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ABSTRACT

The impact of Gram-negative bacteria on sensory characteristics and production of volatile compounds as well as biogenic amines (BA) in the core of an uncooked pressed type model cheese was investigated in the presence of a defined complex microbial consortium. Eleven strains of Gram-negative bacteria, selected on the basis of their biodiversity and *in vitro* BA-production ability, were individually tested in a model cheese. Four out of 6 strains of *Enterobacteriaceae* (*Citrobacter freundii* UCMA 4217, *Klebsiella oxytoca* 927, *Hafnia alvei* B16 and *Proteus vulgaris* UCMA 3780) reached counts close to 6 log CFU g^{-1} in the model cheese. In core of cheeses inoculated with Gram-negative bacteria, only slight differences were observed for microbial counts (*Enterococcus faecalis* or *Lactobacillus plantarum* count differences below 1 log CFU g^{-1}), acetate concentration (differences below 200 mg kg^{-1}) and texture (greater firmness) in comparison to control cheeses. Cheese core colour, odour and volatile compound composition were not modified. Although ornithine, the precursor of putrescine, was present in all cheeses, putrescine was only detected in cheeses inoculated with *H. alvei* B16 and never exceeded 2.18 mmol kg^{-1} cheese dry matter. Cadaverine was only detected in cheeses inoculated with *H. alvei* B16, *K. oxytoca* 927, *Halomonas venusta* 4C1A or *Morganella morganii* 3A2A but at lower concentrations (<1.05 mmol kg^{-1} cheese dry matter), although lysine was available. Only insignificant amounts of the detrimental BA histamine and tyramine, as well as isopentylamine, tryptamine or phenylethylamine, were produced in the cheese model by any of the Gram-negative strains, including those which produced these BA at high levels *in vitro*.

1. Introduction

Microbial diversity in raw milk including yeasts, moulds, Gram-positive and Gram-negative bacteria is a core element in the cheese-making process. It is needed to maintain sensory quality and diversity while ensuring microbiological safety. In raw milk, a wide range of Gram-negative bacteria (Lafarge et al., 2004) was found at relatively high population levels (up to 10^5 CFU ml^{-1}) (Ercolini et al., 2009; Munsch-Alatossava and Alatossava, 2006). In a study by Coton et al. (2012), among 173 Gram-negative strains isolated from milks and various cheeses, 68 Gram-negative species were identified and nearly half belonged to the *Enterobacteriaceae* family. *Enterobacteriaceae* counts can reach 10^6–10^7 CFU g^{-1} in the core of uncooked pressed cheeses during the first days of ripening and then slowly decrease until the end of ripening (Pintado et al., 2008; Tornadijo et al., 2001). In the cores of hard or uncooked pressed cheeses such as Pecorino, Saint-Nectaire or San Simón, they represented from 14% to 56% of the isolates (Chaves-Lopez et al., 2006;

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Delbes et al., 2007; Tornadijo et al., 2001). On the surface of European smear cheeses, Gram-negative bacteria were shown to account for 18–60% of bacterial isolates (Larpin-Laborde et al., 2011; Mounier et al., 2005; Maoz et al., 2003).

The actual contribution of Gram-negative bacteria in regards to the development of organoleptic characteristics in cheese is still unclear. Most Gram-negative bacteria in cheese are considered as spoilage flora as they can be responsible for defects in cheese texture and flavour (Sørhaug and Stepaniak, 1997). However, some species were found to influence organoleptic properties; for example one *Proteus vulgaris* strain was found to produce significant amounts of volatile compounds in a model cheese medium (Deetae et al., 2009b) and some *Hafnia alvei* strains can be used as ripening agents (Alonso-Calleja et al., 2002).

High counts of *Enterobacteriaceae* are considered indicative of poor hygienic quality in milking and cheese-making processes but the safety status of most food-borne Gram-negative bacteria is poorly documented. One aspect of the risks associated with cheese bacteria concerns their ability to form biogenic amines (BA). *Enterobacteriaceae* may produce BA in cheese which may be detrimental to consumer health (Marino et al., 2000; Martuscelli et al., 2005). Cheese-associated strains of *Citrobacter freundii*, *H. alvei*, *Morganella morganii*, *Klebsiella oxytoca*, *Enterobacter* sp. and *Serratia liquefaciens* have been found to produce significant amounts of BA *in vitro* (Coton et al., 2012; Marino et al., 2000; Pattono et al., 2008). In the study by Coton et al. (2012), cadaverine was the most frequent BA produced *in vitro*, followed by isoamylamine, histamine and putrescine. However, the ability to form biogenic amines *in vitro* may not reflect their actual production in cheese. In this context, cadaverine was frequently found in variable amounts in blue cheeses (up to 1.86 mmol kg^{-1}) (Marino et al., 2000), hard cooked cheeses (Bütikofer et al., 1990) as well as in uncooked pressed cheeses (up to 6 mmol kg^{-1} in Asino cheese), especially cheeses with high *Enterobacteriaceae* counts (Innocente et al., 2009; Pintado et al., 2008; Schirone et al., 2011; Schneller et al., 1997). Indeed, even within a cheese type, environmental and microbiological factors can influence BA-production (Buňková et al., 2010).

The aim of the present work was to develop a methodology to evaluate the potentialities and risks associated with Gram-negative bacteria within uncooked pressed cheese ecosystems, in terms of cheese sensory characteristics and biogenic amines production. An uncooked pressed type cheese prepared with a defined microbial consortium and validated as a model favorable to the growth of Gram-negative bacteria, was used to investigate the impact in the cheese core of 11 strains of Gram-negative bacteria selected from the collection studied by Coton et al. (2012). Microbial growth, sensory characteristics, production of organic acids, volatile compounds and biogenic amines in cheese in the presence of each Gram-negative strain were assessed.

