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ORIGINAL RESEARCH

# Characterisation of the technological behaviour of mixtures of mesophilic lactic acid bacteria isolated from traditional cheeses made of raw milk without added starters

ÁNGEL ALEGRÍA,[1] PABLO GONZÁLEZ,[2] SUSANA DELGADO,[1] ANA BELÉN FLÓREZ,[1] ANA MARÍA HERNÁNDEZ-BARRANCO,[3] ANA RODRÍGUEZ[2] and BALTASAR MAYO[1]*

[1]*Departamento de Microbiología y Bioquímica,* [2]*Departamento de Tecnología y Biotecnología, and* [3]*Servicios Científico-Técnicos, Instituto de Productos Lácteos de Asturias (IPLA-CSIC), Paseo Río Linares s/n, 33300 Villaviciosa, Asturias, Spain*

*In this work, the technological behaviour in milk of a set of* Lactococcus lactis *strains, alone or in combination with strains of* Leuconostoc *spp. and* Lactobacillus *spp. isolated from traditional, raw milk cheeses made without commercial starters, was investigated. Small, mixture-specific differences during milk fermentation were recorded for growth, milk acidification and production of organic acids, volatile compounds, free amino acids and biogenic amines. Four combinations appropriate for use as dairy starters were tested in pilot-scale cheese trials. Two mixtures produced cheeses of high flavour and taste quality; these could be confidently used as starter cultures.*

**Keywords** Starters, Lactic acid bacteria, *Lactococcus lactis*, *Lactobacillus*, *Leuconostoc*, Nisin.

## INTRODUCTION

Strains of *Lactococcus lactis* subsp. *lactis* and subsp. *cremoris* are important components of mesophilic starter cultures used in the manufacture of many fermented dairy products (Mills *et al.* 2010). The main function of starter bacteria during fermentation is the production of lactic acid at an acceptable rate. Moreover, during ripening, they also contribute to the development of flavour via their proteolytic action (Smit *et al.* 2005) and improve food safety through the synthesis of substances with antimicrobial activity, such as organic acids, $H_2O_2$ and bacteriocins (Topisirovic *et al.* 2006). *Lc. lactis* is commonly combined with strains of *Leuconostoc* spp. and other species of lactic acid bacteria (LAB) (mainly mesophilic lactobacilli); these LAB contribute to the production of key flavour compounds (Hemme and Foucaud-Scheunemann 2004; Settanni and Moschetti 2010).

Traditional dairy products made from raw milk without the addition of commercial cultures provide a reservoir of biodiversity from which new LAB strains might be selected to improve or replace currently used starters and adjunct cultures (Kelly and Ward 2002; Rademaker *et al.* 2006; Brandsma *et al.* 2008). New strains are particularly needed for traditional cheeses under quality labels such as that of Protected Designation of Origin (PDO), to preserve their originality. Autochthonous strains would ensure the safety and typical sensorial properties of the cheeses, while avoiding the uniformity of the industrial cheeses made with commercial cultures. Much effort has already been invested in the identification, typing and characterisation of candidate strains (Ayad *et al.* 2000; Flórez *et al.* 2006; Alegría *et al.* 2009). In industrial-scale milk fermentations, however, single-strain starters are of little use as they can easily be rendered unserviceable by bacteriophages, the activities of which lead to flavour and texture failures and food safety problems (Garneau and Moineau 2011). Closed fermentation vats, the propagation of starters in antiphage, calcium-depleted media, and the selection of phage-resistant variants have all been explored as ways of reducing the economic losses

*Author for correspondence. E-mail: baltasar.mayo@ipla.csic.es

caused. However, starters composed of phage-unrelated strains, and the rotation of starter mixtures, continue to be the simplest and most cost-effective way of protecting against phage attack (Garneau and Moineau 2011). Traditional dairy product reservoirs might also provide new LAB strains that could form part of such mixtures and rotations (van Hylckama Vlieg *et al.* 2006; Mills *et al.* 2010). In the search for new starters, much effort has been spent to characterise wild LAB strains from traditional dairy products, while analyses of LAB mixtures are scarce.

In this work, starter (lactococci) and nonstarter (leuconostocs and lactobacilli) LAB strains isolated from traditional, Spanish cheeses made of raw cow's milk without added starters were used in combination as starter mixtures. Their growth in milk was then examined, along with their associated acidification rates, the degree of milk acidification reached and their production of organic acids, volatile compounds and free amino acids. The suitability of the most promising combinations for the manufacture of cheese was tested in pilot-scale cheese manufacturing trials.

## MATERIAL AND METHODS

### Strain propagation and design of starter mixtures

A set of LAB strains has been gathered from different batches of five traditional cheese varieties made from raw cow's milk without added starters, two of which (Cabrales and Casín) carry a PDO label. These strains, which had been isolated and characterised in previous reports (Mayo *et al.* 1990; Herrero *et al.* 1996; Sánchez *et al.* 2000; Delgado *et al.* 2002), were chosen as the base material for the design of nine experimental starter blends (Table 1). These mixtures involved combinations of strains of the subspecies *lactis* and *cremoris* and the *lactis* biovar diacetylactis (mixtures S1, S2, S3, S4 and S5), plus strains of the nonstarter LAB species *Leuconostoc* (mixture S6) or strains of both *Leuconostoc* spp. and *Lactobacillus* spp. (mixture S7). Mixtures S8 and S9 were composed exclusively of *Lc. lactis* subsp. *lactis* strains that produce the bacteriocins nisin and lactococcin 972, respectively (Table 1). The undefined commercial starter Flora Danica (Chr. Hansen, Hørsholm, Denmark) was used as a control mixture.

Stock mixtures were produced by pooling identical volumes of individual overnight cultures raised in M17 (Oxoid, Basingstoke Hampshire, UK) (lactococci), Brain Heart Infusion (BHI; Merck, Darmstad, Germany) (leuconostocs) or de Man, Rogosa and Sharpe (MRS; Merck) (lactobacilli) broth at 30, 25 and 37 °C, respectively. These mixtures were then centrifuged and washed with sterile saline (NaCl 0.9%), and the pellets were suspended (at one-tenth of the original fluid volume) in UHT semiskimmed milk (CAPSA,

**Table 1** Bacterial composition of the nine experimental starter combinations studied in this work

| *Starter combination* | *Bacterial species/types* | *Strains in the mixture* |
|---|---|---|
| S1 | *Lactococcus lactis* subsp. *lactis* | A36, A88, AA15, AA22, AA29, AA26, AA2, CAS3, GE1 |
| S2 | *L. lactis* subsp. *lactis* | AA15, AA29, AA2, AA26, CAS3, GE1 |
| | *L. lactis* subsp. *lactis* biovar diacetylactis | AA6, AA27, L74 |
| S3 | *L. lactis* subsp. *cremoris* | AA23, C8d, 206b, AA9, AA23, GE14, L39, LC144, P1A12, P2C1, P2E10 |
| S4 | *L. lactis* subsp. *lactis* | AA15, AA29, AA2, AA26, CAS3, GE1 |
| | *L. lactis* subsp. *cremoris* | AA23, AA9, AA23, GE14, LC144, L39 |
| S5 | *L. lactis* subsp. *lactis* | AA15, AA29, AA2, AA26, CAS3, GE1 |
| | *L. lactis* subsp. *lactis* biovar diacetylactis | AA6, AA27, L74 |
| | *L. lactis* subsp. *cremoris* | AA23, AA9, AA23, GE14, LC144, L39 |
| S6 | *L. lactis* subsp. *lactis* | AA15, AA29, AA2, AA26, CAS3, GE1 |
| | *L. lactis* subsp. *lactis* biovar diacetylactis | AA6, AA27, L74 |
| | *L. lactis* subsp. *cremoris* | AA23, AA9, AA23, GE14, LC144, L39 |
| | *Leuconostoc* spp. | E6c, BC7, E3a, AB2, AC4, G3a |
| S7 | *L. lactis* subsp. *lactis* | AA15, AA29, AA2, AA26, CAS3, GE1 |
| | *L. lactis* subsp. *lactis* biovar diacetylactis | AA6, AA27, L74 |
| | *L. lactis* subsp. *cremoris* | AA23, AA9, AA23, GE14, LC144, L39 |
| | *Leuconostoc* spp. | E6c, BC7, E3a, AB2, AC4, G3a |
| | *Lactobacillus* spp. | GA102, VI19 |
| S8 | *L. lactis* subsp. *lactis* (nisin producers) | A6, A8, A16, A38, AA16, AA17, AA48, BB9, A28, L30, LC83A |
| S9 | *L. lactis* subsp. *lactis* (lactococcin 972 producers) | Q12, Q16, Q18, T226, T243 |
| FD | Lactococci, Leuconostoc, Lactobacilli | |

FD, Flora Danica (Chr. Hansen, Hørsholm, Denmark), a complex, undefined commercial starter.

