100 μL of cellular suspension, and 80 μL of phosphate buffer were added to the reaction mixture. Samples were incubated at 37 °C for at least 30 min, and the reaction was stopped by adding 600 μL acetic acid (10% v/v). Cells were removed by centrifugation (8200 × g, 5 min) and absorbance of supernatants was determined at $\lambda = 410$ nm (Ultrospec 3000, Amersham BioSciences, Uppsala, Sweden). A unit of enzymatic activity was defined as the amount of enzyme that produced 1 μmol of para-nitroaniline per min at 37 °C and pH 7.0.

2.2. Caciocavallo Pugliese cheese manufacture

Laboratory-scale Caciocavallo cheese-making trials (control trial C and inoculated trial I) were performed according to standard procedures as follows: goatling rennet (40.0 mg kg^{-1}) was added to 300 L of whole pasteurized bovine milk inoculated with 3% (v/v) natural whey culture (pH 3.80). In control trial C, the cheese was processed traditionally, with milk and curd fermentation being carried out by the microflora present in non-selected whey culture, whereas in trial I, for inoculated cheese, known amounts of four non-starter lactobacilli were added as adjuncts to the natural whey inoculum. After about 30 min at 37–38 °C, the coagulum was cut coarsely and heated under whey at 45 °C for 2 h and then held at room temperature.

When the curd reached a pH value of about 5.30, it was manually stretched in hot water (70–80 °C) producing cheeses weighing about 2 kg. Cheeses were dipped in brine (27–30% NaCl) for 18 h and then ripened at 10–12 °C and 75–80% relative humidity for 2 months.

For trial I, two strains of *L. paracasei* subsp. *paracasei* (B44f3t and B25f3) and two strains of *L. parabuchneri* (B51f5 and B23f3) were subsequently grown for 24 h at 37 °C in 10, 100, 1000 and 10 L of MRS broth. The final 10-L growths were centrifuged (2460 × g for 20 min), merged in 1 L of 0.05 M Tris–HCl (pH 7.5) containing 0.1 M CaCl$_2$ buffer and then used as starter adjuncts in otherwise traditional cheese-making, thus obtaining trial I. Each strain was added to obtain a final concentration of about log 7.3 cfu mL^{-1}.

Cheeses from two batches of each trial, C and I, were manufactured at different times using completely independent batches of milk on the same day and were analysed in this study.

2.3. Cheese compositional analysis

Samples of cheeses were analysed for protein (macro-Kjeldahl; IDF, 1964), fat (Gerber method; IIRS, 1955), moisture (oven drying at 102 °C; IDF, 1982) and salt (Fox, 1963) content. All determinations were carried out on three different sections of each cheese and values were averaged.

The pH of the cheeses was determined by direct insertion of a pH probe into the cheeses (pH meter 507 Crison, Crison Instruments S.p.A., Carpi, Modena, Italy).

2.4. Microbiological analyses of cheeses

Samples from both C and I cheeses were collected during the process of pilot-scale cheese-making trials at 0, 30 and 60 days. Twenty-five grams of each cheese sample were dispersed in 2% sodium citrate solution, homogenized, decimally diluted in sterile 0.1% (w/v) peptone saline solution (0.9% NaCl) and plated on specific media for viable counts. The appropriate dilutions of cheese samples were plated in triplicate on different agar media: plate count for total mesophilic bacteria at 30 °C and Rogosa (RG; under anaerobic conditions, AnaeroGene, Oxoid S.p.A., Garbagnate, Milano, Italy) for presumptive lactobacilli at 30 and 42 °C for 48 h; Slanetz and Bartley (SB) for presumptive enterococci at 37 °C for 48 h; Baird Parker (BP) for presumptive staphylococci at 30 °C for 48 h; violet red bile lactose for total coliforms at 37 °C for 24 h; wort agar for yeasts and moulds at 25 °C for 4 d. The ability of growth at 30 °C in a medium containing mannitol (*L. paracasei* subsp. *paracasei*) and arabinose (*L. parabuchneri*) as the sole sugar source, was assayed in order to set up a selective medium for the strains with these fermentative capabilities. A modified Rogosa agar (Rogosa arabinose mannitol, RAM) contained the following substances (in g L^{-1}): tryptone 10.0, yeast extract 5.0, mannitol 10.0, arabinose 10.0, agar 20.0, potassium dihydrogen phosphate 6.0, ammonium citrate 2.0, sodium acetate anhydrous 17.0, magnesium sulphate 0.575, manganese sulphate 0.12, ferrous sulphate 0.034 and sorbitan mono-oleate 'Tween 80' 1.0 mL. All media were purchased from Oxoid S.p.A. (Garbagnate, Milan, Italy) or from Difco.

The viable cell count on RAM agar plates, where glucose is replaced by arabinose and mannitol, expressed the presumptive total heterofermentative lactobacilli population, both facultative and obligate, after 48 h of growth at 37 °C under anaerobic conditions.

