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Variability of lactic acid production, chemical and microbiological characteristics in 24-hour Parmigiano Reggiano cheese

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Abstract The achievement of a sufficient acidity level by starters at early stages of manufacture is essential for a good quality Parmigiano Reggiano (PR) cheese. In this work, the chemical parameters and microbial characteristics of 18 PR cheeses during the moulding phase were analysed to determine how the microbial growth takes place conditioning acidification performances. Based on the mean values of lactic acid content, two groups of cheeses were observed. High numbers of culturable thermophilic bacteria and in particular *Lactobacillus helveticus* able to metabolise galactose but also grow faster in the cheese appeared to be essential conditions to achieve adequate lactic acid content. On the other hand, low acidification and residual galactose, as observed in the second group, may be associated with a lower amount of metabolically active *L. helveticus* and higher relevance of *Streptococcus thermophilus* contents in the cheese. While culturable thermophilic bacteria were fundamental for higher lactic acid production, non-culturable cells did not seem to contribute to the acidification.

Keywords Parmigiano Reggiano unripened cheese · Natural whey starter · Acidification level · Microbial development · Chemical parameters · *Lactobacillus helveticus* · *Streptococcus thermophilus*

1 Introduction

Parmigiano Reggiano (PR) is a protected designation of origin (PDO) Italian hard-cooked cheese, ripened for a period of at least 1 year, during which major microbiological and

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biochemical changes occur. Raw and partially skimmed cow's milk supplemented with a natural thermophilic starter culture are the main sources of lactic acid bacteria (LAB) in this cheese. The starter culture, consisting of naturally acidified cheese whey, is composed mainly of different strains of *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis* and by a minority of *Streptococcus thermophilus* and *Lactobacillus fermentum* (Bottari et al. 2010). During traditional cheese manufacture, the high cooking temperature (approximately 55 °C) selects a thermophilic flora that is crucial for the cheese ripening; these species act directly through their enzymes and indirectly through acidification (De Dea et al. 2008; Gatti et al. 2008). This thermophilic microbiota, which remains in the cooked whey, originates the natural whey starter culture (NWC) that is used in the next day's cheese-making, maintaining a microbiological linkage among batches of production. After vat extraction of the cheese mass, its transformation proceeds with a moulding that may last up to 2 days (<http://www.parmigiano-reggiano.it>). It is during this phase and during the first 24 h in particular that acidification occurs and the main biochemical processes take place in the cheese together with the highest growth of the starter thermophilic bacteria (Fox et al. 1993). Highly acidifying thermophilic LAB in the natural whey starter are the main organisms responsible for this process in which suitable fermentation of lactose leads to lactic acid production. In PR, as in other Grana type cheeses, the LAB from the natural whey starters require approximately 5–20 h, depending on the zone of the cheese, to acidify the curd (Mucchetti and Neviani 2006). In fact, it is well known that the cheese-making technology and the dimension of the wheel (22–24 cm high, 40–45 cm diameter) of these long-ripened cheeses lead to differential development of bacterial growth between the inner and the outer regions of the wheel. Thus, the lactic acid production and, correspondingly, the drop in pH at the appropriate rates and times are key steps in the manufacture of a good-quality cheese. Acidification inhibits the growth of pathogens and food-spoilage bacteria and also enhances the expulsion of whey from the cheese mass during the cheese-making process, producing cheese with the desired moisture level (Mucchetti and Neviani 2006; Powell et al. 2011).

In the first hours of PR cheese manufacturing, slow acidification and the presence of residual fermentable sugar, such as galactose, may occur. Moreover, the large size of a cheese wheel (i.e. 50 kg) leads to a slow cooling, creating a gradient between internal and external zones during the overall moulding phase. In particular, at 24 h after vat extraction, temperatures can reach 35 °C and 27 °C in the internal and external zones, respectively (Giraffa et al. 1998). In the absence of antagonist microbiota, these are favourable conditions for the growth of undesirable bacteria that, if present in the raw milk, can lead to fermentations with gas formation with structural defect consequences to the cheese. Spoilage bacteria such as *Clostridium butyricum*, *Clostridium tyrobutyricum*, *Clostridium sporogenes*, coliforms and yeasts can find optimal conditions for their growth, causing early and late blowing in PR cheese (Bassi et al. 2009). Moreover, also early gas, mainly CO₂, can occur due to the growth of heterofermentative, non-starter LAB (NSLAB) able to metabolise residual galactose (Mucchetti and Neviani 2006).

As stated above, the achievement of a sufficient acidity level by starters at a proper time is essential for a good-quality PR cheese. Thus, the aim of this work was to evaluate the level of acidification of PR cheese at 24 h after vat extraction, during the moulding phase, to correlate that level to microbial development. To reach this goal,

different PR cheeses were subjected to chemical and microbiological analyses. For microbial growth evaluation, different methodological approaches were used.