Siero, Spain). These samples were then frozen at −80 °C for 24 h before lyophilisation in a Virtis Freezemobile 12EL lyophiliser (Sp. Scientific, Gardiner, NY, USA) and stored at −20 °C until use.

## Technological characterisation of the blends

*Growth in milk*

To analyse the acidification capacity of the mixtures, 1 g of the different lyophilised mixtures was suspended in 100 mL of UHT semiskimmed milk from a single batch and incubated overnight at 30 °C. Overnight cultures were then used to inoculate new milk aliquots at an approximate concentration of $10^6$ cfu/mL, which were once again incubated at 30 °C. The pH was directly measured at regular intervals over the first 11 h using a pH meter (Crison Instruments, Barcelona, Spain). The pH after 24 and 48 h of incubation was also recorded. Experiments were performed in triplicate.

The number of total aerobic mesophilic bacteria in the fermented milk produced with each of the mixtures was determined on Plate Count Milk Agar (PCMA; Merck) after 72 h of incubation at 30 °C. Total lactococci were determined on M17 agar (M17A) after 48 h of incubation at 30 °C. *Lc. lactis* subsp. *lactis* biovar diacetylactis were enumerated on Kempler and McKay agar plates (KMA; Kempler and McKay 1980) after incubation at 30 °C for 48 h. In this medium, citrate-fermenting *Lc. lactis* colonies (which are blue in colour) are easily distinguished from nonfermenting colonies (white). *Leuconostoc* spp. were counted on Mayeux, Sandine and Elliker agar (MSEA) (Biokar Diagnostics, Beauvais, France) containing 30 μg/mL vancomycin (Sigma-Aldrich, St. Louis, MO, USA) after incubation at 25 °C for 72 h. Lactobacilli were grown on MRS agar (MRSA) adjusted to pH 5.4 and enumerated after incubation at 30 °C for 72 h.

*Detection and quantification of sugars and organic acids in fermented milk*

The detection of sugars and organic acids in milk was performed by HPLC after growing the mixtures in UHT milk under the same conditions as above (30 °C for 48 h). Experiments were also performed in triplicate. After incubation, 5 mL samples were diluted in 25 mL of 5.4 mM $H_2SO_4$, and the suspension shaken for 1 h at 37 °C before centrifuging at 4500 *g* for 10 min. Supernatants were filtered through a 0.45-nm sterile membrane and frozen at −20 °C until analysis. The separation of sugars and organic acids was performed using an ICSep ICE-ION-300 ion-exchange column (mobile phase 0.0085 N $H_2SO_4$, operating temperature 65 °C, flow rate 0.4 mL/min). Two detectors were connected in series to a Waters liquid chromatograph controlled by Millenium 32 Software (Waters, Milford, MA, USA): a 996 Photodiode Array Detector (Waters) for the determination of organic acids (detection wavelength 210 nm) and a Waters 410 differential refractometer for sugar determination (detection wavelength 280 nm). Quantification was performed using calibration curves for the identified sugars and organic acids. The results were recorded as milligrams of acid or sugar per 100 mL of sample.

*Analysis of free amino acids*

Fermented milks were also analysed in triplicate for free amino acid composition. As LAB are mostly responsible for secondary proteolysis (van den Berg and Exterkate 1993), production of free amino acids was repeated in the presence of a small quantity of calf rennet in milk (similar to that retained in most cheese types). A 1 mL sample of each milk sample was homogenised with 10 mL of 0.2% thiodipropionic acid (TDPA) (Fluka, Madrid, Spain) and 250 mM of internal standard (norvaline; Sigma-Aldrich) using an Ultra-Turrax homogeniser (OMNI International, Watersburry, USA) at 20 000 rpm for 2 min. The homogenate was then placed in an ultrasonic bath for 30 min and centrifuged at 5000 × *g* for 20 min. After the removal of the fat layer, the supernatant was filtered through a 0.45-μm membrane and 3 mL of the filtrate deproteinised by passing it through an AmiconUltra 0.5-mL filter (Millipore, Bedford, CA, USA) by centrifugation at 3000 *g* for 1 h. Twenty microlitres of the sample were derivatised following a protocol described elsewhere (Krause *et al.* 1995). The separation of amino acids was performed by reverse phase (RP)-HPLC using an Alltima HP C18 Hi-Load column (6 mm) (Waters). The gradient and detection conditions were similar to those described by Krause *et al.* (1995). Quantification was performed using dedicated calibration curves for all amino acids and derived compounds. Results were recorded as micrograms of amino acid per millilitre of sample.

*Analysis of volatile compounds*

Fermented milks with and without calf rennet were further analysed for the presence of volatile compounds. As before, analyses were also performed in triplicate. Volatile compounds were identified and quantified by headspace/gas chromatography/mass spectrometry (HS/GC/MS) using an Agilent apparatus with G 1888 HS, 6890 GC and 5975B inert MSD components (Agilent Technologies, Wilmington, DE, USA), equipped with a HP-Innowax column (60 m length/0.25 mm I.D./0.25 μm film) (Agilent). Sample preparation and gas chromatographic separation were performed as described elsewhere (Fernández *et al.* 2011). Briefly, starter mixtures were incubated in milk as above, before adding 100 μL of internal standard (cyclohexanone, 0.36 mg/mL), and then stored at −80 °C until analysis. Peaks were quantified as the relative total ionic count with respect to the internal standard.

## Cheesemaking

One experimental cheese batch was produced from cow's milk with each of the four selected mixtures. A Gouda-like cheesemaking process was followed in the experimental trials (Figure 1). Milk (80 L) was pasteurised at 63 °C for 30 min, poured into four 20-L vats and cooled to 32 °C. Each of the vats was then inoculated with a freshly prepared starter aliquot (via overnight incubation of the lyophilised mixture in UHT milk at 30 °C) at 1% v/v (approximate concentration of 6 $\log_{10}$ cfu/mL). Calcium chloride was added to a final concentration of 0.02% (w/v). Rennet with a calculated strength of 1:6000 was also added to the milk at a concentration of 0.017% (v/v) when the pH reached 6.65. The curd was cut to pea-sized grains and heated to 36 °C before draining and moulding. The curd was then subjected to hydraulic pressure at 1.5 bar for 2 h. Four cheeses of around 500 g were obtained from each trial. Cheeses were removed from their moulds when they reached a pH of 5.5 and immersed for 25 min in saturated brine (20° Baumé) at 4 °C.