2.5. Molecular typing and taxonomic identification of biotypes

To follow the adjunct strains during ripening of Caciocavallo Pugliese cheese, 30 colonies randomly isolated on RAM agar plates from samples C and I after 0, 30 and 60 days of ripening were characterized for their fingerprints, modifying a molecular protocol previously described (Baruzzi, Morea, Matarante, & Cocconcelli, 2000), using the REP 1R-I and REP 2-I primers containing the nucleotide inosine (I) as described by Versalovic et al. (1991), by applying the "two step REP-PCR" protocol described below. Bacterial cells were harvested by centrifugation at $3000 \times g$ for 5 min, and bacterial pellets were rinsed with sterile water. Genomic DNA was isolated by using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA) according to the protocol provided by the manufacturer. DNA quantity and quality were determined by electrophoresis with known amounts of lambda DNA, marker VI (Roche S.p.A., Milan, Italy), as a standard.

Amplification consisted of an initial denaturation at 94 °C for 1 min, followed by 10 cycles of 94 °C for 30 s, 35 °C for 30 s, and 72 °C for 1 min and 30 additional cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; the annealing and extension of the last 30 cycles were elongated for 1 and 5 s per cycle, respectively. A final extension at 72 °C for 5 min was added. PCR amplification was carried out in a Thermal Cycler 9700 (Perkin-Elmer, Alameda, CA, USA). *Taq* polymerase, deoxynucleoside triphosphates and DNA molecular weight markers were purchased from Sigma Chemical Co. and gel-filtration purified oligonucleotides were obtained from Sigma-Genosys Ltd. (Cambs, UK). The amplified fragments, separated by electrophoresis on a 2% agarose gel, were sized using Quantity One, version 4.3.1 software (Bio-Rad Laboratories s.r.l., Milan, Italy).

The cheese isolates were amplified together with the added strains in order to compare their electrophoretic patterns; control reaction mixtures lacking the DNA template were also included in each experiment. When different isolates, from each sample, gave the same electrophoretic pattern as the adjunct strains, they were grouped and only one isolate from each group was identified by partial amplification and sequencing of 16S rRNA genes for bacteria (Klijn, Weerkamp, & de Vos, 1995) in order to assess their taxonomic positions. DNA sequences were obtained using an ABI PRISM big dye terminator cycle sequencing kit (PE Applied Biosystems, Monza, Italy) and both the forward and reverse primers for 16S rDNA were used. The reaction products were analysed on an Applied Biosystems 310 automated DNA sequencer (PE Applied Biosystems). Taxonomic strain identification was performed by comparing the obtained sequences with those present in a Basic BLAST search (Altschul et al., 1997).

2.6. Enzymatic activities in water-soluble cheese extracts

Sterile, dialysed water-soluble extracts of the cheeses, prepared by modifying the method of Kuchroo and Fox (1982), were assayed for enzyme activity. The modifications concerned the dialysis (dialysis tubing, cut-off 12 kDa; Sigma Chemical Co.) of the extracts for 24 h at 4 °C against 0.05 M phosphate buffer, pH 7.0, to eliminate interference from salt and peptides in samples, and sterile filtration (0.22 μm pore size, Syrfil Filter, Nucleopore, Costar Co., Cambridge, UK) to avoid interference due to cellular activity (Gobbetti et al., 1997).

Aminopeptidase and proline iminopeptidase activities were determined as described by Gobbetti et al. (1997), using Leu-*p*NA, Glu-*p*NA and Pro-*p*NA as substrates. A unit of enzymatic activity was defined as above (see

Section 2.1). The pH and temperature used in this and subsequent analyses were selected as optimal for enzyme activities in Caciocavallo Pugliese cheese. Leu- and Glu-pNA were used as substrates to partially differentiate the activity of aminopeptidase type N (PepN) and A (PepA), respectively (Christensen, Dudley, Pederson, & Steele, 1999).

Dipeptidase and tripeptidase activities were measured by the Cd-ninhydrin method (Folkertsma & Fox, 1992) using Leu–Leu and Leu–Leu–Leu as substrates, respectively. A unit of enzymatic activity was defined as the amount of enzyme that produced an increase in absorbance at 505 nm of 0.01 min^{-1}. Endopeptidase activity was determined using Z-Gly-Pro-NH-trifluoromethylcoumarin (Sigma Chemical Co.), measuring the fluorescence at excitation and emission wavelengths of 400 and 505 nm, respectively. A unit of enzymatic activity was the amount of enzyme that produced 1 μmol of amido-trifluoromethyl-coumarin per min.

Proteinase activity was measured by the method of Twinning (1984), using fluorescence isothiocyanate-labelled casein as substrate, with incubation at 37 °C for 12 h. A unit of proteinase activity was defined as the amount of enzyme that produced an increase in fluorescence of 0.01 min^{-1}.