2. Materials and methods

2.1. Model cheese manufacture and ripening

Pasteurized milk (72 °C, 20 s) was used to prepare 500 g model cheeses using an uncooked pressed cheese process. The entire cheese-making process was carried out using sterile tools and equipment. Milk was placed into vats and warmed up to 33 °C. The starter culture *Streptococcus salivarius* ssp. *thermophilus* 1a was isolated from the MY800 commercial starter culture (Rhodia S.A., Paris La Défense, France) commonly used to prepare uncooked pressed cheeses. It was inoculated in all vats ($\sim 10^7$ CFU ml^{-1}). A technological consortium (see Section 2.2) was also added to all vats at a concentration of 10^3 CFU ml^{-1} and 10^2 CFU ml^{-1} for each bacterial and yeast strain, respectively. Rennet was added to all vats at 1.51 g l^{-1} (Beaugel 520 mg l^{-1} of chymosin). Coagulation proceeded for approximately 45 min and then the curd was cut, prepressed and placed in molds. Salt (2% of dry matter) was added and the curd was drained under 3-bar pressure for 24 h. The cheeses were coated with wax containing 1000 ppm of natamycin (Coquard, Villefranche sur Saône, France) to prevent the development of moulds and yeasts on the cheese surface. They were then ripened in sterile stainless steel boxes in a ripening room at 9 °C for 28 days. Samples of milk were taken aseptically from each vat prior to the addition of the rennet as well as samples from the cheeses (corresponding to one half of each cheese) were taken on days 1, 8, 18 and 28. The cheese rind (5 mm thick) was discarded. Subsamples were stored either at −80 °C for volatile aroma compounds analysis or at −20 °C for further microbiological tests and determination of biochemical parameters (organic acids, free amino acids index and biogenic amine profile).

2.2. Microbial strains

The technological consortium consisted of 8 bacteria (*Lactococcus lactis* Tan4, *Enterococcus faecalis* SB1, *Lactobacillus plantarum* FH3, *Leuconostoc mesenteroides* MSE7, *Str. salivarius* spp. *thermophilus* MY800 1a, *Arthrobacter arilaitensis* Mu107, *Corynebacterium casei* CRBM9, *Staphylococcus equorum* RPF6) and 2 yeasts (*Debaryomyces hansenii* OGA10, *Yarrowia lipolytica* Sn4Co3). It was chosen to mimic a representative community comprising the major microbial groups commonly found in raw milk cheese. The strains were selected depending on their ability to develop together in the model cheese as determined in preliminary trials (data not shown). All strains were obtained from the INRA UR545 collection (Aurillac, France), except for Mu107 (INRA UMR782, Thiverval-Grignon, France).

Eleven Gram-negative strains originating from the work collection studied by Coton et al. (2012) (Table 1) were selected on the basis of species biodiversity and risk factors determined *in vitro*, in particular BA production (Table 3; Coton et al., 2012; Imran et al., pers. com.), as well as for their ability to grow together in presence of the technological consortium in the model cheese in preliminary trials (data not shown). Thus, eight strains that clearly became established in the cheese core during ripening (PCA Q6.3, CV 30.6, UCMA 4217, 3A2A, 927, B16, UCMA 3780, 4C1A) and three strains whose establishing was doubtful (B39, Pi18, PCAi D6.5) were selected for the experiment. They came from the INRA UR545 collection (PCA Q6.3, CV 30.6, Pi18, PCAi F2.5), INRA UMR782 collection (B16, B39, 3A2A, 4C1A), University of Caen – MILA collection (UCMA 3780, UCMA 4217) or Aérial collection, Illkirch, France (927). They were grown in Brain Heart Infusion medium incubated at 30 °C for 24 h. All Gram-positive and Gram-negative bacteria and yeast pure strains were stored in 5%-glycerol semi-skimmed UHT milk at −80 °C until use.

2.3. Experimental design

In all cheese-making experiments, the tested Gram-negative strain was inoculated into milk at a concentration of 10^3 CFU ml^{-1}. Milk batches inoculated only with the technological consortium were included in each experiment on each day of manufacture (control cheeses).

2.3.1. Experiment 1: impact of Gram-negative strains at the end of cheese ripening

A unique model cheese was made with each of the 11 Gram-negative strains on the same day and the trial was replicated once two days later. Microbial counts as well as profiles for organic acids, volatile compounds and biogenic amines were determined, and sensory analyses performed, on the core of the model cheese at the end of ripening (28 days). Six Gram-negative strains that reached the highest counts and/or were BA-producers in cheese

Table 1
Counts and effect of Gram-negative strains on microbial counts and organic acids in model cheese core after 28 days (experiment 1).

Strain	Species	Family	Gram negative counts[a]	Cheese class[b]	Microbial counts and organic acids per cheese class[a]								
					E. faecalis (SB)			*Lb. plantarum* (FH)			Acetate		
					Mean	SD		Mean	SD		Mean	SD	
UCMA 4217	*Citrobacter freundii*	*Enterobacteriaceae*	7.1	1, 1									
927	*Klebsiella oxytoca*	*Enterobacteriaceae*	5.5	1, 1	6.34	0.32	a	7.19	0.30	b	2.20	0.18	c
B16	*Hafnia alvei 1*	*Enterobacteriaceae*	6.5	1, 1									
PCA Q6.3	*Pantoea agglomerans*	*Enterobacteriaceae*	3.2	1, 2									
Pi18	*Chryseobacterium* sp. close to *C. bovis*	*Flavobacteriaceae*	<2	1, 2									
3A2A	*Morganella morganii*	*Enterobacteriaceae*	3.0	2, 2	6.08	0.24	a	6.08	0.20	a	1.57	0.34	a
4C1A	*Halomonas venusta/alkaliphila/hydrothermalis*	*Halomonadaceae*	3.2	2, 3									
B39	*Halomonas* sp.	*Halomonadaceae*	<2	3, 3									
UCMA 3780	*Proteus vulgaris*	*Enterobacteriaceae*	4.0	3, 3									
CV30.6	*Pseudomonas putida*	*Pseudomonadaceae*	3.0	3, 3	6.87	0.25	b	7.05	0.31	b	1.85	0.18	b
PCAi D6.5	*Stenotrophomonas maltophilia*	*Xanthomonadaceae*	<2	3, 3									
Control	No Gram-negative strain added		<2	3, 3									
					***			***			**		

[a] Counts in log CFU g^{-1}, organic acid concentrations in g per kg of cheese. Only the microbial groups and organic acids for which a significant effect of cheese class was observed are indicated in the table. SB: Slanetz and Bartley agar. FH: facultatively heterofermentative lactobacilli agar.
[b] Class determined after hierarchical classification based on Gram-positive bacteria counts in cheeses inoculated with each Gram-negative strain. Digits separated by a comma indicate the cheese class to which each replicate 1 and 2 from experiment 1 fell into. SD, Standard deviation. Letters a, b, and c in the same column indicate homogeneous statistical processing groups that were significantly different at $P < 0.1$*, $P < 0.05$** or $P < 0.01$*** according to the Newman Keuls test, with a < b < c.