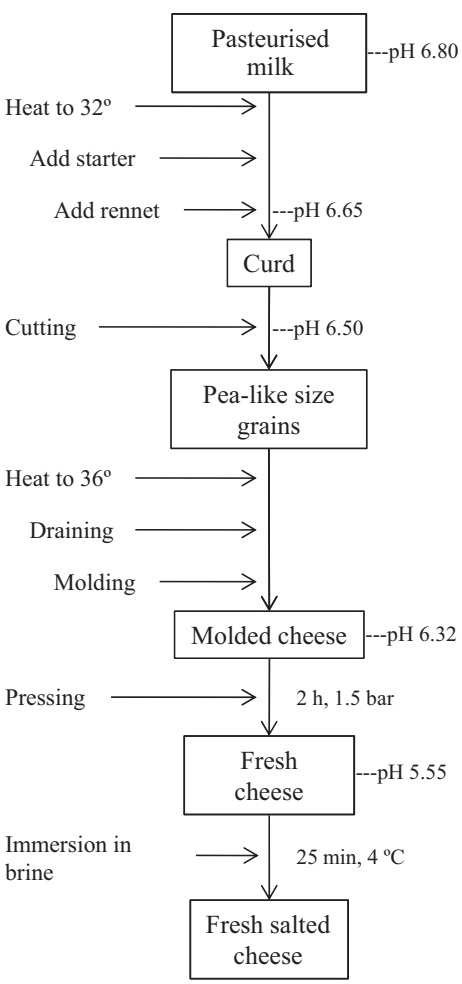


**Figure 1** Flow scheme for the manufacturing process of experimental cheeses. Approximate duration of manufacturing steps and temperature and pH changes of the cheese matrix through the process are indicated.

## Milk, curd and cheese analysis

*Microbial counts*

Duplicate samples of cheese milk, curd and cheeses aged 3, 7, 15 and 30 days were subjected to basic microbial and chemical analysis. Ten gram samples of milk, curd and cheese were homogenised with 90 mL of a 2% (w/v) sodium citrate solution at 45 °C in a Colworth Stomacher 400 (Seward Ltd., London, UK) (for 3 × 1 min). Ten-fold dilutions were then made in Maximum Recovery Diluent (Scharlau, Barcelona, Spain) and plated in duplicate on general and selective media. Total aerobic mesophilic bacteria, lactococci, lactobacilli and leuconostocs were counted as described above. Enterococci were counted on Slanetz and Bartley agar (SBA) (Merck) after incubation at 44 °C for 24 h. Enterobacteria and coliforms were grown on violet red bile glucose agar (VRBGA) and violet red bile lactose agar (VRBLA) (both from Merck), respectively, using the overlay pour plate technique. Bacteria were enumerated after 48 h of incubation at 30 °C. Staphylococci were grown on Baird-Parker agar (BPA) (Merck) supplemented with egg yolk tellurite solution (Merck). Black colonies with or without egg yolk clearance halos were recorded after 24 h of incubation at 37 °C. Yeasts and moulds were enumerated on yeast extract glucose chloramphenicol agar (YGCA) (Merck) after incubation at 25 °C for 3–5 days.

*Biochemical analysis*

Standard procedures of the International Dairy Federation (IDF) were followed to determine in duplicate basic chemical properties (protein, fats and total solid contents). Total nitrogen (TN) and other nitrogen fractions were determined by the Kjeldahl method (IDF 1993) using a Kjeldatherm KT 20S digestion apparatus and a Vapodest 50 titramatic distillation system (Gerhardt GmbH, Bonn, Germany). Total protein was calculated as 6.38 times the TN content (the usual conversion coefficient for dairy proteins). The fat content of milk and cheese in percentage was determined using a butyrometer, following the methods of Gerber (IDF 1981) and van Gulik (IDF 2008), respectively. Total solids for milk and curd/cheese were calculated by drying samples at 102 ± 2 °C until a constant weight was reached (IDF 1982 and IDF 1987, respectively). Sodium chloride (NaCl) was determined using a Chloride Analyzer (Corning Medical and Scientific, Halstead, UK) following the manufacturer's recommendations.

*Sensory analysis*

Ripe cheeses (30 days old) were finally subjected to sensory analysis by a trained panel of 20 experts (10 men and 10 women aged between 24 and 65 years) recruited from

among the IPLA staff. The terms describing the attributes of the cheeses were grouped into five categories (main descriptors): appearance (shape, size, rind colour, rind thickness, rind rugosity, inner colour, inner homogeneity and presence of eyes), odour (strength, cleanliness), taste (balance, spiciness, acidity, saltiness, bitterness, sourness, rancidness, and the presence of strange flavours), texture (firmness, creaminess, grain presence, and adherence) and overall impression. All these descriptors were rated on a hedonistic scale from 1 (very unsatisfactory) to 5 (very satisfactory).

### Statistical analysis

Averages and standard deviations were calculated for each parameter. Bacterial counts, chemical compounds and sensorial values were subjected to one-way ANOVA with the SPSS 11.0 software program (SPSS Inc., Chicago, IL, USA) using starter mixtures as a factor.

## RESULTS AND DISCUSSION

All the starter combinations coagulated the milk after 24 h of incubation at 30 °C. The final pH obtained ranged from 4.12 (combinations S2 and S4) to 4.37 (combination S9) (Table 2). In all cases, the pH fell a little further by 48 h of incubation. The acidification curves for the different combinations, produced over 24 h (Figure 2), were all similar to that of the Flora Danica commercial starter, except for combination S9 (which contained the lactococcin 972 producers). The latter took much longer to acidify the milk, and the final pH after 24 and 48 h was higher.

Small, mixture-specific differences were recorded for most microbial parameters. After 24 h of incubation in milk, total counts for the different starter combinations in M17 reached values >9 $\log_{10}$ cfu/mL except for combinations S5 and S9 (8.88 and 8.87 $\log_{10}$ cfu/mL respectively) (Table 2). Prolongation of the incubation period to 48 h under the same conditions greatly reduced the viability of the cells in all combinations (between 1 and almost 3 $\log_{10}$ cfu/mL). *Lc. lactis* subsp. *lactis* biovar diacetylactis strains reached, in all four combinations with this bacterium (S2, S5, S6 and S7), 1 $\log_{10}$ cfu/mL below that of the noncitrate fermenting lactococci. In the incubations involving combinations S6 and S7, the strains of *Leuconostoc* and *Lactobacillus* reached cell densities approximately half that of the lactococci at 24 h (Table 2). But, while the leuconostocs fell in number between 24 and 48 h, the lactobacilli increased in this period from 3.60 to 5.21 $\log_{10}$ cfu mL. Batch-to-batch differences have repeatedly been reported in small-scale cheese manufacture. Nevertheless, general trends in cell numbers and growth rates similar to those observed in this work have been reported elsewhere, using as starters either wild strains isolated from traditional dairy products (Ayad *et al.* 2000; Centeno *et al.* 2001; Randazzo *et al.* 2008; Franciosi *et al.* 2009; Nieto-Arribas *et al.* 2009) or commercial cultures (Centeno *et al.* 2001; Nieto-Arribas *et al.* 2009).