2.7. Assessment of proteolysis

Water-soluble extracts of the cheeses were prepared according to the method of Kuchroo and Fox (1982); the pH of the extracts was adjusted to 4.6 with 0.1 M HCl. The pH 4.6-soluble nitrogen fractions of the cheeses were analysed by urea polyacrylamide gel electrophoresis (Urea-PAGE) using a Protean II vertical slab gel unit (Bio-Rad Laboratories Ltd., Watford, UK) and a stacking gel system, as described by Andrews (1983). The gel was stained directly with Coomassie brilliant blue G250 and destained in 10% glacial acetic acid and 40% methanol.

The peptide profile of the pH 4.6 soluble extracts was determined by using a reverse phase—fast protein liquid chromatography (RP-FPLC) system (mod. Akta FPLC, Amersham Biosciences, Uppsala, Sweden) equipped with a UV detector operating at 214 nm. Samples were diluted 1:5, and trifluoroacetic acid (TFA) was added to give a final concentration of 0.05%. After centrifugation (12,800 × g, 10 min), supernatants were filtered (0.22 μm) and injected into the column, using a 2 mL loop. Elution was performed at a flow rate of 1 mL min^{-1} with a gradient (5–100%) of acetonitrile in 0.05% TFA. The concentration of acetonitrile was linearly increased from 5% to 46% between 16 and 62 min and from 46% to 100% from 62 to 72 min (Rizzello et al., 2005).

Total and individual free amino acid (FAA) concentrations in the water-soluble extracts of cheeses were determined by a Biochrom 30 series Amino Acid Analyser (Biochrom Ltd., Cambridge Science Park, UK) using a sodium cation-exchange column (20 cm length × 0.46 cm

internal diameter). Cysteic acid, methionine sulphoxide, methionine sulphone, tryptophan and ornithine at known concentrations were added to a standard amino acid mixture (Sigma Chemical Co.) and used to evaluate the concentration of individual amino acids in the samples. Before analysis, proteins and peptides from the water-soluble extracts were precipitated by the addition of 5% (v/v) cold solid sulphosalycilic acid (SSA), held at 4 °C for 1 h, and centrifuged at 15,000 × g for 15 min. The supernatants were filtered (0.22 μm) and diluted (1:5) with 0.2 M sodium citrate loading buffer, pH 2.2. Amino acids were post-column derivatized with ninhydrin reagent and detected by absorbance at 440 nm (proline) or 570 nm (all the other amino acids).

2.8. Statistical analyses

Statistical evaluations were carried out by the Duncan method to determine statistical differences between the treatments. Data were then considered significantly different if $P < 0.05$.

The reported values for enzymatic activities of water-soluble extracts and for free amino acid levels in Caciocavallo Pugliese cheeses are the average of three replicates of two batches of cheese production.

3. Results and discussion

3.1. Peptidase activities of lactic acid bacteria from Caciocavallo cheese

The peptidase activity of some lactic acid bacteria previously isolated from Caciocavallo Pugliese cheese (Gobbetti et al., 2002) is shown in Table 1.

Showing peptidase activities of strains not included in the following cheese experiments was judged necessary in order to provide for tangible evidence for the choice of NSLAB. Some strains of *L. paracasei* subsp. *paracasei* and *L. parabuchneri* exhibited high proline iminopeptidase activity. Strains capable of hydrolysing peptides containing proline residues are useful, since hydrophobic peptides containing at least one proline residue were found to be responsible for a bitter taste in cheese (Habibi-Najafi & Lee, 1996). It is established that, in ripened cheeses, enzymes from cheese-related NSLAB can contribute to flavour development through the action of their intracellular enzyme systems (El Soda et al., 2000) even though *Lactococcus lactis* species are considered the most studied lactic acid bacteria able to contribute to cheese flavour.

Considering the dominant microflora in Caciocavallo Pugliese cheese ripening, two *L. paracasei* subsp. *paracasei* and two *L. parabuchneri* strains, with good peptidase activities, were chosen to be used as adjuncts in a traditional Caciocavallo Pugliese production trial. Among the available *L. parabuchneri* strains indicated in Table 1, only B23f3 and B51f5 were isolated previously from cheese samples at 42 and 60 days (data not shown) and therefore

Table 1
Peptidase activity (U mg^{-1}) of lactic acid bacteria from Caciocavallo Pugliese cheese[a]