(UCMA 4217, 3A2A, 927, B16, UCMA 3780, 4C1A), were selected for the next experiment.

2.3.2. Experiment 2: kinetic impact of Gram-negative strains

These six strains (UCMA 4217, B16, UCMA 3780, 3A2A, 927 and 4C1A) were individually tested in cheese at two-day intervals. Three model cheeses were made per tested strain on the same day. Evolution of microbial counts and organic acids as well as BA concentrations in the cheese core were determined at day 1, 8, 18 and 28 while the volatile compound profile was analyzed only at the end of ripening (28 days).

2.4. Cheese analyses

2.4.1. Microbial analyses

Str. salivarius ssp. *thermophilus*, *Lc. lactis*, *Lb. plantarum*, *Ln. mesenteroides*, *E. faecalis* and yeast populations were enumerated under the conditions described by Callon et al. (2005), on the following agar media respectively: M17, Turner–Sandine–Elliker (TSE), facultatively heterofermentative lactobacilli (FH), Mayeux–Sandine–Elliker (MSE), Slanetz and Bartley (SB) and oxytetracyclin–glucose (OGA). Gram-positive catalase-positive bacteria (*A. arilaitensis*, *C. casei*, *S. equorum*) and *E. faecalis* were enumerated on Cheese Ripening Bacteria Medium (CRBM) as described by Denis et al. (2001) and on Rabbit Plasma Fibrinogen Agar (RPFA) (EN ISO 6888-2). To discriminate between these four species, isolates (approx. 10 colonies, including at least two of each colony morphotype, per CRBM and RPFA plate, at each ripening time) were checked for their ability to grow on BHI agar in the presence of five selective agents as described by Deetae et al. (2009a). Gram-negative bacteria were enumerated on Plate Count Agar supplemented with 1% milk, 5 mg l^{-1} vancomycin and 5 mg l^{-1} crystal violet as inhibitors of Gram-positive bacteria (PCAI) (Delbes et al., 2007) and incubated at 30 °C for 48 h as well as on cetrimide-fucidin-cephalosporin (CFC) agar. All media were purchased from Biokar Diagnostics (Pantin, France). Milk and cheese pH values were determined with a 926 VTV pH meter with an Ingold 406 MX penetration electrode (Mettler-Toledo S.A., Viroflay, France).

2.4.2. Determination of organic acid concentrations

Concentrations of citric, malic, succinic, lactic, fumaric, acetic and propionic acids, which are commonly found in milk and cheeses, were determined. Cheese samples (5 g) were homogenized for 2 min in 10 mL distilled water with an Ultra-Turax (9500 rpm). After incubation at 50 °C for 2 h, the suspensions were homogenized again (Ultra-Turax). Samples were then deproteinized by mixing with 5 mL trichloracetic acid 24% and allowed to stand for 1 h before centrifugation at 40 000 × g for 20 min at 4 °C. Supernatants were filtered through 0.20 μm pore-size filters (PTFE). Organic acids were separated isocratically by high-performance liquid chromatography (HPLC) (Dionex, France) on a cation-exchange column (Aminex HPX-87, Bio-Rad, Marnes-la-Coquette, France) maintained at 35 °C (Oven LC30) under 0.6 mL min^{-1} of sulfuric acid (0.01 N) (GP50 gradient pump). Detection was done at 210 nm (UVD 170S). Results were expressed in mg per kg of cheese.

2.4.3. Sensory analyses

Twelve trained assessors compared the colour (lightness), odour (overall intensity) and texture (softness to the touch) of trial cheeses inoculated with each of the 11 Gram-negative strains (experiment 1) to those of control cheeses (24 cheeses in total) in 44 blind triangular differences tests (ISO 4120). For each triangular test, either the assay or the control cheese sample was doubled. The assessors had to indicate which sample was different from the other two and to make comments on perceived sensations. Percentages of sensory term citations among the assessors were calculated. At least 24 responses were required for each test. All samples were served at 15 ± 2 °C. Two replicates were tested for each cheese sample.

2.4.4. Volatile aroma compounds analysis

Analyses of volatile compounds were carried out at the end of ripening on cheese samples from experiments 1 and 2 using dynamic headspace extraction, gas chromatography and mass selective detection, as described by Sympoura et al. (2009). One gram of cheese was rapidly homogenized with 9 g of anhydrous sodium sulfate (Na_2SO_4) in a mortar. Four grams of the powdery mixture was placed on 0.2 g of glass wool in a cylindrical glass extraction cartridge (28 mm diameter × 47 mm height; Etablissements Maillières Frères, Aubière, France). Dynamic headspace extraction was performed using Tekmar apparatus (Telmar-Dohrmann, Cincinnati, OH). An Agilent 5890 gas chromatograph (Agilent Technologies, Palo Alto, CA), equipped with a DB MS capillary column (30 m × 0.32 mm i.d. × 1 μm, J&W Scientific, Agilent Technologies) was used for the analysis of volatile compounds with a MSD HP 5973 mass selective detector. The chromatography

parameters were the same as described by Sympoura et al. (2009). However, the oven temperature was programmed as follows: 9 min isothermal at 5 °C, 4 °C min^{-1} increasing to 215 °C. All volatile compounds were identified according to the comparison of their mass spectra with those obtained from two databanks (Wiley 275K and NBS 75k). Peak absolute areas (arbitrary units) were calculated from the total ion current (TIC).

2.4.5. Free amino acids and biogenic amines determination

The overall concentration of free amino acids (FAA) was evaluated using a cadmium-ninhydrine colorimetric assay determined on the water soluble fraction of the cheese core (Folkertsma and Fox, 1992). The FAA index was expressed as mmoles glycine equivalent in 100 g dry matter, as glycine was used instead of leucine for the calibration curve. Samples were analyzed twice on two different days. Two colorimetric assays were performed on each water soluble fraction.