Table 3 shows the production or consumption of organic acids in the incubations with the different strain combinations. The table also shows the postfermentation lactose, glucose and galactose contents, as well as the results obtained with the Flora Danica complex microbial starter (Lodics and Steenson 1989). All the combinations produced large quantities of lactic acid, and small amounts of pyruvic, formic, uric, acetic and butyric acids, while hippuric, orotic and citric acids were consumed. Surprisingly, this last organic acid was not differentially used by combinations

**Table 2** Acidification of UHT milk and growth recoveries of the nine starter combinations in different culture media

| | | | *Microbial counts $Log_{10}$ cfu per g or per mL* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *pH of milk*[a] | | *M17A* | | *KMA* | | | | *MSEA* | | *MRSA* | |
| *Starter mixture* | *24 h* | *48 h* | *24 h* | *48 h* | *24 h L. lactis*[b] | *24 h L. diacetylactis*[b] | *48 h L. lactis* | *48 h L. diacetylactis* | *24 h* | *48 h* | *24 h* | *48 h* |
| S1 | 4.14 | 4.10 | 9.31 | 6.60 | – | – | – | – | – | – | – | – |
| S2 | 4.12 | 4.09 | 9.85 | 7.70 | 9.12 | 8.51 | 7.30 | 6.70 | – | – | – | – |
| S3 | 4.14 | 4.12 | 9.06 | 6.78 | – | – | – | – | – | – | – | – |
| S4 | 4.12 | 4.08 | 9.26 | 7.32 | – | – | – | – | – | – | – | – |
| S5 | 4.16 | 4.15 | 8.88 | 7.88 | 8.33 | 7.52 | 7.80 | 6.74 | – | – | – | – |
| S6 | 4.13 | 4.10 | 9.32 | 8.56 | 8.09 | 7.04 | 7.39 | 5.93 | 3.99 | 3.78 | – | – |
| S7 | 4.14 | 4.11 | 9.27 | 7.48 | 8.62 | 7.26 | 6.74 | 6.00 | 4.83 | 4.34 | 3.60 | 5.21 |
| S8 | 4.15 | 4.13 | 9.17 | 8.08 | – | – | – | – | – | – | – | – |
| S9 | 4.37 | 4.26 | 8.87 | 8.64 | – | – | – | – | – | – | – | – |
| FD | 4.14 | 4.09 | 9.29 | 8.44 | 8.56 | 6.95 | 7.40 | 6.35 | 3.47 | 4.58 | 4.20 | 5.83 |

–, not detected.

[a]Incubations were performed in triplicate and average results are shown; pH of uninoculated milk 6.68.

[b]*L. lactis, Lactococcus lactis* subsp. *lactis* or subsp. *cremoris; L. diacetylactis, L. lactis* subsp. *lactis* biovar diacetylactis.

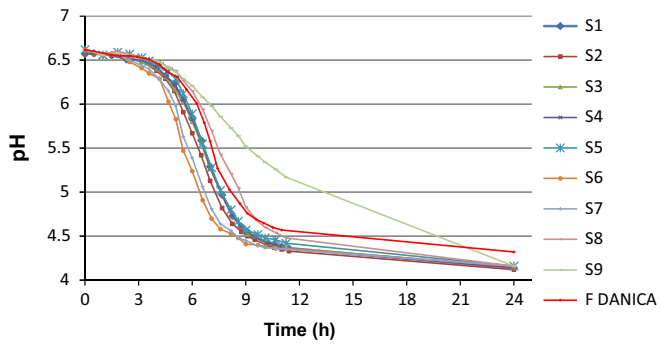


**Figure 2** Acidification curves in UHT milk of the nine experimental starter combinations and that of the commercial starter Flora Danica (Chr. Hansen) used as a control.

containing strains of *Lc. lactis* subsp. *lactis* biovar diacetylactis and *Leuconostoc* spp. (S2, S5, S6 and S7). The utilisation of milk citrate is linked to the production of diacetyl, a key odourant in many fermented dairy products. The consumption of all the citric acid in the milk (around 30 mg 100 per mL) and the production of more acetic acid (8.52 mg 100 per mL) were the main differences observed for the commercial Flora Danica starter with respect to the experimental mixtures. Lactose and galactose were the only sugars detected in milk after 48 h of incubation. With small combination-specific differences, all strains consumed lactose, releasing some galactose.

The consumption and production behaviours determined the final organic acid profile of the milks fermented with the different mixes. Lactic acid is the main end product of the metabolism of LAB, although these bacteria are known to produce small quantities of other organic acids such as acetic, formic and butyric acids (Passerini *et al.* 2013). Orotic acid is an intermediary in the synthesis of nucleotides (Østile *et al.* 2003); it may also act as a growth promoter for some LAB species. Pyruvic acid is a key intermediate in the metabolism of LAB (Mayo *et al.* 2010). Its presence at the end of fermentation may result from the (partial) lysis of cells and its consequent release into milk, or from its secretion during precursor/product exchanges (Mortera *et al.* 2013).

Table 4 shows the relative abundance of the different volatile compounds detected after growth in milk at 30 °C for 48 h. In agreement with the results obtained for organic acids, the greatest acetic acid content was obtained with the Flora Danica commercial starter. The same was true for the contents of diacetyl and acetoin. However, the experimental combinations produced more ethanol than the Flora Danica starter. Small differences were observed between the different mixtures for all other compounds, but no clear correlation and statistical significance between odour compounds and bacterial types was detected.

The free amino acid (FAA) content of the milk after 48 h of incubation was analysed as a measure of the proteolytic capacity of the starter combinations. Table 5 shows the postfermentation content in amino acids and related compounds. Experimental mixtures produce FAA profiles similar to each other, but different to that of Flora Danica (FD), for which most FAAs were shown to be statistically different to all others. The content of most amino acids, but especially glutamic acid (Glu), lysine (Lys) and proline (Pro), increased with all combinations. The aspartic acid (Asp) content, however, changed little, and glycine (Gly) concentrations were always lower after fermentation. The free

**Table 3** Presence of sugars and organic acids detected by HPLC after growth of the starter combinations at 30 °C for 48 h in UHT-treated milk

| | *Sugar*† | | | *Organic acid*† | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Starter mixture* | *Lactose* | *Glucose* | *Galactose* | *Orotic acid* | *Citric acid* | *Pyruvic acid* | *Lactic acid* | *Formic acid* | *Uric acid* | *Acetic acid* | *Butyric acid* | *Hippuric acid* |
| S1 | 694$^{a}$ | – | 5.43$^{a}$ | 0.46$^{a}$ | 27$^{a}$ | 1.69$^{a}$ | 154$^{a}$ | 1.1$^{a}$ | 0.27$^{a}$ | 1.5$^{a}$ | 0.71$^{a}$ | –$^{a}$ |
| S2 | 674$^{a}$ | – | 4.77$^{a}$ | 0.47$^{a}$ | 26$^{a}$ | 1.75$^{a}$ | 149$^{a}$ | 1.1$^{a}$ | 0.26$^{a}$ | 1.7$^{a}$ | 0.54$^{a}$ | –$^{a}$ |
| S3 | 697$^{a}$ | – | 4.51$^{ab}$ | 0.61$^{b}$ | 27$^{a}$ | 1.69$^{a}$ | 149$^{a}$ | 0.8$^{a}$ | 0.26$^{a}$ | 1.3$^{b}$ | 0.46$^{a}$ | –$^{a}$ |
| S4 | 707$^{a}$ | – | 5.10$^{b}$ | 0.41$^{a}$ | 27$^{a}$ | 1.59$^{a}$ | 156$^{a}$ | 1.1$^{a}$ | 0.28$^{a}$ | 1.3$^{b}$ | 0.62$^{a}$ | –$^{a}$ |
| S5 | 715$^{a}$ | – | 4.61$^{b}$ | 0.64$^{b}$ | 28$^{a}$ | 1.22$^{bc}$ | 156$^{a}$ | 1.1$^{a}$ | 0.28$^{a}$ | 1.5$^{ab}$ | 0.56$^{a}$ | –$^{a}$ |
| S6 | 715$^{a}$ | – | 4.72$^{a}$ | 0.38$^{a}$ | 28$^{a}$ | 2.50$^{a}$ | 155$^{a}$ | 1.1$^{a}$ | 0.28$^{a}$ | 1.4$^{a}$ | 0.88$^{b}$ | –$^{a}$ |
| S7 | 636$^{ab}$ | – | 4.17$^{b}$ | 0.31$^{a}$ | 25$^{a}$ | 2.37$^{a}$ | 138$^{b}$ | 1.0$^{a}$ | 0.24$^{a}$ | 1.3$^{b}$ | 0.74$^{ab}$ | –$^{a}$ |
| S8 | 691$^{a}$ | – | 4.15$^{b}$ | 0.73$^{b}$ | 27$^{a}$ | 2.17$^{a}$ | 143$^{ab}$ | 1.2$^{a}$ | 0.27$^{a}$ | 1.5$^{a}$ | 0.53$^{a}$ | –$^{a}$ |
| S9 | 667$^{a}$ | – | 5.69$^{a}$ | 0.60$^{b}$ | 25$^{a}$ | 1.80$^{bc}$ | 123$^{c}$ | 1.0$^{a}$ | 0.25$^{a}$ | 1.9$^{a}$ | 0.29$^{c}$ | 0.38$^{b}$ |
| FD | 597$^{b}$ | – | 6.49$^{c}$ | 1.04$^{c}$ | –$^{b}$ | 0.96$^{c}$ | 163$^{a}$ | 0.3$^{b}$ | 0.24$^{a}$ | 8.6$^{c}$ | 0.73$^{ab}$ | –$^{a}$ |
| Milk‡ | 851 | 0.94 | 1.91 | 1.23 | 28.97 | 0.04 | 1.50 | – | 0.21 | – | – | 0.61 |

†Concentration in mg per 100 mL.