2 Materials and methods

2.1 Cheese manufacturing

Eighteen PR cheeses were sampled from seven different dairies in the PDO cheese production area. Five dairies supplied two cheeses, and two dairies four cheeses. PR cheeses (33–35 kg) were made according to the method approved by the Consortium (Council Regulation, 1992; <http://www.parmigiano-reggiano.it>). Vats (copper tanks) of 1,200 L capacity were filled with 1,100 kg of raw cow's milk obtained by combination (1:1) of partially skimmed evening milk and whole morning milk. The milk from the evening milking was partially skimmed after overnight natural creaming (8–10 h at 12–18 °C). The natural whey cultures (NWC) used as starters (2.5–3.2% v/v) were obtained by incubating the cooked whey of the previous day's cheese-making at a gradient of temperature from approximately 50 °C to 35–20 °C for 18–24 h. Calf rennet powder was added, and coagulation was performed at 32–35 °C (1 mL coagulates 1 L milk at 35 °C in 4 min). After the coagulation, the curd was cut, then stirred and cooked for 5–15 min at 54–56 °C. After waiting 40–80 min to allow precipitation of the broken-up curd particles, the resulting cheese mass was extracted from the vat and cut into two twin wheels of approximately 38 kg each. The wheels were placed into moulds and were moulded for 48 h before being salted.

2.2 Cheese samples

Samples of cheese were collected aseptically at 24 h after vat extraction, during the moulding phase, and were kept at 4 °C until arrival at the laboratory. Representative portions of internal (i.e. central) and external (i.e. ~0.5 cm from the cheese rind) zones were obtained from these cheese sections. To obtain a sample representative of the whole wheel, the two portions were mixed in equal masses and grated before the analysis. As soon as the samples arrived, they were subjected to microbiological analysis, moisture and pH determinations and stored at –20 °C for further analysis within 2 days.

2.3 Microbial counts

Ten grams of the grated cheese samples were suspended in 90 mL of 20 g.L⁻¹ of trisodium citrate (pH 7.5) (Sigma-Aldrich, St. Louis, MO, USA) and homogenised for 2 min in a blender (Seward, London, UK) at 230 rpm. Serial decimal dilutions in sterile quarter-strength Ringer solution (Oxoid, Basingstoke, UK) were made and spread-plated on the following media and incubated under the following conditions: MRS (Man Rogosa Sharpe) agar, pH 5.4 (Oxoid), at 42 °C and 25 °C for 72 h (for the recovery of thermophilic and mesophilic lactobacilli, respectively) under anaerobic conditions (Anaerogen^{TM}; Oxoid); M17 agar (for the recovery of streptococci) (Oxoid) at 42 °C for 72 h under aerobic conditions and whey agar medium (WAM)

(Gatti et al. 2003) at 42 °C for 72 h under anaerobic conditions (Anaerogen^{TM}; Oxoid) (for enumeration of lactic acid bacteria arising from the NWC). Plates were made in triplicate and results were expressed as log cfu per gram of cheese.

Additionally, an aliquot (15 mL) of each cheese homogenate in trisodium citrate was used for total and viable direct microscopic counts. The LIVE/DEAD *Baclight*^{TM} Bacterial Viability kit (Molecular Probes, Eugene, OR, USA) was used according to the manufacturer's instructions. The cheese homogenates (15 mL) were centrifuged (10,000 rpm, 10 min, 4 °C). Each resulting pellet was washed twice in 15 mL of 20 g.L⁻¹ sodium citrate (pH 7.5) (Sigma-Aldrich), then resuspended in 15 mL of sterile water and 10-fold diluted. Subsequently, 1 mL of each sample was used for viability counts as directed by the supplier. Samples stained with LIVE/DEAD were filtered onto black polycarbonate filters (0.2 µm pore size) (Millipore Corp., Billerica, MA, USA), visualised using an epifluorescence microscope (Nikon 80i, Tokyo, Japan) and counted as described by Bottari et al. (2010). Three separate replicates were examined for each sample.

2.4 Determination of cheese community structure

Microbial composition was evaluated by fluorescence in situ hybridisation (FISH) and length heterogeneity-PCR (LH-PCR).

2.4.1 FISH

FISH was performed on the 10-fold dilutions of the cheese samples as previously described by Bottari et al. (2010). The Lbh1 FITC-labelled probe specific for *L. helveticus* and the St4 Cy3-labelled probe specific for *S. thermophilus* were used. Both probes were synthesised and labelled by Eurofins MWG (Ebersberg, Germany). After addition of 10 µL of the hybridisation buffer (0.9 mol.L⁻¹ NaCl, 0.01% SDS, 20 mmol.L⁻¹ Tris-HCl, pH 7.2, 45% formamide) containing 10 ng of each probe, slides were incubated in a dark humid chamber at 45 °C for 4 h. Slides embedded in mounting oil were evaluated using a Nikon Eclipse 80i epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a C-SHG1 100-W mercury lamp.

2.4.2 DNA extraction and LH-PCR

To obtain information about the community members in the cheese samples, genomic DNA was extracted from the cheese samples and subjected to LH-PCR fingerprinting analysis. Five grams of grated cheese was mortared, and 450 mg was used for DNA extraction using the General Rapid Easy Extraction System DNA kit (InCura S.r.l., Cremona, Italy) according to the manufacturer's instructions.