Free amino acids were analyzed in cheese extracts by HPLC. Cheese extracts were prepared as described by Yvon et al. (1998). Amino acids were then analyzed by HPLC after a derivatization procedure. Briefly, derivatization was carried out on a mixture of 400 µl of cheese filtrate, 40 µl of internal standard consisting of NorLeucine (0.3 g l^{-1}) and 560 µl of MilliQ water. To 10 µl of the above solution, 70 µl of borate buffer and 20 µl of AQC reagent (6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate) were mixed and then placed at 55 °C for 10 min. The amino acids were analyzed by reverse phase HPLC on a NovaPack C18 column (3.9 mm × 150 mm) by using a fluorescence detector (UltiMate 3000 Fluorescence detector, Dionex) with λ_{ex} 250 nm and λ_{em} 395 nm. The flow rate was 1 ml min^{-1} and the elution was done at 37 °C. Eluant A was an AccQ.Tag buffer (Waters) and eluant B was acetonitrile. The elution started with 100% of eluant A and the ratio of eluant B was increased from 0 to 17% over 39 min. The concentration of each amino acid was determined from a standard curve.

Nine BA (tryptamine, 2-phenylethylamine, isopentylamine, putrescine, cadaverine, histamine, tyramine, spermidine and spermine) were analyzed in cheese cores after dansyl derivation and separation by reverse phase high-performance-liquid-chromatography (Dionex, France) (Bütikofer et al., 1990). The identity of the peaks was determined by comparing their retention times and spectral profiles (diode array detection) to those of standard molecules analyzed in the same conditions. Samples were analyzed twice on two different days.

2.5. Statistical analyses

All statistical analyses were performed with the Statistica software (version 6; Statsoft, Maisons-Alfort, France). Microbial count data from the duplicate 28-day old cheeses from experiment 1 were subjected to principal-component analysis followed by an ascending hierarchical classification (AHC) analysis and by K-means clustering, in order to determine classes of cheeses inoculated with the various Gram-negative strains. The data (microbial counts, organic acids and volatile compound concentrations) were then processed by monofactorial (cheese classes defined by AHC) variance analysis (ANOVA). Data from experiment 2 (triplicate dataset of microbial counts and organic acid concentrations in cheeses on day 1, 8, 18 and 28) were subjected to repeated measures (four sampling dates) ANOVA to assess the effect of inoculation with Gram-negative strains on microbial counts and organic acid concentrations in cheeses over the whole ripening period. Biogenic amine concentration data in 28-day old cheeses from experiments 1 and 2 were processed by monofactorial (biogenic amine-producing Gram-negative strain) ANOVA. In those comparisons where a significant effect after any variance analysis was observed, differences between means were tested using a Newman–Keuls test.

3. Results

3.1. Counts of Gram-negative bacteria in the core of the model cheese

The 11 selected Gram-negative strains were individually tested in the cheeses (experiment 1, Table 1). Gram-negative bacteria counts after 28 days were below the detection level (2 log CFU g^{-1}) in control cheeses as well as in cheeses inoculated with *Halomonas* sp. B39, *Chryseobacterium* sp. Pi18 or *Stenotrophomonas maltophilia* PCAI D6.5 (Table 1). Average counts in cheeses inoculated with *Pantoea agglomerans* PCA Q6.3, *Pseudomonas putida* CV 30.6, *Halomonas venusta* 4C1A, or *M. morganii* 3A2A were similar, around 3 log CFU g^{-1} ± 0.5 log. They were highest in the cases of addition of *P. vulgaris* UCMA 3780 (4 log CFU g^{-1}), *K. oxytoca* 927 (5.5 log CFU g^{-1}), *H. alvei* B16 (6.5 log CFU g^{-1}) and *C. freundii* UCMA 4217 (7.1 log CFU g^{-1}) (Table 1).

The four strains that reached the highest counts at 28 days (*C. freundii* UCMA 4217, *K. oxytoca* 927, *H. alvei* B16 and *P. vulgaris* UCMA 3780) were selected for the second experiment. Among the remaining strains, the two strains that produced biogenic amines in cheese in the first experiment (*M. morganii* 3A2A and *H. venusta* 4C1A, see below) were also selected. Their growth was monitored during cheese ripening (Fig. 1). *K. oxytoca* 927 reached the highest levels of all strains (around 7.4 log CFU g^{-1}) in the cheese core on day 1 but then slightly decreased and stabilized to around 6 log CFU g^{-1} from day 18 until the end of ripening. The growth of *H. alvei* B16 was lower at the beginning (6.7 log CFU g^{-1} on day 8) then it reached a 0.8 log higher level in comparison to any of the other Gram-negative strains on days 18 and 28. *P. vulgaris* UCMA 3780 and *C. freundii* UCMA 4217 stabilized at an intermediary level of 5.7 log CFU g^{-1} from day 1 until the end of ripening. Finally, the levels of *H. venusta* 4C1A and *M. morganii* 3A2A remained close to 5.5 log CFU g^{-1} from day 1 to day 18 but had dropped to below 4 log CFU g^{-1} by day 28.

Although *P. vulgaris* UCMA 3780 counts in 28-day cheese were lower in experiment 1 (4 log CFU g^{-1}) than in experiment 2 (5.7 log CFU g^{-1}), and conversely *C. freundii* UCMA 4217 counts were higher in experiment 1 (7.1 log CFU g^{-1}) than in experiment 2 (5.7 log CFU g^{-1}), the four *Enterobacteriaceae C. freundii* UCMA 4217, *K. oxytoca* 927, *H. alvei* B16 and *P. vulgaris* UCMA 3780 showed the highest counts of the tested Gram-negative strains in both experiments.

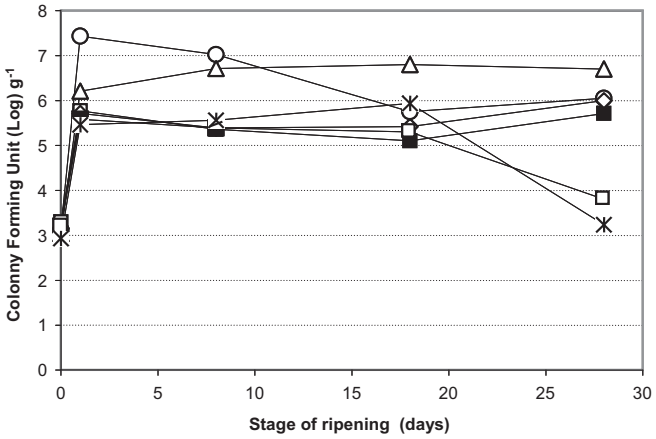

Fig. 1. Gram-negative counts (log CFU g^{-1}) during ripening (experiment 2) in cheeses inoculated with *K. oxytoca* 927 (○), *H. alvei* B16 (△), *C. freundii* UCMA 4217 (■), *P. vulgaris* UCMA 3780 (◇), *M. morganii* 3A2A (□), *H. venusta* 4C1A (×). Values are the means of 3 cheeses at each different ripening stage. For greater clarity, error bars are not included.