Strains	Synthetic peptides			
	Ala-*p*-NA	Pro-*p*-NA	Leu-*p*-NA	Lys-*p*-NA
L. delbrueckii B15Z	12.5[ef]±0.6	6.7[f]±0.3	17.6[g]±0.3	15.4[i]±0.2
L. gasseri B24W	12.3[ef]±0.7	7.1[f]±0.5	15.7[h]±0.7	15.3[i]±0.3
L. helveticus B26W	15.7[de]±0.2	7.9[e]±0.5	20.5[f]±0.2	22.3[h]±0.6
L. parabuchneri B14f3	23.1[bc]±0.5	9.3[cd]±0.1	39.5[c]±0.5	42.1[b]±0.5
L. parabuchneri B23f3	25.4[b]±0.2	8.9[cd]±0.1	38.3[c]±0.1	33.4[e]±0.7
L. parabuchneri B48f3	18.5[d]±0.5	9.6[bc]±0.2	18.6[g]±0.2	28.7[g]±0.5
L. parabuchneri B9f5	18.7[d]±0.3	7.1[f]±0.4	45.4[a]±0.8	45.3[a]±0.3
L. parabuchneri B10f5	20.8[bd]±0.3	9.7[b]±0.3	24.3[e]±0.6	31.5[f]±0.5
L. parabuchneri B51f5	32.9[a]±0.5	10.1[ab]±0.5	39.3[c]±0.2	42.5[b]±0.8
L. paracasei subsp. *paracasei* B25f3	18.7[d]±0.4	8.7[d]±0.1	41.2[b]±0.7	36.5[d]±0.2
L. paracasei subsp. *paracasei* B44f3t	20.4[cd]±0.8	10.8[a]±0.2	35.7[d]±0.8	38.6[c]±0.4
Pediococcus pentosaceus C9f5	8.7[f]±0.5	3.5[g]±0.2	12.5[i]±0.1	10.1[l]±0.4

[a]Values are the average of three replicates (±standard deviation) of two cell growths. In the same column, values without a common superscript letter were significantly different ($P < 0.05$).

they could survive during Caciocavallo processing and ripening.

3.2. Compositional analysis

No significant differences were found between C and I cheeses. Average values for protein and fat content were ca. 23 and 24.5%, respectively. These values are in agreement with those reported by Gobbetti et al. (2002). Percentages of moisture and salt content were ca. 42% and 2.5%, respectively. The pH of the cheeses on day 0 were 5.02 for trial C and 5.27 for trial I. After 60 days of ripening both trial cheeses reached pH values close to 5.32.

3.3. Isolation and identification of strains from complex dairy natural population

In order to monitor the persistence of one or more strains added to complex microflora such as those present in dairy products, it is essential to have an efficient tool for their selective enumeration. Since NSLAB usually increase from a low number in fresh curd to reach levels of up to 10^7–10^8 cfu g^{-1} in cheese during ripening, the present study initially focused on evaluating a method aimed at selectively distinguishing the lactobacilli added as starter adjuncts from the remaining isolates. The method used was based on assaying the ability of strains to ferment specific sugars and develop strain-specific REP-PCR fingerprints.

The use of a selective medium (RAM), described above, made it feasible to isolate heterofermentative lactobacilli including presumptive *L. paracasei* subsp. *paracasei* and *L. parabuchneri* strains. Well-isolated colonies were then grouped by using the "two step REP-PCR" protocol, which proved to be a successful means for the selective identification of adjunct strains from total complex microflora. A similar approach using specific sugars and REP-PCR was previously used to monitor adjunct
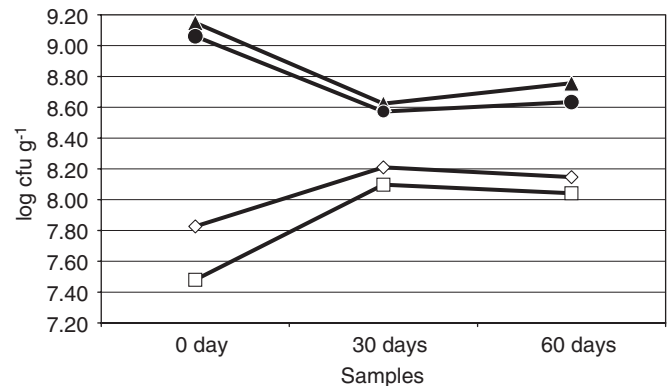

Fig. 1. Growth kinetics of mesophilic lactobacilli in inoculated trial I; closed symbols and control (trial C; open symbols) Caciocavallo Pugliese cheese during 60 days of ripening. Bacterial counts on Rogosa (RG: ◇; ▲) and Rogosa Arabinose Mannitol (RAM: □; ●) agar plates. The reported values represent the average of viable cell counts from three replicates of two batches. The standard deviations ranged from ±0.36 (RAM-I) to ±0.58 (RG-C).

L. paracasei and *L. curvatus* strains in Cheddar cheese (Dasen, Berthier, Grappin, Williams, & Banks, 2003).

3.4. Incorporation of autochthonous Lactobacillus strains into Caciocavallo cheese

The microbial characteristics of samples C and I from the two batches did not significantly differ ($P < 0.05$). In cheese C samples, the counts for both mesophilic and thermophilic lactobacilli populations were similar, remaining stable during ripening at about 7.2 log cfu g^{-1}. The thermophilic lactobacilli of cheese I decreased in 60 d from 7.1 to 6.1 log cfu g^{-1}. Since the counts of mesophilic lactobacilli were always higher than those of thermophiles, as described below, the evaluation of growth kinetics of added strains and the comparison of natural strains present

in cheese C were carried out on mesophilic lactobacilli populations.