The V1 and V2 16S rRNA gene regions were amplified using primers 63F and 355R (Lazzi et al. 2004). The forward primer was 5' end-labelled with 6-carboxyfluorescein (6-FAM) dye. Amplicons were then separated by capillary electrophoresis in an automated sequencer (Applied Biosystems, Foster City, CA, USA). The PCR and capillary electrophoresis conditions were as described by Bottari et al. (2010). The fragment sizes (base pairs) were determined using GeneMapper software version 4.0 (Applied Biosystems) and the local Southern method to generate a sizing

curve from the fragment migration of the internal size standard (GS500 LIZ®; Applied Biosystems) using a threshold of 150 fluorescence units. Amplicon lengths were attributed to bacterial species according to published LH-PCR databases (Gatti et al. 2008; Lazzi et al. 2004).

2.5 Compositional analyses

pH measurements were performed using a potentiometer (Crison Instruments, Barcelona, Spain) and measuring directly in the cheese paste. Moisture content was determined by drying the sample at 102 °C (IDF 4A 1982), and fat was measured using Gerber's method as modified by Siegfeld (Savini 1946). Ash content was determined for the cheese and for water-soluble extracts of the cheese using the gravimetric method according to Savini (1946) after calcination of the cheese samples in a muffle furnace at 530 °C. Water-soluble extracts of the cheese were obtained as reported by Metzger et al. (2001). Lactose, lactic acid and galactose contents were determined using an enzymatic test kit according to Giudici et al. (1996).

Total nitrogen, pH 4.6-soluble nitrogen (SN), 12% trichloroacetic acid-soluble nitrogen (TCASN) and 5% phosphotungstic acid-soluble nitrogen (PTASN) fractions were separated using the procedure proposed by Gripon et al. (1975), and their values were assessed using the Kjeldahl method. Ammonia nitrogen (N-NH_3) was determined according to Savini (1946). Using the nitrogen fractions, nitrogen values for the high molecular weight components (Peptone $\text{N} = \text{SN} - \text{TCASN}$), low molecular weight peptides (Peptide $\text{N} = \text{TCASN} - \text{PTASN} - \text{N-NH}_3$) and free amino acids (Amino acid $\text{N} = \text{PTASN}$) were obtained.

2.6 Amino acid determinations

Each sample of grated cheese (10 g) was suspended in 45 mL of 0.1 N HCl. D,L-Nor-Leu (0.5 mL of a 5 mmol.L⁻¹ solution) was added as an internal standard. The mixture was homogenised for 2 min with an Ultraturrax T 50 basic homogeniser (IKA, Staufen, Germany) and centrifuged for 60 min at 4 °C and 3,220×g. The solution was filtered through a 45-µm filter, and 3 mL of the filtrate was dried. The residue was dissolved in 2 mL of a 0.1% solution of formic acid and ultrafiltered for 30 min at 7,370×g (using a molecular cut-off of 10 kDa). The filtrate was dried and suspended in 300 µL of bidistilled water. The derivatisation reaction was carried out using 10 µL of the solution and the AccQ-Fluor™ reagent kit (Waters, Milford, MA, USA) following the manufacturer's instructions. The mixture was analysed by HPLC using an Alliance 2695 separation module at a flow rate of 1 mL.min⁻¹, using an AccQ-Tag C18 column (3.9 × 150 mm) thermostated at 37 °C. Elution conditions were eluent A: AccQ-Tag™ added to phosphate buffer in 1 L of bidistilled water, eluent B: CH₃CN/H₂O 60:40, gradient: 0–1 min linear gradient from 100% to 97% A, 1–13 min linear gradient from 97% to 93% A, 13–18 min linear gradient from 93% to 90% A, 18–38 min linear gradient from 90% to 67% A, 38–51 min isocratic 67% A, 51–52 min linear gradient from 67% to 0% A, 52–61 min isocratic 0% A and reconditioning (25 min). Detection was performed using a Waters 2475 fluorimetric detector, $\mu_{\text{abs}} = 250$ nm and $\mu_{\text{em}} = 395$ nm. Quantification was performed using a calibration curve obtained using the internal standard method.

2.7 Natural whey starter cultures

Eighteen NWC used for each cheese production were also analysed. Aliquots (10 mL) of NWC were collected and kept at 4 °C until arrival to the laboratory. Serial decimal dilutions in sterile quarter-strength Ringer solution (Oxoid) were made for microbial counts. Samples were spread-plated on WAM (Gatti et al. 2003) and incubated at 42 °C for 72 h under anaerobic conditions (Anaerogen^{TM}, Oxoid). Plates were made in triplicate and results were expressed as log cfu per milliliter. Total and viable direct counts were carried out to 1 mL of a second-diluted NWC, previously washed twice with sterilised distilled water, according to the manufacturer's instructions (Molecular Probes). FISH analysis, DNA extraction and LH-PCR were performed as previously described by Bottari et al. (2010). pH was measured with a potentiometer and titratable acidity determined by titration with 0.25 M NaOH using the Soxhlet–Henkel method (Anon 1963). D-Lactic acid and L-lactic acid contents were determined using an enzymatic test kit according to Pellegrino et al. (1996).

2.8 Statistical analysis

Data were analysed using ANOVA multivariate (PASW statistics 18.0.0, Armonk, NY, USA) according to the following general linear model: $y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$, where y_{ij} = dependent variable, μ = common mean, α_i = effect of lactic acid content $i=1, 2$ and ε_{ij} = residual error. Significances of differences were tested using Fisher's LSD (least significant difference) method.