3.2. Impact of Gram-negative bacteria on microbial counts and organic acid concentrations

After 28 days of ripening, the different cheeses inoculated with one of the 11 Gram-negative strains (experiment 1), clustered in three classes in an ascending hierarchical classification based on microbial counts (Gram-positive bacteria and yeasts) (Table 1). Control cheeses fell into class 3. Class 1 clustered cheeses which showed significantly lower counts in *E. faecalis* than the class 3 cheeses, higher counts of *Lb. plantarum* than the class 2 cheeses and the highest concentrations of acetate. Cheeses in class 2 showed lower counts of *E. faecalis* than the class 3 cheeses, the lowest counts of *Lb. plantarum* as well as the lowest concentrations of acetate. No difference in neither any other microbial population count nor any other organic acid concentration was detected between classes (data not shown). Both duplicate cheeses inoculated with *Halomonas* B39, *P. putida* CV 30.6, *P. vulgaris* UCMA 3780 or *S. maltophilia* PCAi D6.5 clustered in class 3 along with the control cheeses. Both cheeses inoculated with *C. freundii* UCMA 4217, *K. oxytoca* 927 or *H. alvei* B16 clustered in class 1. Cheeses inoculated with *M. morganii* 3A2A fell into class 2.

Microbial counts and organic acid concentration data from 1, 8, 18 and 28-day old cheeses from experiment 2 were subjected to repeated ANOVA measures. Table 2 sums up the significant effects induced by inoculation with different Gram-negative strains over the entire ripening period. In particular, cheeses inoculated with *C. freundii* showed lower counts for *E. faecalis* and to a lesser extent for *Lb. plantarum* than control cheeses. Cheeses inoculated with *P. vulgaris* showed lower counts for *S. equorum*, *Lb. plantarum* than control cheeses. Cheeses inoculated with *Halomonas* 4C1A showed higher counts for *E. faecalis* and lower amounts of acetate than control cheeses. Cheeses inoculated with *M. morganii* showed the lowest counts for *Lb. plantarum*. Finally, cheeses inoculated with *K. oxytoca* showed the highest amounts of acetate and the lowest amounts of citrate. No significant difference was observed between the control cheeses and cheeses inoculated with *H. alvei*.

Hence, in both experiments 1 and 2, cheeses inoculated with *C. freundii* showed lower counts for *E. faecalis* than control cheeses, those inoculated with *M. morganii* showed the lowest counts for *Lb. plantarum* and the lowest amounts of acetate and those inoculated with *K. oxytoca* showed the highest amounts of acetate. *P. vulgaris* (UCMA 3780) only had a significant effect on microbial counts in cheeses from experiment 2, where this strain reached a higher count. However, even when significant, most differences in microbial counts or organic acid concentrations between cheeses inoculated with Gram-negative bacteria and control cheeses can be considered low (below 1 log CFU g^{-1} and below 200 mg kg^{-1} for acids).

3.3. Effect of Gram-negative bacteria on sensory characteristics and volatile compounds

According to the results obtained after the sensory analyses (experiment 1, triangular tests), each assay cheese was significantly different ($p < 5\%$) from the control cheeses after 28 days of ripening (data not shown). Cheese texture was the main difference observed with assay cheese firmness considered different to that of the control cheeses (to various extents depending on the strain considered). Cheeses inoculated with *H. alvei* B16, *M. morganii* 3A2A or *H. venusta* 4C1A were cited as firmer than control cheeses by 79, 71, and 69% of the trained assessors respectively. Inoculation with *Halomonas* sp. (B39), *Chryseobacterium* sp. (Pi18), *S. maltophilia* (PCAI D6.5), *P. agglomerans* (PCA Q6.3), *P. putida* (CV 30.6), *P. vulgaris* (UCMA 3780), or *C. freundii* (UCMA 4217) showed increased cheese firmness for 51 to 64% of assessors. Finally, cheeses inoculated with *K. oxytoca* 927 were only cited as firmer by 43% of the assessors. Concerning cheese odour, the assessors detected a change in odour only for cheeses inoculated with *P. vulgaris* UCMA 3780. Indeed, a less instense odour was detected by 47% of the assessors for the two cheese replicates analyzed in comparison to control cheeses. Volatile compounds were isolated from model cheeses from experiments 1 and 2 at day 28 of ripening by dynamic headspace. A limited number (26) of volatile compounds were detected. They belonged to 5 chemical families (ketones, alcohols, esters, aldehydes and terpenes). Volatile sulphur compounds were not detected or were present only as traces. The main compounds isolated from cheese headspaces were 3-hydroxy-2-butanone, 3-methyl-1- butanol, 1-butanol, ethyl caprylate, ethyl butyrate, ethyl caproate, hexanal, nonanal and heptanal. There were no qualitative or quantitative differences in the volatile compounds identified in any of the model cheeses (controls and assays). Overall, the Gram-negative bacteria did not markedly affect the core colour or odour of the model cheese and in the majority of cases only slightly affected its texture.

3.4. Production of biogenic amines by Gram-negative bacteria in model cheese

BA profiles were determined in cheeses from experiments 1 and 2. At 28 days of ripening, all the analyzed amines except tryptamine were detected in each of the experimental cheeses including

Table 2
Microbial counts and organic acid concentrations over the whole ripening period in cheeses inoculated with Gram-negative strains (experiment 2).