Fig. 1 shows the viable counts of the presumptive mesophilic lactobacilli from cheeses I and C, collected during the ripening of Caciocavallo Pugliese.

In cheese C, total mesophilic lactobacilli dominated in day 0 sample in comparison with heterofermentative lactobacilli enumerated on RAM, but during ripening the differences between these populations decreased and after 60 d they cannot be considered significantly different, reaching values close to $8.0 \log \mathrm{cfu\,g}^{-1}$.

Cheese made with *L. paracasei* subsp. *paracasei* and *L. parabuchneri* adjuncts always contained higher levels of presumptive mesophilic lactobacilli in comparison with cheese C. In cheese I, counts of total and heterofermentative lactobacilli were always very similar. At day 0, total mesophilic lactobacilli were more than $9.0 \log \mathrm{cfu\,g}^{-1}$ decreasing to ca. $8.6 \log \mathrm{cfu\,g}^{-1}$ at 30 d. The high count of mesophilic lactobacilli at day 0 demonstrated the growth of added strains during milk and curd fermentations and their survival at the stretching and brining steps. The permanence of a lactobacilli population close to $8.6 \log \mathrm{cfu\,g}^{-1}$ for the first 30 d of ripening could indicate an equilibrium between viable and unviable lactobacilli in the curd. Furthermore, after 60 d of ripening, the ratio between heterofermentative lactobacilli, enumerated on RAM, and total lactobacilli, enumerated on Rogosa, showed similar values (0.75 and 0.79) in both trials.

The results of viable counts of mesophilic lactobacilli from cheeses I and C were strengthened by REP-PCR analyses performed on 360 isolates. Fig. 2 shows the distribution of adjunct strains and naturally occurring non-starter strains during the processing and ripening of inoculated cheese: the electrophoretic patterns corresponding to the adjunct lactobacilli were all detected in cheese I

at the beginning of ripening, whereas during ripening some strains seemed to disappear. In the cheese sample collected at day 0, *L. paracasei* B44f3t was the dominant strain followed by the two strains of *L. parabuchneri* and *L. paracasei* B25f3. After 30 days, only three of the adjunct lactobacilli were recovered: *L. parabuchneri* B51f5 and *L. paracasei* subsp. *paracasei* B44f3t, present in similar amounts, followed by *L. parabuchneri* B10f5. Only the two strains of *L. paracasei* subsp. *paracasei* (B44f3t and B25f3) were recovered at the end of ripening. Naturally occurring non-starter mesophilic lactobacilli from natural whey culture were recovered from inoculated Caciocavallo Pugliese cheese during ripening in higher amounts than adjunct strains, indicating that their growth was not hindered by adjunct strain growth. Since the goal of this work was to evaluate the contribution to proteolysis of adjunct NSLAB in Caciocavallo Pugliese cheese, the survival to stretching and brining steps was a prerequisite. For this reason, molecular tools were applied only to recognize adjunct NSLAB strains, while the composition of natural lactobacilli populations in trial C and I were not analysed.

It could be surmised that *L. paracasei* subsp. *paracasei* B25f3, found in the 60 d cheese sample, was also present in the 30 d cheese sample, but it was not recovered under our experimental conditions, because the analysis for the presence of a strain in a sample is influenced by the random pick-up of colonies from plates.

Sequence analysis, carried out for each cheese sample on one biotype exhibiting the same electrophoretic pattern as the added strains, always confirmed that it belonged to either *L. paracasei* subsp. *paracasei* or *L. parabuchneri* species.

No REP-PCR electrophoretic patterns from isolates collected during the processing and ripening of cheese C were similar to those of adjunct strains.

To the best of our knowledge, *Lactobacillus* strains had not been added previously to Caciocavallo Pugliese cheese, although lactobacilli had been added before to other cheeses such as Cheddar or Pecorino and had been subsequently found to remain at high levels throughout maturation (Broome, Krause, & Hickey, 1990; Crow et al., 2001; Lynch et al., 1996; McSweeney et al., 1994). In our study, the use of a medium with specific sugars and the "two-step REP-PCR" protocol based on REP primers, were used to monitor adjuncts strains in cheese I and to ascertain their absence in cheese C.