3 Results

The 18 samples of 24-h cheese were grouped on the basis of lactic acid content into LLA (low lactic acid, ≤ 1.8) and HLA (high lactic acid, > 1.8) groups, for which lactic acid was expressed as grams per 100 g of cheese dry matter (DM) (Table 1). Among the cheese samples examined, 7(39%) belonged to the LLA group and 11 (61%) to the HLA group. In particular, two dairies made cheeses that were included in LLA group, three dairies in HLA group and two dairies produced cheeses that belonged to both cheese groups.

3.1 Cheese composition

The basic compositions of the cheese samples are reported in Table 2. No differences between LLA and HLA cheeses were observed for moisture, protein and fat content ($\text{g} \cdot 100 \text{ g}^{-1}$ cheese DM). However, the LLA group was characterised by ash contents lower ($P \leq 0.05$) than those of the HLA group. In addition, water-soluble ash (expressed as percent of cheese ash) was lower ($P \leq 0.05$) in the LLA group.

The lactose, galactose and lactic acid contents ($\text{mmol} \cdot \text{kg}^{-1}$ DM) and pH values of the 24-h cheeses are shown in Table 2. Compared to HLA cheeses, LLA cheeses were characterised by higher ($P \leq 0.05$) values for galactose and lower ($P \leq 0.05$) values for lactic acid. The same cheeses were also analysed 12 h after moulding at vat extraction, and the lactic acid content was not different between the LLA and HLA cheese samples

Table 1 The values of lactic acid content (as % cheese dry matter, DM) recorded in the 18 cheese samples are reported

ID sample	Lactic acid (g.100 g ⁻¹ DM)	Classification
1	0.53	LLA
2	0.94	LLA
3	1.49	LLA
4	1.67	LLA
5	1.67	LLA
6	1.70	LLA
7	1.80	LLA
8	1.90	HLA
9	1.95	HLA
10	1.95	HLA
11	2.08	HLA
12	2.19	HLA
13	2.22	HLA
14	2.22	HLA
15	2.19	HLA
16	2.28	HLA
17	2.35	HLA
18	2.35	HLA
Average value	1.86	

The average lactic acid content in cheese was 1.86 g.100 g⁻¹. Samples with lactic acid content below the average value were assigned to LLA while those with a lactic acid above the average value were categorised as HLA

(52.16±14.42 vs. 54.38±15.54 mmol.kg⁻¹ DM) (data not shown). The proportion of D-lactic acid (and thus also of L-lactic acid) was not different between HLA and LLA cheeses. Regarding acidification, the LLA and HLA group showed 12 h after moulding almost identical pH values (mean 5.420±0.200 and 5.444±0.060, respectively) (data not shown), but differed significantly after 24 h ($P\leq 0.05$). The pH values were lower in HLA than in LLA cheeses, with an average difference of 0.18 units. No differences between nitrogen fractions of LLA and HLA cheeses were observed (data not shown), except for the ripening index (pH 4.6-soluble nitrogen, as percent of total nitrogen) that was higher in HLA than in LLA cheeses ($P\leq 0.05$).

3.2 Amino acid composition

Total free amino acid concentrations (expressed as milligrams per 100 g of cheese protein) were significantly higher in 24-h cheese samples in the HLA group than those in the LLA group (Table 2). Moreover, significant differences could be detected in the concentrations of some amino acids such as Gly, His, Arg, Ala, Pro and Lys that were higher in HLA cheese samples. Their values ranged from 27% for Lys to 49% for His (data not shown). The content of free amino acids was also assessed for the same cheese samples just after the vat extraction (12 h). At that point of the cheese-making process, no differences in the contents of the single amino acids or their sum were observed between LLA and HLA cheeses (data not shown).

Table 2 Composition and pH values of 24-h cheeses according to lactic acid content [LLA, ≤ 1.8 ; HLA, >1.8 g.100 g⁻¹ cheese dry matter (DM)]

Composition	LLA		HLA		P^b
	Mean	SD ^a	Mean	SD ^a	
Moisture (g.100 g ⁻¹)	38.70	0.98	38.55	0.83	NS ^c
Protein (g.100 g ⁻¹ DM)	48.75	1.06	48.18	2.79	NS
Fat (g.100 g ⁻¹ DM)	44.73	1.12	44.61	5.07	NS
Ash (g.100 g ⁻¹ DM)	4.77	0.15	4.94	0.16	≤ 0.05
Lactose (mmol.kg ⁻¹ DM)	0.87	1.46	0.41	0.61	NS
Galactose (mmol.kg ⁻¹ DM)	43.39	10.54	17.31	11.15	≤ 0.05
Lactic acid (mmol.kg ⁻¹ DM)	115.38	53.05	239.07	17.98	≤ 0.05
D-Lactic acid (%)	47.21	4.79	39.08	11.44	NS
Ripening index ^d	3.90	1.09	4.88	0.89	≤ 0.05
Sum FAAs ^e (mg.100 g ⁻¹ of cheese protein)	674.94	123.96	805.22	123.19	≤ 0.05
Water-soluble ash/ash (%)	30.79	2.22	44.79	1.77	≤ 0.05
pH	5.401	0.154	5.220	0.196	≤ 0.05

^a Standard deviation^b P value^c Not significant, $P > 0.05$ ^d Soluble nitrogen at pH 4.6/total nitrogen (%)^e Free amino acids**Table 3** Microbial counts of 24-h cheese samples according to lactic acid content [LLA, ≤ 1.8 ; HLA, >1.8 g.100 g⁻¹ cheese dry matter]