	Strain	*E. faecalis* (SB)			*Lb. plantarum* (FH)			*S. equorum* (RPF)			Acetate			Citrate		
		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD	
C. freundii	UCMA 4217	5.33	0.21	a	5.86	0.69	b	3.71	0.17	b	1111	850		915	429	b
H. alvei	B16	5.54	0.25	b	6.10	0.91	c	3.79	0.47	b	1166	859		811	541	ab
P. vulgaris	UCMA 3780	5.71	0.23	b	5.66	0.65	a	2.20	1.10	a	1073	860		903	469	b
Control		5.72	0.34	b	6.02	0.74	c	3.89	0.26	b	1201	931		667	571	a
		**			**			*			NS			*		
H. venusta	4C1A	5.43	0.69	b	5.98	0.93	b	4.08	1.10		940	726	a	769	350	b
M. morganii	3A2A	5.15	0.52	a	5.62	0.56	a	4.17	0.57		925	695	a	742	340	b
K. oxytoca	927	4.99	0.54	a	5.92	0.91	ab	3.92	0.48		1212	888	c	600	451	a
Control		5.07	0.45	a	5.86	0.71	ab	3.85	0.47		1090	783	b	667	410	ab
		**			*			NS			***			**		

Counts in log CFU g^{-1}. SB: Slanetz and Bartley agar. FH: facultatively heterofermentative lactobacilli agar. RPF: Rabbit Plasma Fibrinogen agar. Organic acid concentrations in mg kg^{-1} of cheese. Values are the means of 12 samples obtained from 3 cheeses at 4 different ripening times. SD, Standard deviation. Only the microbial groups and organic acids for which a significant effect of cheese class was observed are indicated in the table. Letters a, b, and c in the same column indicate homogeneous statistical processing groups that were significantly different at $P < 0.1$*, $P < 0.05$** or $P < 0.01$*** after repeated measurements ANOVA analysis and according to the Newman Keuls test, with a < b < c. NS, non significant.

control cheeses (experiments 1 and 2) but only putrescine, cadaverine, and tyramine were detected at concentrations above the quantification limit (defined as three times the detection limit). Tyramine was found in equivalent amounts ($\sim$0.3 mmol kg^{-1} cheese dry matter) in the assay and control cheeses of experiments 1 and 2 (results not shown). For cadaverine, cheeses manufactured with *H. alvei* B16, *K. oxytoca* 927 and to a lower extent, those manufactured with *H. venusta* 4C1A or *M. morganii* 3A2A contained significantly higher concentrations than control cheeses (Table 3). For putrescine, the only cheeses containing statistically higher amounts of putrescine (1.53 mmol kg^{-1} cheese dry matter as average) in comparison to control cheeses were those manufactured with *H. alvei* B16.

Fig. 2 shows the evolution of cadaverine and putrescine concentrations and of the free amino acid index during ripening in cheeses inoculated with the 4 BA-producing strains (experiment 2). Cadaverine was produced from day 1 and its concentration then increased at a fairly constant rate until day 28 (Fig. 2A). Observed differences in cadaverine concentration between cheeses elaborated with different Gram-negative strains, were constant all along the ripening process. By contrast, in cheeses manufactured with *H. alvei*, putrescine concentration increased slowly until day 7 and then increased at a similar rate as cadaverine (at least between day 8 and 28).

The evolution of the free amino acids index was roughly parallel to cadaverine production regardless of the Gram-negative strain (Fig. 2B). On day 1, phenylalanine ($\sim$0.30 mmol kg^{-1}) was the major FAA in all cheeses, followed by threonine, arginine and glutamic acid (data not shown). Histidine, leucine and tyrosine were detected as traces (<0.1 mmol kg^{-1}), whereas lysine, and ornithine were not detected. By contrast, after 28 days of ripening, lysine was the major FAA in control cheeses (Table 3), followed by leucine, phenylalanine, alanine, glutamic acid and ornithine. Low amounts of histidine and arginine were detected, whereas tyrosine was not detected. Lysine concentration was significantly lower in cheeses inoculated with *H. alvei* or *K. oxytoca* (1.40 and 1.28 mmol kg^{-1} respectively) than in control cheeses (4.07 mmol kg^{-1}) (Table 3). In addition, ornithine was not detected in cheeses inoculated with *H. alvei*, while it was present in concentrations above 1.14 mmol kg^{-1} in the other cheeses analyzed.

4. Discussion

Enterobacteriaceae counts in cheese usually reach a peak during the first week of ripening. After this period, counts may decrease with time, depending on *Enterobacteriaceae* species, cheese type and cheese part (core or surface) (Morales et al., 2004; Nuñez et al., 1985; Tornadijo et al., 2001). In our work, most tested *Enterobacteriaceae* reached high counts (>6 log CFU g^{-1}) at day 1 in the model cheese core. The relative evolution during ripening of *K. oxytoca* and *H. alvei* was similar to that described by Tornadijo et al. (2001) in the core of San Simón pressed cheese made from raw milk. In our model cheeses as in San Simón cheese, *K. oxytoca* was predominant up to 2 weeks, its level then decreasing slightly, while *H. alvei* increased in proportion from week 2 up to the end of ripening. *H. alvei* has also been found dominant among Gram-negative bacteria in ripened Alberquilla hard cheese (Abriouel et al., 2008). In our work, *C. freundii* was present until the end of ripening in our model cheese as observed in Saint-Nectaire uncooked pressed cheese (Delbes et al., 2007), though it was not detected beyond the first week in San Simón cheese (Tornadijo et al., 2001). We observed that the levels of *H. venusta* and *M. morganii* decreased after 18 days in the model cheese, possibly due to antagonistic interactions with microbial populations in the technological consortium. Finally, *Chryseobacterium* sp. (Pi18) and *S. maltophilia* (PCAi D6.5), which were isolated from Saint-Nectaire cheese and raw milk respectively, did not seem to be able to establish themselves in the model cheese. But in the strain collection studied by Coton et al. (2012), *Chryseobacterium* and *Stenotrophomonas* were among the most frequent genera found, mainly in milk but also in uncooked pressed cheese core samples. The culture and storage conditions of the pure strains prior to their use for cheese-making may have been unfavorable to their adaptation and growth in the model cheese. Also, the limited ten-species consortium may have been less favorable to colonization by these strains than a more complex microbial community such as those of raw milk cheese. In particular, Mounier et al. (2008), showed that bacterial development on the surface of smear cheeses was affected by the yeast species present. In that instance, the Gram-positive *Leucobacter* sp. grew only in cheeses containing the yeast *Geotrichum candidum*, while *H. alvei* dominated the bacterial flora regardless of the yeast species present.