Total bacterial count did not differ from that enumerated on selective medium. Staphylococci decreased from 6.3 to $5.5 \log \mathrm{cfu\,g}^{-1}$ during ripening in both trial C and I. Presumptive enterococci remained stable at ca. $6.2 \log \mathrm{cfu\,g}^{-1}$ for both cheese trials. Total coliforms decreased slightly from ca. 4.3 to $2.9 \log \mathrm{cfu\,g}^{-1}$ in both trials after 60 days of ripening. The moulds and yeasts were never higher than $2.8 \log \mathrm{cfu\,g}^{-1}$ in both cheeses. These microbial populations did not seem to be influenced by adjunct lactobacilli.
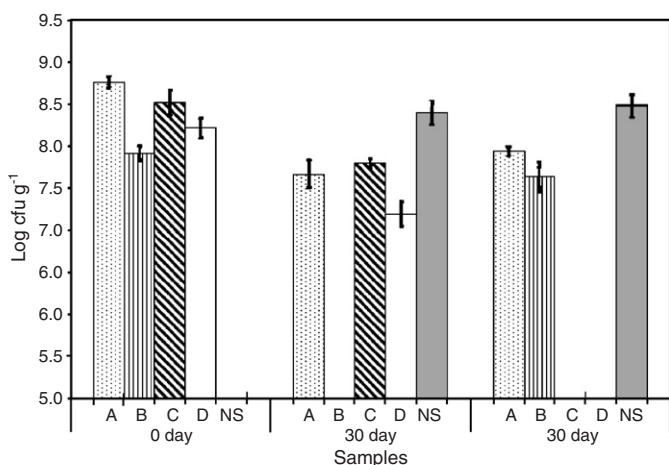

Fig. 2. Relative amounts of adjunct strains and natural non-starter mesophilic lactobacilli during ripening of inoculated Caciocavallo Pugliese cheese. (A) *L. paracasei* subsp. *paracasei* B44f3t; (B) *L. paracasei* subsp. *paracasei* B25f3; (C) *L. parabuchneri* B51f5; (D) *L. parabuchneri* B10f5; NS) naturally occurring non-starter (NS) mesophilic lactobacilli.

Table 2
Enzymatic activities of water-soluble extracts of Caciocavallo Pugliese cheeses (control C and inoculated I) after 60 days of ripening[a]

Enzyme	C	I
Aminopeptidase type N	$82.2^b \pm 0.7$	$165.1^a \pm 0.8$
Aminopeptidase type A	$15.7^b \pm 0.4$	$58.8^a \pm 0.4$
Proline iminopeptidase	$12.6^b \pm 0.2$	$31.5^a \pm 0.2$
Dipeptidase	$92.1^b \pm 0.8$	$177.4^a \pm 0.5$
Tripeptidase	$66.3^b \pm 0.3$	$155.8^a \pm 0.2$
Endopeptidase	$14.3^b \pm 0.5$	$42.7^a \pm 0.5$
Proteinase	$11.3^a \pm 0.2$	$12.5^a \pm 0.6$

[a]Values represent the average ($\pm$ standard deviation) of enzymatic activity ($U\,g^{-1}$) of two batches of water soluble extracts in triplicate. Values in the same row without a common superscript letter were significantly different ($P < 0.05$).

3.5. Enzyme activity in cheese extracts

Activities of proteolytic enzymes in the curd which are liberated after cell lysis or which are derived from the coagulant are shown in Table 2. It is evident that all the peptidase activities in cheese I were always higher (ca. 2.5 fold, on average) than in cheese C, probably due to the presence of adjunct autochthonous NSLAB strains.

Peptidase activities of NSLAB and their amino acid-catabolizing enzymes give a great contribution to the flavour of mature cheese (Crow et al., 2001; Gobbetti et al., 1999; Williams, Noble, Tammam, Lloyd, & Banks, 2002). However, as in trial I total lactobacilli population was always higher than added NSLAB, it cannot be unequivocally ascertained that the higher peptidase activities found in cheese I came only from added strains.

Proteinase activity was not significantly different in the two trials C and I, thus indicating that NSLAB adjunct strains did not influence primary proteolysis that is predominantly due to rennet and milk enzymes. This is in accordance with the fact that NSLAB possess very low proteinase activity (Fox, 1996).

3.6. Proteolysis

Fig. 3 shows the urea-PAGE electrophoresis of the pH 4.6 soluble nitrogen fraction of both trials C and I at 0, 15, 30, 40 and 60 days of ripening. Peptides contained in this fraction are mainly due to secondary proteolysis, in which the peptidase activity of adjunct NSLAB strains could play a considerable role. No qualitative differences could be observed between the samples, whilst the intensity of all bands diminished as maturation progressed. This indicates that medium to high MW peptides released upon primary proteolysis were further hydrolysed to shorter peptides and finally to amino acids. Some amino acids exceeding cellular requirements are extruded and most amino acids are liberated into the curd upon cell lysis, thus contributing to the flavour and aroma of mature cheese. Moreover, in all cases the bands are less intense in cheese I than in cheese
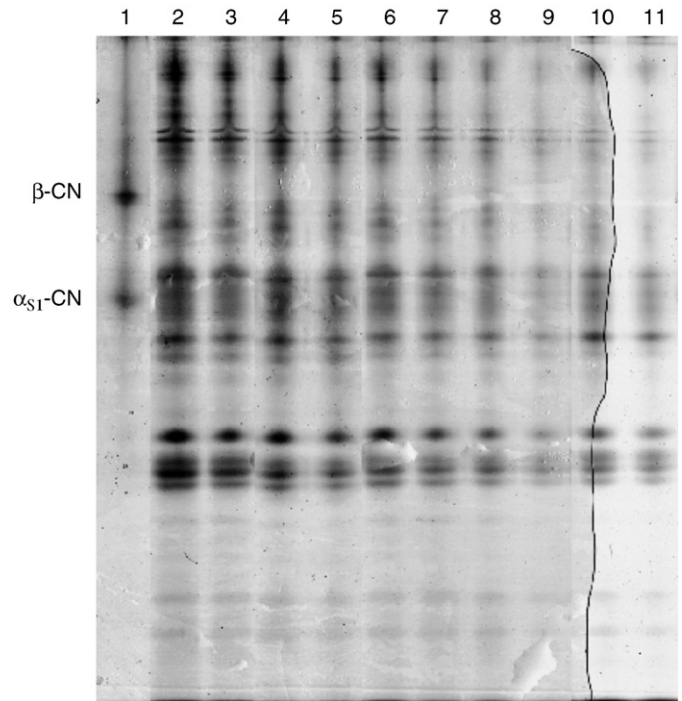