Composition	Units of measurement	LLA		HLA		P^b
		Mean	SD ^a	Mean	SD ^a	
Total	Log cells.g ⁻¹	8.48	0.40	8.66	0.34	NS ^c
Mesophilic lactobacilli	Log cfu.g ⁻¹	1.44	1.02	1.52	0.77	NS
Thermophilic lactobacilli	Log cfu.g ⁻¹	6.95	0.50	8.21	0.38	≤ 0.05
Streptococci	Log cfu.g ⁻¹	4.46	0.71	5.47	1.06	≤ 0.05
Cultivable in WAM	Log cells.g ⁻¹	6.63	0.80	7.89	0.28	≤ 0.05
Viable	Log cells.g ⁻¹	8.14	0.41	8.44	0.30	NS
Non-viable	Log cells.g ⁻¹	8.18	0.42	8.19	0.45	NS

Mesophilic lactobacilli: MRS at 25 °C, in anaerobiosis; thermophilic lactobacilli: MRS at 42 °C in anaerobiosis, *Streptococci* population: M17 at 25 °C, aerobiosis. WAM (whey agar medium) at 42 °C under anaerobic conditions for enumeration of lactic acid bacteria arising from natural whey starter. Total, viable and non-viable were performed by direct microscopic counts

^a Standard deviation^b P value^c Not significant, $P > 0.05$

3.3 Microbial counts

Direct microscopic counts showed high total cell numbers in all samples (greater than $8 \log \text{ cells.g}^{-1}$), and no significant differences between the two groups were observed (Table 3). Cultivable mesophilic lactobacilli counts (evaluated in MRS agar, pH 5.4, at 25 °C) were similar for both LLA and HLA samples. However, thermophilic lactobacilli counts (MRS at 42 °C) showed significant differences between the LLA and HLA groups (Table 3). Moreover, both the streptococcus population and the WAM-cultivable thermophilic bacterial population, used for the enumeration of LAB arising from the NWC, showed differences ($P \leq 0.05$) between the two groups of cheeses. Therefore, only thermophilic microbial counts in the three growth media (MRS, M17 and WAM) showed higher cell densities in the HLA cheese group. Viability counts were performed using fluorescence microscopy. In contrast to the cultivable counts, no significant differences were observed between the LLA and HLA groups.

3.4 Determination of cheese community structure

Bacterial community structures of the 24-h cheeses were characterised using LH-PCR and FISH. Among the 18 cheese samples, three different LH-PCR profiles were observed (Fig. 1). Profile A was characterised by peaks at 330 ± 1 bp (attributed to *L. delbrueckii* subsp. *lactis* or subsp. *bulgaricus*), 334 ± 1 bp (*L. helveticus*) and double peaks at 342 ± 1 bp and 345 ± 1 bp (attributed to *L. fermentum*). A peak at 305 ± 1 bp was also detected (non-attributed).

Profile B was characterised by the presence of only two peaks at 330 ± 1 bp and 334 ± 1 bp, attributed to *L. delbrueckii* subsp. *lactis* and *L. helveticus* species, respectively, according to the database. Finally, profile C presented peaks at 330 ± 1 bp and 334 ± 1 bp, attributed to *L. delbrueckii* subsp. *lactis* and *L. helveticus*, respectively, and a peak at 320 ± 1 bp attributed to *S. thermophilus*. Other peaks at 305 bp and 323 bp were also detected but not attributed to any species in the database.

Most cheese samples (45%) were characterised by profile A and 33% displayed profile B, whereas profile C was revealed in 22% of the 24-h cheese samples. No correlation between profile types and lactic acid content groups was observed. However, only profile C was represented by the HLA cheeses.

Bacterial identification using FISH analysis was performed using probes specific for viable *L. helveticus* and *S. thermophilus* species. The signal intensity of cells hybridised with oligonucleotide probes is directly related to the cellular rRNA content, which is a useful indicator of viability (Bottari et al. 2006). An evaluation of the relative abundance of the two targeted viable species was performed for each sample according to Bottari et al. (2010). Figure 2 shows two representative pictures of FISH analysis of LLA and HLA cheese groups. Red fluorescent cocci were *S. thermophilus* hybridised cells, bright green fluorescent rods were *L. helveticus* hybridised cells and dim green non-fluorescent rods cells were lactobacilli cells with very low metabolic activity. It was observed that the LLA cheese group was characterised by a majority of *S. thermophilus* hybridised cells with respect to *L. helveticus* hybridised cells with intense fluorescence. On the contrary, HLA cheese samples showed a majority of *L. helveticus* hybridised cells with respect to *S. thermophilus* hybridised cells.