On the other hand, Gram-negative bacteria may also interact positively or negatively with the other micro-organisms in the consortium. Counts of *A. arilaitensis*, *Brevibacterium aurantiacum* and *H. alvei* significantly decreased in the presence of *P. vulgaris* (UCMA 3780) in a smear-type model cheese (Deetae et al., 2009a). In the core of our model cheese, the same *P. vulgaris* strain slightly reduced the counts of *S. equorum* and *Lb. plantarum*. Several other Gram-negative strains among those that reached the highest counts (*C. freundii*, *M. morganii*, *K. oxytoca*) also slightly affected the development of the consortium micro-organisms and organic acid production in the model cheese. However, since freezing the cheese samples prior to analysis could have altered the balance between culturable strains, it can not be excluded that the effect of

Table 3
Concentrations of biogenic amines and amino acids in cheeses inoculated with Gram-negative bacterial strains after 28 days of ripening (experiments 1 and 2).

Species	Strain	BA production *in vitro* from Coton et al., 2012	BA						Amino acids					
			Cadaverine			Putrescine			Mean[a]					
			Mean[a]	Min.	Max.	Mean[a]	Min.	Max.	Arg	Hist	Leu	Lys	Orn	Phe
C. freundii	UCMA 4217	*Cad (15.1). IsoP (8.9). His (5.8). Tyr (0.1). Put (0.6)*	0.04^{a}	0.03	0.04	0.13^{a}	0.02	0.20	nd	nd	nd	nd	nd	nd
H. alvei	B16	*Cad (14.6). Isop (8.4). His (6.0). Phe (1.1). Put (0.7). Try (0.3)*	0.81^{b}	0.68	0.94	1.53^{b}	0.65	2.18	0.33	0.37	2.02^{a}	1.40^{a}	0.00^{a}	2,52^{a}
P. vulgaris	UCMA 3780	*Cad (0.1). Isop (0.5). His (0.1). Phe (0.1). Put (1.0). Try (0.7)*	0.02^{a}	0.02	0.03	0.03^{a}	0.02	0.03	nd	nd	nd	nd	nd	nd
H. venusta	4C1A	*Cad (12.2). His (7.4). IsoP (6.8). Phe (1.2). Put (1.2). Try (0.6)*	0.53^{b}	0.42	0.67	0.09^{a}	0.06	0.14	0.60	0.66	2.40^{b}	3.32^{b}	1.48^{b}	3,12^{b}
M. morganii	3A2A	*Cad (14.4). His (8.8). IsoP (8.6). Put (1.4). Phe (1.6). Try (0.7)*	0.56^{b}	0.42	0.65	0.11^{a}	0.05	0.18	0.79	0.55	1.95^{a}	4.14^{b}	1.14^{b}	2,45^{ab}
K. oxytoca	927	*Cad (12.1). IsoP (0.4)*	0.74^{b}	0.47	1.05	0.02^{a}	0.01	0.03	0.70	0.66	2.67^{b}	1.28^{a}	1.90^{c}	2,52^{ab}
Control			0.01^{a}	0.00	0.02	0.02^{a}	0.01	0.03	0.62	0.52	2.08^{a}	4.07^{b}	1.50^{b}	2,05^{a}

In brackets, biogenic amine production *in vitro* expressed in mmol l^{-1} of culture supernatant. *Cad* = cadaverine, *His* = histamine, *IsoP* = Isopentylamine, *Phe* = phenylethylamine, *Put* = putrescine, *Try* = tryptamine.
In-core concentrations of biogenic amines and amino acids are expressed in mmol kg^{-1} of cheese dry matter. nd: not determined.
Results having a different superscript letter are statistically different at the $P = 0.01$ level according to Newman Keuls test.
[a] Mean values are the means of 3 cheeses from 3 independent batches. Arg = arginine, Hist = histamine, Leu = leucine, Lys = lysine, Orn = ornithine, Phe = phenylalanine.

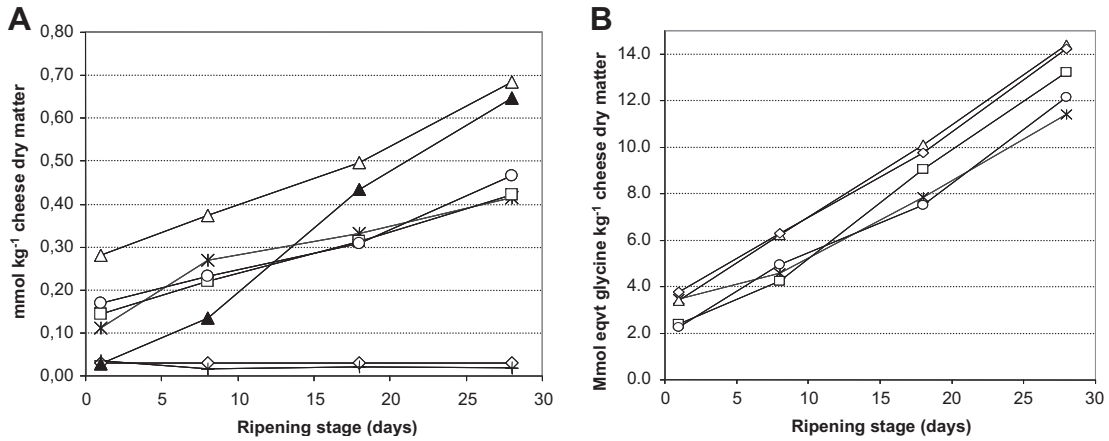

Fig. 2. Production of cadaverine and putrescine (A) and evolution of the free amino acids index (B) during ripening in the cores of cheeses inoculated with one Gram-negative strain. Cadaverine content (A) and free amino acids index (B) in control cheeses (◇), cheeses inoculated with *K. oxytoca* 927 (○), *H. alvei* B16 (△), *M. morganii* 3A2A (□), *H. venusta* 4C1A (×). Putrescine content (A) in control cheeses (+) and in cheeses inoculated with *H. alvei* B16 (▲). Values of biogenic amines in mmol kg^{-1} of cheese dry matter and of free amino acids index in mmole glycine equivalent per kg of cheese dry matter. Values are the means of duplicate analyses of cheeses from a single batch per strain (experiment 2; see also Table 3) over the ripening period.

Gram-negative strains on the development of the consortium micro-organisms could be underestimated or biased.