‡Milk before starter addition; data are an average of the results obtained from three different milk samples used for growing the starters.

–, not detected; to perform statistical analysis, a value of 0 was considered.

Means in columns without common superscripts are significantly different ($P < 0.05$).

**Table 4** Relative abundance of volatile compounds after growth of the starter combinations at 30 °C for 48 h in UHT-treated milk

| | *Volatile compound*† | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Starter mixture* | *Acetaldehyde* | *Carbon disulfide* | *2-Methyl propanal* | *2-Propanone* | *3-Methyl butanal* | *Ethanol* | *Diacetyl* | *2-Methyl-1-propanol* | *3-Methyl-1-butanol* | *Acetoin* | *Acetic acid* |
| S1 | –[a] | 1.3[a] | –[a] | 0.30[a] | 0.14[a] | 3.71[a] | 0.08[a] | –[a] | –[a] | 0.04[a] | 0.46[ab] |
| S2 | –[a] | 3.7[b] | –[a] | 0.37[a] | 0.68[b] | 3.66[a] | 0.08[a] | 0.04[ab] | 0.09[a] | 0.03[a] | 0.35[ab] |
| S3 | –[a] | 2.0[ab] | 0.37[ab] | 0.12[b] | 1.25[bc] | 2.65[b] | 0.04[a] | 0.08[b] | 0.34[b] | 0.03[a] | 0.26[a] |
| S4 | –[a] | 1.2[a] | –[a] | 0.32[a] | 0.05[a] | 3.69[a] | 0.05[a] | –[a] | –[a] | 0.04[a] | 0.55[ab] |
| S5 | 0.20[ab] | –[c] | 0.56[b] | 0.17[b] | 1.81[c] | 3.23[a] | 0.07[a] | 0.08[b] | 0.53[b] | 0.06[a] | 0.43[ab] |
| S6 | –[a] | –[c] | –[a] | 0.38[a] | 0.27[a] | 3.86[a] | 0.13[ab] | –[a] | –[a] | 0.07[a] | 0.64[b] |
| S7 | 0.15[ab] | –[c] | –[a] | 0.39[a] | 0.11[a] | 4.14[a] | 0.24[b] | –[a] | –[a] | 0.13[a] | 0.69[b] |
| S8 | 0.23[ab] | –[c] | –[a] | 0.29[a] | 0.07[a] | 3.95[a] | 0.02[a] | –[a] | –[a] | 0.03[a] | 0.17[a] |
| S9 | 0.52[c] | –[c] | –[a] | 0.36[a] | 0.44[ab] | 3.97[a] | 0.04[a] | –[a] | 0.09[a] | 0.08[a] | 0.54[ab] |
| FD | –[a] | –[c] | –[a] | 0.42[a] | 0.10[a] | 1.26[c] | 0.35[b] | –[a] | –[a] | 2.44[b] | 1.38[c] |
| Milk | 0.19 | 0.5 | – | 0.30 | 0.04 | 0.85 | 0.05 | – | – | 0.08 | 0.25 |

†Concentration referred to the internal standard (cyclohexanone 0.36 mg/mL), to which a value of 1.00 was given.

–, not detected; to perform statistical analysis, a value of 0 was considered.

Means in columns without common superscripts are significantly different ($P < 0.05$).

amino acid content of cheese is used as an index of maturation (Vicente *et al.* 2001). Proteolysis during cheese ripening also strongly affects the sensory properties of cheeses, because free amino acids are major precursors of many aroma and taste compounds (Smit *et al.* 2005). Compared to commercial starters, wild *Lactococcus* strains have been reported to produce large amounts of volatile compounds via the degradation of branched-chain amino acids (BCCAs) (Leu, Ile, Val) (Urbach *et al.* 1997; Ayad *et al.* 1999; Dhaisne *et al.* 2013). BCCAs have a very low taste threshold and have been associated with malty and burnt notes in dairy products (Smit *et al.* 2005). However, when properly balanced with other odourants, they seem to be essential in generating the typical aroma profile of certain traditional cheeses (Curioni and Bosset 2002). Compounds derived from BCCAs (2-methylbutanal, 3-methylbutanal, 2-methylbutanol, etc.) were the majority type in the present work.

Small (and similar) quantities of histamine (Him), a harmful compound produced from the decarboxylation of histidine (His), were detected in all samples, including those of nonfermented controls. Tyramine and putrescine (other biogenic amines), however, were not detected. This strongly suggests that the tested strain combinations carry no biogenic amine producers. Indeed, many of the present strains have already been shown not to produce these compounds (Ladero V, Martín MC, Redruello B, Mayo B, Flórez AB, Fernández M, and Alvarez MA, unpublished). Interestingly, all the combinations produced certain levels of γ-aminobutyric acid (GABA), an inhibitory neurotransmitter associated with relaxing and antianxiety effects (Abdou *et al.* 2006).

The production of volatile compounds and free amino acids was repeated in the presence of small quantities of calf rennet (similar to that retained in most cheese types). As rennet is responsible for the majority of primary proteolysis (Irigoyen *et al.* 2000, van den Berg and Exterkate 1993), the aim was to test whether its presence in milk would modify their formation during fermentation. Under these conditions, the production of volatile compounds (data not shown) was unexpectedly similar to that recorded without rennet (no significant differences). The free amino acid contents recorded in the presence of rennet were also very similar to those obtained without it, although most amino acid concentrations were slightly higher (between 1 and 4.5%) but statistically not significant in most cases under the latter condition (data not shown). The proteolytic activity of the rennet may have been too low for it to have any appreciable effect over such a short incubation time.

Although they may show good technological characteristics, candidate starter mixtures still need to be assayed under real cheesemaking conditions to determine whether they meet manufacturing and ripening requirements. Based on their acidification rates (Figure 2) and overall aromatic profile while fermenting milk (mild acidic, clean, buttery flavour), four combinations S1, S4, S6 and S8 were selected for their use as starters in experimental trials for the production of a Gouda-like cheese (see Figure 1). Table 6 shows the composition and development of the majority and indicator bacterial groups detected during manufacturing and ripening of the cheeses made with all starter combinations. The numbers and types of micro-organisms found were normal for small-scale, artisanal products. Whether present in the milk or not, the cheeses became contaminated during manufacturing by certain numbers of enterococci, staphylococci (including *Staphylococcus aureus* in some samples, which might represent a health issue), coliforms and fungi.