Cheese samples	Microbial counts		Community structure by LH-PCR				Acidification level
	Cultivable in WAM ^a Log cfu.g ⁻¹	<i>L. helveticus</i>	<i>L. delbrueckii lactis</i>	<i>S. thermophilus</i>	<i>L. fermentum</i>	Unknown ^b	
1	6.69	72.27%	17.35%	nd ^c	8.36%	2.02%	LLA
2	7.43	71.83%	14.67%	nd	3.78%	9.72%	LLA
3	6.61	44.93%	28.68%	nd	4.74%	21.65%	LLA
6	7.02	53.92%	27.27%	nd	2.11%	16.70%	LLA
7	7.33	89.95%	8.65%	nd	1.39%	nd	LLA
10	8.25	65.12%	24.40%	0.59%	2.15%	7.75%	HLA
12	7.65	44.17%	14.02%	nd	34.83%	6.98%	HLA
18	7.88	70.49%	4.39%	nd	8.94%	16.18%	HLA
4	5.09	66.58%	33.42%	nd	nd	nd	LLA
5	6.26	65.96%	34.04%	nd	nd	nd	LLA
9	8.32	75.33%	24.67%	nd	nd	nd	HLA
11	8.23	73.36%	16.26%	nd	nd	10.38%	HLA
13	7.76	84.72%	12.60%	nd	nd	2.68%	HLA
15	7.61	87.75%	8.49%	nd	nd	3.76%	HLA
8	7.64	61.77%	25.74%	0.69%	nd	11.79%	HLA
14	8.03	56.72%	24.58%	1.71%	nd	16.98%	HLA
16	7.55	57.31%	36.42%	1.08%	nd	5.19%	HLA
17	7.83	54.19%	37.45%	1.33%	nd	7.03%	HLA

^aWAM: Whey agar medium

^bUnknown: LH-PCR peaks that are not attributed to any species in the database

^cnd: not detected. The value is below the detection limit

Fig. 1 Microbial counts and community structures assessed by LH-PCR of 18 cheese samples. Three representative electropherogram profiles (A–C) of DNA fragments amplified by PCR from 24-h cheese samples are shown. The x-axis shows the peak size in base pairs, and the y-axis shows the peak intensity in relative fluorescence units. *Numbers* represent the species, identified according to a published LH-PCR database (Lazzi et al. 2004), as follows: 1, *L. delbrueckii* subsp. *lactis*; 2, *L. helveticus*; 3, *L. fermentum*; 4, *S. thermophilus* (305 and 323 are unattributed peaks)

a FISH LLA **b** FISH HLA

Fig. 2 FISH of two representative 24-h cheese samples (respectively sample 1 and sample 9) classified as LLA (**a**) and HLA (**b**). The simultaneous use of probes St4 (red label) and Lbh1 (green label) revealed the presence of a majority (++) of hybridised *S. thermophilus* cells with respect to *L. helveticus* cells (**a**). A lower amount (+) of hybridised *L. helveticus* cells was also present. While a relative majority (++) of hybridised *L. helveticus* cells were revealed in HLA, a lower amount of hybridised *S. thermophilus* cells (+) were also present (**b**)

4 Discussion

During the first 24 h of the moulding phase, acidification by conversion of lactose into lactic acid is the main biochemical process that takes place in cheese. Acid production at the appropriate rate and time is a key step in the manufacture of a good-quality cheese (Fox et al. 1993; Powell et al. 2011). In PR, the growth of the natural whey starter LAB is responsible for the acidity reached in cheese at 24 h. The chemical parameters and microbial characteristics of the 18 PR cheeses, sampled from seven dairies at 24 h after vat extraction, were analysed in order to correlate their level of acidification to microbial development. The cheeses were divided into two groups according to their lactic acid contents detected at 24 h during moulding, termed LLA and HLA (low and high lactic acid content cheeses, respectively).

4.1 Compositional differences between LLA and HLA cheeses

Regarding the contents of moisture, fat and protein, the values were consistent with those reported by Panari et al. (2003) for PR cheese at 24–48 h after vat extraction. In addition to higher lactic acid content, HLA group was characterised by lower values for galactose and pH and higher contents of ash, water soluble ash and total amino acid in comparison with the LLA group. The lower pH values reported in the HLA group may be responsible for greater solubilisation of the calcium phosphate bound to their casein matrices (Lucey and Fox 1993) leading to higher proportions of water-soluble ash in those cheeses. The different extents of mineral solubilisation observed in this work may lead to differences in the evolution of cheese ripening in LLA and HLA cheeses (Lucey et al. 2005).

In a previous study, which was carried out on six PR cheeses at 23 h after vat extraction, Pecorari et al. (2003) reported lactic acid content and pH values comparable with those observed for HLA cheeses but clearly different when compared to LLA cheeses. Furthermore, in contrast to the present study, Pecorari et al. (2003)

detected a lower amount of galactose ($0.009 \text{ g} \cdot 100 \text{ g}^{-1}$ 23-h cheese) compared with the amount we found in the HLA group ($0.19 \text{ g} \cdot 100 \text{ g}^{-1}$) and, in particular, in LLA cheeses ($0.48 \text{ g} \cdot 100 \text{ g}^{-1}$). The residual galactose and lactose in early cheese can be the consequence of non-exhaustive fermentative activities of the starter LAB (Mucchetti and Neviani 2006). However, the natural whey starters utilised for each cheese production batch were considered of good quality with regard to their microbiological and chemical parameters (Table 4).