Fig. 3. Urea-PAGE electrophoregram of the pH 4.6 soluble nitrogen fraction of cheeses. Lane 1, bovine sodium caseinate; 2 and 3, cheeses C and I at day 0 of ripening; 4 and 5, cheeses C and I after 15 days of ripening; 6 and 7, cheeses C and I after 30 days of ripening; 8 and 9, cheeses C and I after 40 days of ripening; 10 and 11, cheeses C and I after 60 days of ripening.

C, suggesting a more intense and rapid secondary proteolysis when selected adjunct NSLAB strains had been used.

Complex profiles were obtained by RP-FPLC of the pH 4.6 soluble extracts from the cheeses, indicating a heterogeneous mixture of proteolytic products (Fig. 4). Profiles of cheeses I and C at the beginning of ripening (Fig. 4A and B, respectively) were almost identical. It is well known that curd undergoes gradual proteolysis as maturation proceeds and therefore, it is expected that any differences in the degree of proteolytic activity would not be observed in the very first phases of ripening. Profiles of cheeses I and C after 60 days of ripening (Fig. 4C and D, respectively) showed higher peaks than those for pH 4.6 soluble extracts of cheeses at day 0, particularly in the most hydrophilic zone of the gradient (from 0 to 10 min, peak 1). Differences were even more accentuated for cheese I (Fig. 4C), whose chromatogram presented very high peaks in the medium-high hydrophobic zone of the gradient (from 40 to 60 min, peaks 2 and 3). These results are in agreement with the concentration of the total FAAs (Table 3). In fact, FAA content was quite similar in both trials C and I analysed at the beginning of ripening, whereas after 60 days of ripening, cheese I was found to contain more FAA than cheese C ($20.9\,g\,kg^{-1}$ vs. $15.3\,g\,kg^{-1}$). During ripening, the concentration of most amino acids increased, and this was particularly evident for methionine, glycine and, above all, ornithine.
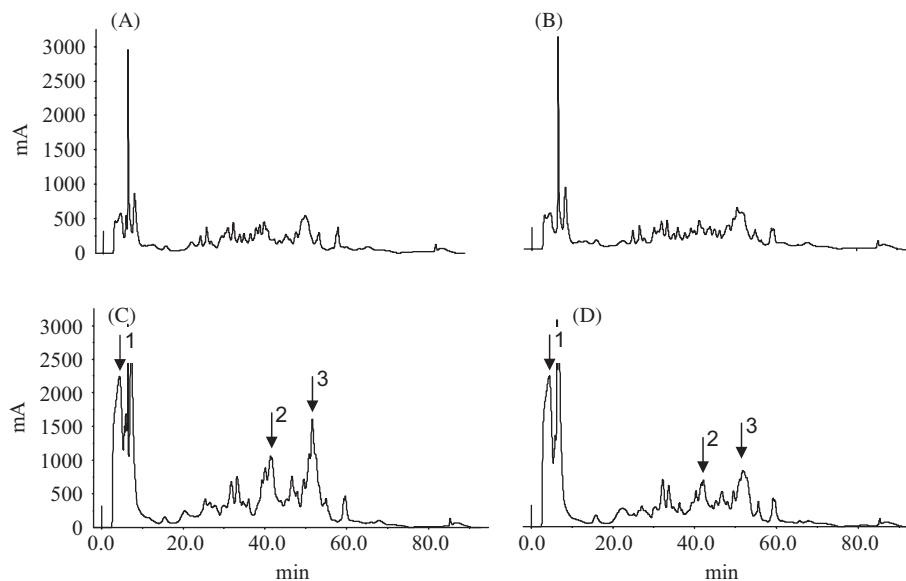
Fig. 4. RP-FPLC profiles of the pH 4.6 soluble fraction of cheese I after 0 (A) and 60 days of ripening (C), and of cheese C after 0 (B) and 60 days of ripening (D). Peaks indicate hydrophilic (1) and medium-high hydrophobic (2 and 3) zones of the acetonitrile gradient.