**Table 5** Amino acids and derived compounds detected by HPLC after growth of the starters combinations at 30 °C for 48 h in UHT-treated milk

| | *Amino acid (μg/mL of milk)* | | | | | | | | | | | | | | | | | | | | | *Derived compound* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Starter mixture* | *αAla* | *βAla*† +Arg | *Asn* | *Asp* | *Cys* | *Cys-Cys* | *Gln* | *Glu* | *Gly* | *His* | *Ile* | *Leu* | *Lys* | *Met*‡ | *Phe* | *Pro* | *Ser* | *Thr* | *Trp* | *Tyr* | *Val* | $NH_4^+$ | *Gaba* | *Him* |
| S1 | $1.05^{a}$ | $0.46^{a}$ | $0.41^{ab}$ | $0.38^{a}$ | 0.21 | 14.65 | 0.10 | 7.37 | 0.17 | 1.19 | 0.24 | $1.20^{a}$ | $3.77^{a}$ | $0.14^{a}$ | $0.92^{a}$ | $4.13^{a}$ | $0.70^{a}$ | $1.01^{a}$ | $-^{a}$ | $1.97^{ab}$ | $0.70^{a}$ | $4.75^{a}$ | $1.80^{a}$ | $0.09^{a}$ |
| S2 | $0.91^{ab}$ | $0.44^{a}$ | $0.45^{b}$ | $0.43^{a}$ | $0.47^{a}$ | $14.4^{ab}$ | $0.16^{a}$ | $1.50^{a}$ | $0.14^{a}$ | $1.17^{ab}$ | $0.25^{a}$ | $1.11^{a}$ | $3.88^{ac}$ | $0.13^{a}$ | $0.91^{a}$ | $3.60^{a}$ | $0.69^{a}$ | $1.01^{a}$ | $-^{a}$ | $1.95^{ab}$ | $0.66^{a}$ | $4.53^{a}$ | $6.29^{b}$ | $0.09^{a}$ |
| S3 | $0.55^{b}$ | $0.48^{a}$ | $0.34^{a}$ | $0.36^{a}$ | $0.67^{a}$ | $10.5^{b}$ | $0.12^{a}$ | $6.22^{b}$ | $0.13^{a}$ | $0.61^{b}$ | $0.17^{a}$ | $0.82^{a}$ | $2.73^{b}$ | $0.10^{a}$ | $1.23^{a}$ | 2.71b | $0.69^{a}$ | $0.83^{a}$ | $-^{a}$ | $1.63^{b}$ | $0.43^{b}$ | $4.60^{a}$ | $1.33^{a}$ | $0.06^{a}$ |
| S4 | $1.20^{a}$ | $0.52^{a}$ | $0.54^{b}$ | $0.41^{a}$ | $0.21^{b}$ | $17.1^{a}$ | $0.17^{a}$ | $8.47^{b}$ | $0.20^{ab}$ | $1.28^{a}$ | $0.44^{b}$ | $1.45^{a}$ | $4.20^{c}$ | $0.20^{a}$ | $1.13^{a}$ | $3.87^{a}$ | $0.76^{a}$ | $1.12^{a}$ | $-^{a}$ | $2.01^{ab}$ | $0.86^{a}$ | $4.80^{a}$ | $1.70^{a}$ | $0.12^{a}$ |
| S5 | $0.83^{ab}$ | $0.53^{a}$ | $0.48^{b}$ | $0.39^{a}$ | $0.45^{a}$ | $12.4^{b}$ | $0.15^{a}$ | $2.34^{a}$ | $0.17^{a}$ | $0.92^{ab}$ | $0.26^{a}$ | $1.10^{a}$ | $3.67^{a}$ | $23.4^{b}$ | $1.37^{a}$ | $3.93^{a}$ | $0.93^{a}$ | $1.23^{a}$ | $-^{a}$ | $2.59^{a}$ | $0.67^{a}$ | $4.99^{a}$ | $5.81^{b}$ | $0.07^{a}$ |
| S6 | $1.42^{a}$ | $0.56^{a}$ | $0.60^{bc}$ | $0.44^{a}$ | $0.29^{ab}$ | $20.0^{a}$ | $0.10^{a}$ | $8.48^{b}$ | $0.22^{ab}$ | $1.50^{a}$ | $0.30^{ab}$ | $1.33^{a}$ | $4.70^{c}$ | $23.5^{b}$ | $1.22^{a}$ | $5.01^{c}$ | $0.99^{a}$ | $1.21^{a}$ | $-^{a}$ | $2.82^{a}$ | $0.76^{a}$ | $5.24^{a}$ | $3.29^{ab}$ | $0.11^{a}$ |
| S7 | $1.20^{a}$ | $0.48^{a}$ | $0.49^{b}$ | $0.42^{a}$ | $0.19^{b}$ | $16.9^{a}$ | $0.12^{a}$ | $9.15^{b}$ | $0.18^{a}$ | $1.31^{a}$ | $0.24^{a}$ | $1.19^{a}$ | $3.95^{ac}$ | $20.1^{b}$ | $0.98^{a}$ | $5.11^{c}$ | $0.82^{a}$ | $0.99^{a}$ | $-^{a}$ | $2.38^{a}$ | $0.66^{a}$ | $4.46^{a}$ | $1.27^{a}$ | $0.08^{a}$ |
| S8 | $0.96^{a}$ | $0.51^{a}$ | $0.36^{a}$ | $0.38^{a}$ | $0.16^{b}$ | $9.4^{b}$ | $-^{b}$ | $7.51^{b}$ | $0.16^{a}$ | $0.78^{b}$ | $0.11^{ac}$ | $0.49^{b}$ | $2.73^{b}$ | $19.1^{b}$ | $0.77^{a}$ | $2.97^{b}$ | $0.82^{a}$ | $1.06^{a}$ | $-^{a}$ | $1.44^{b}$ | $0.40^{b}$ | $3.64^{ab}$ | $1.06^{a}$ | $0.07^{a}$ |
| S9 | $3.59^{c}$ | $0.49^{a}$ | $0.93^{c}$ | 0.40 | $0.15^{b}$ | $8.8^{b}$ | $0.17^{a}$ | $7.27^{b}$ | $0.37^{b}$ | $0.56^{b}$ | $0.05^{c}$ | $0.04^{c}$ | $2.81^{b}$ | $21.1^{b}$ | $0.83^{a}$ | $2.52^{b}$ | $1.07^{a}$ | $1.02^{a}$ | $-^{a}$ | $1.53^{b}$ | $0.03^{c}$ | $1.44^{b}$ | $0.09^{c}$ | $0.22^{b}$ |
| FD | $0.63^{b}$ | $0.55^{a}$ | $0.36^{a}$ | $0.40^{a}$ | $0.34^{ab}$ | $19.7^{a}$ | $0.40^{c}$ | $7.94^{b}$ | $0.09^{a}$ | $0.98^{ab}$ | $0.02^{c}$ | $0.18^{bc}$ | $3.60^{a}$ | $21.2^{b}$ | $0.92^{a}$ | $5.68^{c}$ | $0.59^{a}$ | $0.44^{b}$ | $0.12^{b}$ | $1.62^{b}$ | $0.18^{bc}$ | $35.23^{c}$ | $1.02^{a}$ | $0.09^{a}$ |
| Milk | 0.33 | 0.71 | – | 0.51 | 0.30 | 1.1 | 0.08 | 2.91 | 0.67 | 0.04 | 0.06 | 0.06 | 0.45 | 12.9 | 0.05 | 0.24 | 0.15 | 0.15 | – | 0.15 | 0.13 | 2.60 | – | 0.13 |

–, not detected; to perform statistical analysis, a value of 0 was considered.

FD, milk incubated with the commercial starter Flora Danica (Chr. Hansen).

Milk 1–4, 5–9; mixed samples of milk from the uninoculated blanks of the different assays of the corresponding starter mixtures.

Gaba: gamma-aminobutyric acid; Him, histamine.

The biogenic amines tyramine and putrescine were also analysed but never recorded.

†βAla is an enantiomer produced during processing of the sample of the natural protein constituent amino acid L-α-alanine (αAla). In the conditions of analysis, βAla elutes together with the peak of arginine (Arg).

‡Quantification of Met (methionine) is not accurate, as its peak elutes as part of a peak including buffer and internal standard reagents.