4.2 Linking microbial characteristics to acidification level

In this study, LLA and HLA cheeses showed similar lactose consumption but differed in their galactose content, which was significantly higher in the former group. This finding led us to hypothesise that the starter bacteria used to produce LLA cheeses might be characterised by LAB species or strains that consume galactose at low rates or that might not be able to consume it. Results obtained from the culture-dependent approach revealed significantly higher densities of thermophilic lactobacilli population able to grow in MRS and WAM media in HLA cheese group. Interestingly, in contrast to the higher cultivable counts in the HLA cheese group, no differences were found in the numbers of total, viable and non-viable cells between the two groups of samples. This similarity could be ascribed to the presence of bacteria that, although viable (detected only microscopically) in the sample, are not able to proliferate on culture media but could be capable of growth in cheese. However, the relevance of this population during ripening requires further study, and, being non-cultivable, they remain unknown.

For a deeper insight into microbial composition, culture-independent approaches were performed which helped to get more information. FISH and LH-PCR give different results, LH-PCR investigate total microbial DNA (that could arise from viable, non-viable and lysed cells, Gatti et al. 2008), while FISH detects ribosomal RNA of live cells. Thus, a thorough non-quantitative evaluation and comparison of both techniques was made. The dominant species found in the 24-h cheeses by LH-PCR and/or FISH belonged to *L. helveticus*, *L. delbrueckii* subsp. *lactis*, *S. thermophilus* and *L. fermentum* according to Bottari et al. (2010). Most of these species can utilise galactose as a carbon source. However, it is known that galactose consumption is strain dependent in *S. thermophilus* (Robinson 2005). In this regard, although lactose is efficiently transported into the cell and subsequently hydrolysed by an intracellular β -galactosidase, many strains of *S. thermophilus* ferment only the glucose moiety of lactose, while galactose moiety is excreted into the medium in equimolar amounts with the lactose uptake (de Vin et al. 2005). De Vin et al. (2005) found that some *S. thermophilus* strains began to consume the galactose excreted into the medium only after lactose depletion, whereas others were unable to consume the galactose or they did so at different rates and to various extents but never to completion. In this work, the higher proportions of viable *S. thermophilus* hybridised cells, with respect to *L. helveticus* hybridised cells, revealed by FISH, support the hypothesis of a lower activity of *L. helveticus*, galactose positive species, and higher relevance of *S. thermophilus* strains in the residual carbohydrates found in LLA cheeses. Our hypothesis is supported by previous results of Mucchetti et al. (2002). These authors found that higher amounts of unmetabolised galactose remained at 24 h

Table 4 Chemical and microbiological characteristics of natural whey cultures (NWC)

NWC samples	Microbial counts			Community structure by LH-PCR and FISH							Chemical characteristic				
	Log cfu.mL ⁻¹		Log cells.mL ⁻¹		LH-PCR—relative peak areas (%)							FISH ^b			
	Cultivable in WAM ^a	Viable	Non-viable	Total	<i>L.helv</i> ^b	<i>L.deb</i> ^c	<i>S.ther</i> ^d	<i>L.ferm</i> ^e	Unknown ^f	Lh	St	Cheese acidification level	pH	°SH	Lactic acid composition g.100 g ⁻¹
NWC 1	8.36	8.61	7.63	8.66	57.21	42.79	nd ^g	nd	nd	++	+	LLA	3.30	31.10	nr ⁱ
NWC 2	8.36	8.61	7.63	8.66	57.21	42.79	nd	nd	nd	++	+	LLA	3.30	31.10	nr
NWC 3	8.68	8.27	9.17	9.22	43.56	39.69	nd	nd	16.75	+	+	LLA	3.23	31.60	1.38
NWC 4	8.37	9.97	8.81	10.00	39.40	60.60	nd	nd	nd	+	++	LLA	3.42	29.08	nr
NWC 5	8.37	9.97	8.81	10.00	39.40	60.60	nd	nd	nd	+	++	LLA	3.42	29.08	nr
NWC 6	8.68	8.27	9.17	9.22	43.56	39.69	nd	nd	16.75	+	+	LLA	3.23	31.60	1.38
NWC 7	8.29	9.57	8.63	9.61	64.36	28.93	nd	6.71	nd	+	+	LLA	3.35	29.90	nr
NWC 8	7.70	8.52	8.81	8.99	60.85	33.33	nd	nd	5.82	+	–	HLA	nr	nr	1.73
NWC 9	8.29	9.57	8.63	9.61	64.36	28.93	nd	6.71	nd	+	+	HLA	3.35	29.90	nr
NWC10	8.98	9.35	8.87	9.48	44.67	48.61	nd	nd	6.72	++	+	HLA	3.24	31.30	1.62
NWC11	8.98	9.35	8.87	9.48	44.67	48.61	nd	nd	6.72	++	+	HLA	3.24	31.30	1.62
NWC12	8.25	8.99	8.64	9.15	28.82	26.79	nd	nd	44.39	++	+	HLA	3.25	28.70	1.29
NWC13	8.22	9.59	8.81	9.66	72.09	27.91	nd	nd	nd	+	+	HLA	3.25	29.10	nr
NWC14	7.70	8.52	8.81	8.99	60.85	33.33	nd	nd	5.82	+	–	HLA	nr	nr	1.73
NWC15	8.22	9.59	8.81	9.66	72.09	27.91	nd	nd	nd	+	+	HLA	3.25	29.10	nr
NWC16	8.75	9.05	8.96	9.31	32.99	48.60	nd	nd	18.40	++	++	HLA	3.45	27.30	1.44

Table 4 (continued)