Table 3
Concentrations of free amino acids in control (C) and inoculated (I) cheeses after 0 and 60 days of ripening[a]

Amino acid (mg kg^{-1} of cheese)	C—0 day	I—0 day	C—60 days	I—60 days
Cysteic acid	10.6[b]±1.2	17.1[a]±0.9	43.3[a]±1.3	42.5[a]±1.2
Methionine sulphoxide-1	0	0	30.3[b]±0.7	50.9[a]±2.0
Methionine sulphoxide-2	9.9[b]±0.8	17.2[a]±1.2	42.4[b]±1.3	58.9[a]±1.8
Methionine sulphone	28.0[a]±1.1	33.1[a]±0.9	25.1[b]±1.3	53.4[a]±1.5
Aspartic acid	105.8[a]±1.6	95.3[b]±0.7	646.1[b]±1.9	750.6[a]±1.6
Threonine	70.6[a]±0.7	74.3[a]±1.2	449.3[b]±1.7	546.9[a]±1.5
Serine	243.4[a]±1.1	239.1[a]±1.3	612.4[b]±1.4	1390.1[a]±2.0
Glutamic acid	456.1[a]±1.5	460.2[a]±1.4	1969.5[b]±1.8	2344.3[a]±1.2
Glycine	54.6[a]±1.3	61.7[a]±0.7	410.7[b]±1.4	539.0[a]±1.6
Alanine	203.1[a]±1.2	199.2[a]±1.1	640.9[b]±1.9	676.7[a]±1.5
Cysteine	22.0[a]±0.7	20.1[a]±0.9	109.2[b]±1.3	116.9[a]±0.7
Valine	292.9[a]±1.5	302.3[a]±1.8	991.0[b]±1.4	1445.3[a]±1.3
Methionine	92.2[a]±1.6	86.3[a]±1.5	395.0[b]±1.7	423.0[a]±1.9
Isoleucine	130.8[a]±1.3	128.6[a]±1.8	992.0[b]±1.1	1404.8[a]±2.0
Leucine	511.4[a]±1.2	512.1[a]±1.7	1552.6[b]±1.8	1946.6[a]±1.9
Tyrosine	155.5[a]±0.8	166.8[a]±1.4	458.3[b]±1.8	680.1[a]±1.3
Phenylalanine	330.0[a]±1.3	328.9[a]±1.7	1286.3[b]±1.9	1768.1[a]±0.9
Histidine	125.5[a]±1.1	126.7[a]±1.8	267.4[b]±0.7	309.5[a]±1.2
Tryptophan	40.4[b]±1.6	51.3[a]±1.1	127.7[b]±1.1	194.9[a]±1.9
Ornithine	21.5[b]±1.0	37.8[a]±1.5	903.8[b]±1.1	1034.8[a]±1.9
Lysine	417.7[a]±1.3	411.1[a]±1.9	1414.9[b]±1.5	1966.6[a]±1.0
Free ammonium	22.7[b]±1.2	45.0[a]±1.8	202.0[b]±0.9	235.0[a]±1.6
Arginine	148.0[a]±0.9	147.1[a]±1.5	53.9[b]±1.9	134.3[a]±1.5
Proline	315.0[a]±1.4	312.9[a]±1.0	1632.3[b]±1.7	1941.0[a]±1.3
Total free amino acids	3807.8[a]±27.4	3875.3[a]±30.8	15256.7[b]±34.6	20954.4[a]±36.3

[a]Values represent the average (±standard deviation) of free amino acid content (mg kg^{-1}). For each amino acid, values without a common superscript letter were significantly different ($P < 0.05$) within cheese samples having the same ripening time.

After 60 days of ripening, cheese I greatly differed in concentrations of serine, glutamic acid, valine, isoleucine, leucine, phenylalanine and lysine which were much higher than in cheese C, whereas very little difference was found for cysteic acid, alanine, cysteine and methionine. Regarding all remaining individual amino acids, cheese I always

presented higher concentrations than cheese C. Overall, it can be stated that adjunct NSLAB strains significantly contributed to secondary proteolysis, due to the combined action of their intracellular peptidases. These results are in accordance with the findings of other authors, who have observed that NSLAB used as adjunct starters in Cheddar cheese manufacture are involved in the release of amino acids and peptides (Lane & Fox, 1996; McSweeney, Fox, Lucey, Jordan, & Cogan, 1993).

4. Conclusion

The results of this study demonstrate that the *L. paracasei* subsp. *paracasei* strains used are particularly suitable for Caciocavallo cheese applications, surviving the stretching and brining steps and remaining at high levels even after 2 months of ripening. All the peptidase activities assayed, as well as the content of total free amino acids, were consistently higher in cheese I than in cheese C. Further work needs to be carried out to understand how the higher amount of free amino acids influences the development of typical Caciocavallo Pugliese cheese flavours.

Acknowledgements

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