Means in columns without common superscripts are significantly different ($P < 0.05$).

**Table 6** Microbial counts (in $Log_{10}$ cfu per g or mL) of diverse microbial groups along manufacturing and ripening stages of the experimental cheeses made with the starter mixtures S1, S4, S6 and S8

| *Microbial group* | | *Stage of manufacturing and ripening of the cheeses* | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Curd* | | | | *3 days* | | | | *7 days* | | | | *15 days* | | | | *30 days* | | | |
| *(counting medium)* | *Milk* | *S1* | *S4* | *S6* | *S8* | *S1* | *S4* | *S6* | *S8* | *S1* | *S4* | *S6* | *S8* | *S1* | *S4* | *S6* | *S8* | *S1* | *S4* | *S6* | *S8* |
| Total aerobic counts (PCA) | 5.62 | 9.02[a] | 9.08[a] | 9.02[a] | 8.34[b] | 9.41[a] | 9.68[a] | 9.50[a] | 9.29[a] | 9.37[a] | 9.48[a] | 9.56[a] | 9.47[a] | 9.33[a] | 9.45[a] | 9.45[a] | 9.34[a] | 8.92[a] | 9.27[a] | 9.17[a] | 8.96[a] |
| Lactococci (M17A) | 5.62 | 9.07[a] | 8.99[a] | 9.06[a] | 8.42[b] | 9.46[a] | 9.69[a] | 9.59[a] | 9.28[a] | 9.35[a] | 9.45[a] | 9.22[a] | 9.54[a] | 9.25[a] | 9.42[a] | 9.49[a] | 8.98[a] | 8.90[a] | 9.19[a] | 8.92[a] | 8.78[a] |
| Lactobacilli (MRSA) | <1.0 | <2.0[a] | <2.0[a] | <2.0[a] | 2.30[b] | 3.30[a] | 3.30[a] | 4.00[a] | 3.48[a] | 4.78[a] | 4.19[a] | 4.08[a] | 3.65[b] | 4.89[a] | 5.9[ab] | 6.43[b] | 5.28[a] | 5.86[a] | 6.8[ab] | 7.76[b] | 6.5[ab] |
| Leuconostoc (MSEA) | <1.0 | <2.0[a] | <2.0[a] | 3.30[b] | <2.0[a] | <2.0[a] | <2.0[a] | 5.74[b] | <2.0[a] | 3.70[a] | <2.0[b] | 5.86[c] | 3.1[ab] | 3.70[a] | <2.0[b] | 5.76[c] | <2.0[b] | 3.70[a] | 2.60[b] | 5.82[c] | <2.0[b] |
| Enterococci (SBA) | <1.0 | 1.70[a] | <2.0[a] | 1.70[a] | 2.00[a] | 2.48[a] | 2.40[a] | 2.65[a] | <2.0[a] | <2.0[a] | <2.0[a] | 2.78[b] | 2.3[ab] | 2.00[a] | <2.0[a] | 2.48[a] | 2.40[a] | 2.18[a] | 2.70[b] | 2.74[b] | 2.18[a] |
| Staphylococci (BPA) | 1.78 | 4.19[a] | 2.9[b] (2.0)[†] | 3.5[ab] (2.6) | 3.6[ab] (2.8) | 4.19[a] | 4.53[a] | 5.64[b] | 6.13[b] | 4.19[a] (2.9) | 3.57[b] (2.6) | 5.31[c] (2.9) | 5.91[a] (4.9) | 4.19[a] (2.0) | 3.70[a] | 5.00[b] | 5.21[b] | 4.19[a] | 2.88[b] | 5.06[a] | 4.62[a] |
| Enterobacteriaceae (VRBGA) | 2.20 | 5.34[a] | 4.80[a] | 4.60[a] | 4.60[a] | 4.85[a] | 4.76[a] | 4.70[a] | 6.17[b] | 4.34[a] | 5.04[a] | 5.41[a] | 5.74[a] | 4.53[a] | 4.67[a] | 4.89[a] | 5.24[a] | 4.41[a] | 4.24[a] | 4.88[a] | 4.56[a] |
| Coliforms (VRBLA) | 1.70 | 5.30[a] | 4.92[a] | 4.98[a] | 4.71[a] | 5.64[a] | 5.34[a] | 5.17[a] | 6.02[a] | 4.01[a] | 4.59[a] | 5.29[b] | 5.69[b] | 4.36[a] | 4.41[a] | 4.81[a] | 4.89[a] | 3.84[a] | 4.21[a] | 4.58[a] | 4.51[a] |
| Yeasts and moulds (YGCA) | 1.70 (1.7)[‡] | 3.90[a] (3.2) | 2.90[a] | 3.36[a] (3.3) | 1.92[b] (1.9) | (3.8)[a] | 3.49[a] | 2.00[b] | 2.00[b] | 2.88[a] (2.4) | 3.64[a] | 2.81[a] (2.7) | 2.60[b] (2.5) | 2.60[a] | 2.74[a] | 2.40[a] (2.2) | 2.54[a] (2.5) | 2.92[a] | 2.30[a] | 2.40[a] | 2.18[a] (2.2) |

[†]In brackets, numbers of egg yolk clearing colonies in BPA (presumably, *Staphylococcus aureus*).

[‡]Numbers of yeast-like colonies in YGCA.

Means in lines in each manufacturing stage without common superscripts are significantly different ($P < 0.05$).

**Table 7** Biochemical parameters along the manufacturing and ripening stages of the experimental cheeses made with four starter mixtures S1, S4, S6 and S8