NWC samples	Microbial counts		Community structure by LH-PCR and FISH							Chemical characteristic					
	Log cfu.mL ⁻¹	Log cells.mL ⁻¹		LH-PCR—relative peak areas (%)					FISH ^h		pH	°SH	Lactic acid composition g.100 g ⁻¹		
		Cultivable in WAM ^a	Viable	Non-viable	Total	<i>L.helv</i> ^b	<i>L.deb</i> ^c	<i>S.ther</i> ^d	<i>L.ferm</i> ^e	Unknown ^f				Lh	St
NWC17	8.75	9.05	8.96	9.31	32.99	48.60	nd	nd	18.40	++	++	3.45	27.30	1.44	
NWC18	8.25	8.99	8.64	9.15	28.82	26.79	nd	nd	44.39	++	+	3.25	28.70	1.29	

The NWC order corresponds to the 24-h cheese samples (1–18)

^a WAM whey agar medium

^b *L. helveticus*

^c *L. delbrueckii* subsp. *lactis*

^d *S. thermophilus*

^e *L. fermentum*

^f Unknown: LH-PCR peaks that are not attributed to any species in the database

^g nd not detected. Means the value is below the detection limit (10⁴–10⁵ cells.mL⁻¹)

^h FISH signs indicate majority (++) , presence (+) and absence (–) of hybridised *L. helveticus* (Lh) and *S. thermophilus* (St)

ⁱ nr not revealed

after vat extraction in an experimental mini-cheese made with a defined *S. thermophilus* strain as starter than by using (1) undefined natural cultures, (2) *L. helveticus* strains alone or (3) *L. delbrueckii* subsp. *lactis* strains alone (Mucchetti et al. 2002).

The change in relative lactic acid levels found at 24 h compared to 12 h was in good agreement with the presence of lower number of active cells of *L. helveticus* and a majority of *S. thermophilus* cells in the LLA cheese group. In fact, *S. thermophilus* is reported to acidify more rapidly than lactobacilli (Béal and Corrieu 1994; Mucchetti and Neviani 2006), but rapid acidification does not mean high acidification as demonstrated by Spinnler and Corrieu (1989), who concluded that the maximum acidification rate of *L. helveticus* was generally higher than that of *S. thermophilus*.

On the other hand, results lead to hypothesise that *L. helveticus* and other thermophilic lactobacilli are involved in the achievement of high acidification level. Using FISH, it was found that the HLA cheese group showed higher proportions of *L. helveticus* hybridised cells with respect to *S. thermophilus*. As *L. helveticus* is a highly acidifying species (Tuner and Martley 1983) also able to consume galactose, this result agrees with the higher average lactic acid contents and lower pH values found in those cheeses. Moreover, cultivable lactobacilli evaluated in MRS and WAM were significantly higher in HLA with respect to LLA cheese group. Thus, we could hypothesise that other lactobacilli able to grow in these culture media such as *L. delbrueckii* subsp. *lactis* could be responsible for the different acidification level.

Other findings led us to support the hypothesis of a higher population of *L. helveticus* during the early phase. The nitrogen contents and the individual free amino acids highlight some issues concerning cheese proteolysis from a quantitative and qualitative point of view. The ripening index and total amino acid contents were higher in HLA cheeses. Notably, the ripening index was already higher in HLA cheeses at vat extraction (data not shown). Therefore, the different degree of casein solubilisation reported here could be related to microbial development that had already occurred in the vat milk. The higher casein degradation observed in HLA cheese was associated with a majority of viable *L. helveticus* cells together with higher thermophilic lactobacilli counts in the HLA cheese group. Because different *L. helveticus* strains manifest different proteolysis rates (Gatti et al. 1999), the great variability among *L. helveticus* strains found not only in natural whey starters but also in cheese during moulding (Gatti et al. 2003; Giraffa and Neviani 1999) must also be considered.

5 Conclusions

The different lactic acid contents, found in two groups of PR cheese 24 h after vat extraction, were associated with different degrees of mineral solubilisation with possible repercussions for cheese rheology and, consequently, on the development of chemical, biochemical and microbial processes that take place throughout ripening.

Results obtained by the polyphasic approach used in this research allow to conclude that the presence of a high number of *L. helveticus* cells and/or other acidifying thermophilic lactobacilli, able to metabolise galactose and also grow faster in the cheese, appear to be essential conditions to achieve adequate lactic acid content and, thus, correct curd acidification. The presence of residual galactose was associated with low densities of thermophilic lactobacilli and higher proportion of fully

active *S. thermophilus* with respect to *L. helveticus*. In this sense, even though their presence is not necessarily related to their development in the curd, the significance of *S. thermophilus* in the whey starter has to be reconsidered. In particular, the occurrence of *S. thermophilus* strains unable to completely consume galactose is likely an undesired component of the natural mix of LAB strains in whey starter.

Additional hypotheses may further explain differences in lactic acid contents, found in two groups of PR cheese 24 h after vat extraction. The biotype compositions of the whey starter LAB appear to be far more important than the species composition in ensuring their good performance. It should also be noted that residual galactose could also be due to inefficient natural whey starter acidification caused by phage activity not considered in this study.

Even if the approach used in this study provided technologically useful answers for the correct production of PR cheese, it may be extended to all hard-cooked cheeses produced with thermophilic natural cultures.

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