| *Biochemical parameter* | *Milk* | *Stage of manufacturing and ripening of the cheeses* | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Curd* | | | | *3 days* | | | | *7 days* | | | | *15 days* | | | | *30 days* | | | |
| | | *S1* | *S4* | *S6* | *S8* | *S1* | *S4* | *S6* | *S8* | *S1* | *S4* | *S6* | *S8* | *S1* | *S4* | *S6* | *S8* | *S1* | *S4* | *S6* | *S8* |
| pH | 6.6 | $6.1^{a}$ | $6.1^{a}$ | $6.1^{a}$ | $6.2^{a}$ | $5.1^{a}$ | $5.1^{a}$ | $5.1^{a}$ | $5.2^{a}$ | $5.1^{a}$ | $5.1^{a}$ | $5.1^{a}$ | $5.2^{a}$ | $5.1^{a}$ | $5.1^{a}$ | $5.1^{a}$ | $5.2^{a}$ | $5.1^{a}$ | $5.1^{a}$ | $5.1^{a}$ | $5.1^{a}$ |
| Total solids % (w/w) | 12.1 | $39.6^{a}$ | $40.4^{a}$ | $33.3^{b}$ | $36.2^{ab}$ | $49.4^{a}$ | $50.3^{a}$ | $45.7^{b}$ | $47.6^{ab}$ | $53.0^{a}$ | $51.1^{a}$ | $46.7^{b}$ | $49.2^{a}$ | $56.7^{a}$ | $55.8^{a}$ | $50.9^{b}$ | $52.9^{b}$ | $61.2^{a}$ | $56.9^{ab}$ | $51.3^{b}$ | $54.8^{ab}$ |
| Fat % (w/w) | 3.6 | $16.5^{a}$ | $17.5^{a}$ | $14.5^{a}$ | $16.0^{a}$ | $21.5^{a}$ | $22.0^{a}$ | $19.0^{a}$ | $20.0^{a}$ | $22.0^{a}$ | $23.0^{a}$ | $21.0^{a}$ | $20.5^{a}$ | $23.5^{a}$ | $24.0^{a}$ | $24.0^{a}$ | $22.5^{a}$ | $25.5^{a}$ | $24.5^{a}$ | $24.5^{a}$ | $23.0^{a}$ |
| Fat/total solids Index | 29.7 | $41.7^{a}$ | $43.3^{a}$ | $43.6^{a}$ | $44.2^{a}$ | $43.5^{a}$ | $43.8^{a}$ | $41.6^{a}$ | $42.0^{a}$ | $41.5^{a}$ | $45.0^{b}$ | $45.0^{b}$ | $41.6^{a}$ | $41.5^{a}$ | $43.0^{ab}$ | $47.2^{b}$ | $42.5^{ab}$ | $41.7^{a}$ | $43.8^{a}$ | $47.7^{b}$ | $42.0^{a}$ |
| Salt % (w/w) | 0.04 | $0.2^{a}$ | $0.2^{a}$ | $0.2^{a}$ | $0.2^{a}$ | $1.0^{a}$ | $0.9^{a}$ | $0.9^{a}$ | $0.6^{b}$ | $1.4^{a}$ | $1.2^{a}$ | $1.3^{a}$ | $1.1^{a}$ | $1.6^{a}$ | $1.4^{a}$ | $1.6^{a}$ | $1.2^{b}$ | $1.6^{a}$ | $1.5^{a}$ | $1.8^{a}$ | $1.7^{a}$ |
| Nitrogen content (% w/w) | | | | | | | | | | | | | | | | | | | | | |
| Total protein | 3.1 | $16.3^{a}$ | $15.2^{a}$ | $15.5^{a}$ | $17.5^{a}$ | $21.9^{a}$ | $21.8^{a}$ | $20.3^{a}$ | $23.5^{b}$ | $21.5^{a}$ | $22.1^{a}$ | $20.7^{a}$ | $21.3^{a}$ | $22.3^{a}$ | $21.9^{a}$ | $21.9^{a}$ | $21.4^{a}$ | $22.7^{a}$ | $22.1^{a}$ | $23.1^{a}$ | $23.0^{a}$ |
| Casein | 2.6 | $15.6^{ab}$ | $14.4^{a}$ | $14.6^{a}$ | $16.6^{b}$ | $20.8^{a}$ | $20.6^{a}$ | $18.7^{a}$ | $21.4^{a}$ | $19.8^{a}$ | $20.5^{a}$ | $18.3^{a}$ | $18.5^{a}$ | $20.3^{a}$ | $19.8^{a}$ | $18.5^{a}$ | $16.7^{b}$ | $20.3^{a}$ | $19.3^{a}$ | $18.9^{a}$ | $18.0^{b}$ |
| Soluble nitrogen | 0.5 | $0.7^{a}$ | $0.8^{ab}$ | $0.8^{ab}$ | $0.9^{b}$ | $1.1^{a}$ | $1.2^{a}$ | $1.6^{ab}$ | $2.1^{b}$ | $1.6^{a}$ | $1.6^{a}$ | $2.4^{b}$ | $2.8^{b}$ | $2.0^{a}$ | $2.1^{a}$ | $3.5^{b}$ | $4.7^{c}$ | $2.5^{a}$ | $3.8^{ab}$ | $4.3^{ab}$ | $5.0^{b}$ |
| Nonprotein nitrogen | 0.2 | $0.2^{a}$ | $0.2^{a}$ | $0.2^{a}$ | $0.2^{a}$ | $0.8^{a}$ | $0.8^{a}$ | $1.0^{a}$ | $0.7^{a}$ | $1.2^{a}$ | $1.4^{a}$ | $1.4^{a}$ | $1.1^{a}$ | $1.8^{a}$ | $1.8^{a}$ | $2.0^{a}$ | $1.7^{a}$ | $2.3^{a}$ | $2.3^{a}$ | $2.6^{a}$ | $2.5^{a}$ |

Means in lines in each manufacturing stage without common superscripts are significantly different ($P < 0.05$).

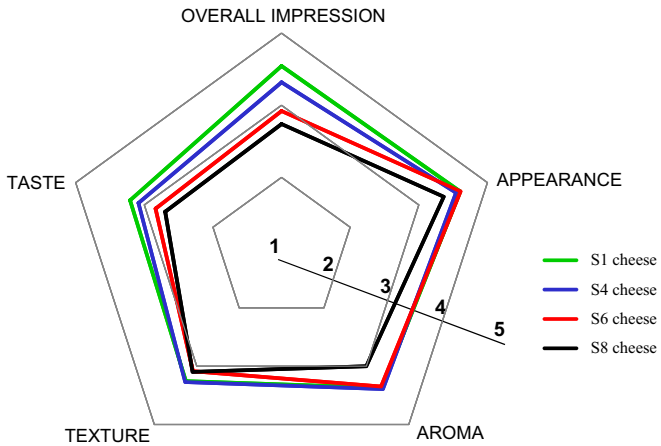


**Figure 3** Radial diagram showing the average scores for the five main cheese descriptors during the evaluation of the sensory characteristics of experimental cheeses. The five points of the hedonistic scale are also indicated.

Their populations reached only discrete numbers compared to those of the dominant lactococci (starter) (which reached >9 $\log_{10}$ cfu/g) between days 3 and 7. Lactobacilli formed the subdominant population in all mature cheeses. Although undetectable in the pasteurised milk, they developed slowly but firmly to reach cell densities of around 7.0 $\log_{10}$ cfu/g of cheese.

Smaller numbers of Gram-positive populations were recorded during ripening when nisin producers were used as starter components (Table 6). The inhibition of Gram-positive bacteria (including experimentally added pathogens) by nisin-producing starters has been reported (Roberts *et al.* 1992; Rodríguez *et al.* 1998; Rilla *et al.* 2004). Lactococci that produce bacteriocins other than nisin have also been used as starters or adjunct cultures to improve food quality and safety (O'Sullivan *et al.* 2003; Dal Bello *et al.* 2012), which argues in favour of the experimental assessment of the little-acidifying combination S9 (lactococcin 972 producers) in future work.

The chemical variables (including the nitrogen fractions) used as indices of ripening showed similar trends for all combinations (Table 7). Although an identical cheesemaking protocol was followed in all four batches, the small manufacturing scale makes the process alike to those of farmhouse cheeses, in which unpredictable differences (amount of rennet, whey drainage, applied pressure, position in the ripening room, etc.) lead to ample variations in the value of chemical and microbial parameters. Their values fall within the normal limits for artisanal cheeses (Estepar *et al.* 1999; Arenas *et al.* 2004; Horne *et al.* 2005; Ballesteros *et al.* 2006). The sensorial properties of the cheeses produced in the four manufacturing trials were scored by a panel of 20 experts. The results are shown in Figure 3. Except for the cheese made with combination S8, all had a similar appearance and aroma. The texture of all cheeses was also very similar, although those made with S6 and S8 received slightly lower scores. Large differences were found, however, in terms of taste and overall impression, for which cheeses made with combinations S1 and S4 scored best. This suggests that these two combinations can be confidently used as starters, at least in artisanal-scale cheesemaking. Other trials should be performed to check the suitability of the untested bacterial combinations as starters in specific situations. That containing the lactococcin 972 producers might be of particular interest. Acid production by this mixture is slower, but it may still be sufficient, resulting in a more protective culture than that offered by the nisin producers (S8).

## CONCLUSIONS

In summary, the present results, and those reported by other authors, show that technologically useful LAB strains can be obtained from traditional cheeses. The acidification efficiency of most of the LAB starter combinations tested – a key technological variable – proved to be highly similar to that of the successful commercial culture Flora Danica. Individual strains of the blends can be selected as components of mixed-strain commercial starters. Alternatively, the studied mixtures could be used as defined-strain starters, suitable for most artisan and traditional cheeses. In this sense, large-scale cheese manufacturing trials involving combinations S1 and S4 will shortly be undertaken. Native bacterial strains from prestigious cheeses with a PDO status, such as Cabrales (coded A or AA) and Casín (coded CAS), might be used as starter components to create product-specific cultures, investing these cheese varieties with an extra added value.

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