The Gram-negative bacteria studied, especially the *Enterobacteriaceae*, had no significant positive nor adverse effect on the colour or odour of the model cheese, but slightly affected its texture. They also had no impact on volatile compound content, which may partly explain the lack of differences in odour. The main volatile compounds detected in all our model cheeses have also been identified in uncooked pressed Saint-Nectaire cheese made from raw milk (Sympoura et al., 2009; Picque et al., 2011). However, a lower number of volatile compounds were detected in our model cheese (26) than in Saint Nectaire cheese (37, Picque et al., 2011), probably mainly because of the less complex microflora used and the shorter ripening period (28 *vs* 56 days, respectively). The main volatile aroma compounds detected in our model cheeses are associated with a buttery note (such as 3-hydroxy-2-butanone) or fruity notes (such as 3-methyl-1- butanol, ethyl caprylate, ethyl caproate and hexanal) (Poveda et al., 2008).

The *in vitro* BA-production ability of Gram-negative strains (Coton et al., 2012; Table 3) neither quantitatively nor qualitatively predicted their respective capacities for producing BA in our model cheese. Indeed, in our conditions, cadaverine and putrescine were the only BA produced in significant amounts in the model cheeses where a Gram-negative *in vitro* BA-producer was added. Cadaverine was produced by four of the five *in vitro* BA-producing strains (*H. alvei* B16, *K. oxytoca* 927, *H. venusta* 4C1A, *M. morganii* 3A2A). No cadaverine was produced by *C. freundii* UCMA 4217 even though this strain reached counts similar to that of *H. venusta* 4C1A or *M. morganii* 3A2A in the cheese and has a similar *in vitro* producing capacity. Similarly, only *H. alvei* B16 produced significant amounts of putrescine in cheese, while the three other *in vitro* BA producing strains showed a similar (*C. freundii* UCMA 4217) or higher (*H. venusta* 4C1A, *M. morganii* 3A2A) *in vitro* putrescine-production capacity. None of the tested Gram-negative bacteria were able to produce tyramine in large amounts *in vitro*. Tyramine production at similar rates in the trial and control cheeses may be explained by the presence of one or several tyramine-producing microorganisms in the technological consortium present in all the cheeses. In particular, *E. faecalis* strains possess the tyrosine decarboxylation pathway (Connil et al., 2002) and have been shown to produce tyramine in various cheeses (Bonetta et al., 2008; Komprda et al., 2008). No other BA produced *in vitro* (isopentylamine, histamine, phenylethylamine, and tryptamine) was produced in significant amount in our cheeses. No appreciable amount of histamine was produced in the cheese model by any of the 5 strains which produced it in high amount *in vitro* (*C. freundii* UCMA 4217, *M. morganii* 3A2A, *H. alvei* B16, *P. vulgaris* UCMA 3780, *H. venusta* 4C1A) (Coton et al., 2012). Even when significant, the observed cheese BA content can be considered low, as it never exceeded 100 mg kg^{-1} cheese (1.1 mmol kg^{-1} of putrescine, taking into account dry matter content, in cheese manufactured with *H. alvei* B16) and was lower than the level of 200–500 mg kg^{-1} cheese generally admitted as unsafe for the consumer for tyramine and histamine. Furthermore, putrescine and cadaverine don't have the same physiological impact on the consumer than histamine and tyramine, although they can be potentiators of these detrimental BA (Taylor and Lieber, 1979).

Cheese proteolysis is a dynamic process and cheese content in precursor amino acid, specific for each BA, results from a complex balance between their production during ripening and their use for BA synthesis or other metabolic pathways (García-Palmer et al., 1997). Concentrations of lysine and ornithine in 28-day old control cheeses (~4 mmol kg^{-1} and 1.5 mmol kg^{-1} respectively) were in the same range as those found in one-month old Terrincho uncooked pressed cheeses made from raw milk (0.6 to 3.3 mmol kg^{-1} for lysine and 0.42 to 2.71 mmol kg^{-1} for ornithine) (Pintado et al., 2008). However, the availability of precursor amino acids did not always explain the level of BA-production in cheese by *in vitro* BA-producers. Indeed, amino acid decarboxylases such as those that convert L-lysine to cadaverine or L-ornithine to putrescine can be constitutive or induced (Kanjee et al., 2011; Fritz et al., 2009). In *Escherichia coli* and other *Enterobacteriaceae*, inducible lysine decarboxylases (LDC) are most effective under mild acid stress conditions (~pH 5) in a lysine-rich environment. *In vitro* at pH 5.8, the LDC of *E. coli* was found barely active at lysine concentrations below 0.5 mM and fully active at levels exceeding 5 mM (Fritz et al., 2009).

The absence of histamine in the model cheeses may be explained by the low amounts of histidine found in all assay and control cheeses. The absence of isopentylamine and 2-phenylethylamine was more surprising, especially in the cheeses inoculated with *H. alvei* B16, since their respective precursors leucine and phenylalamine were present in cheese from day 1 and reached concentrations above 2 mmol kg^{-1} in all cheeses at the end of ripening. Concentrations of putrescine in 28-day old cheeses inoculated with *H. alvei* B16 (0.65 to 2.18 mmol kg^{-1}) were in the same range as those found in one month-old Gouda or Maasdam cheeses made from pasteurized milk inoculated with *H. alvei* LN1 (0.29 to 2.0 mmol kg^{-1})

(Joosten, 1988), but were about half of those found in one-month old Terrincho cheeses (0.93 to 5.06 mmol kg^{-1}) (Pintado et al., 2008). The depletion of ornithine in cheeses inoculated with *H. alvei* B16, in contrast with control cheeses which contained 1.5 mmol kg^{-1} after 28 days, suggested that this AA may be limiting for the synthesis of putrescine by this strain. Concentrations of cadaverine in cheeses inoculated with *H. alvei* B16 (0.69–0.94 mmol kg^{-1}) were about half of those found in Gouda cheeses of the same age inoculated with *H. alvei* LN1 (1.6 to 2.0 mmol kg^{-1}) as well as those found in Terrincho cheeses (0.47–2.34 mmol kg^{-1}). Production of cadaverine by *H. alvei* LN1 in Gouda cheese was likely limited by lysine availability, since the addition of lysine to give a concentration of 3.5–7.0 mmol kg^{-1} in cheese enhanced cadaverine production up to 6.9 mmol kg^{-1} (Joosten, 1988). Thus, although some lysine (1.4 mmol kg^{-1}) was still available in cheeses inoculated with *H. alvei* B16 after 28 days of ripening, lysine concentration could have been too low for the decarboxylation to be efficient.

In addition, cheese physico-chemical characteristics and microbial composition may affect the metabolism of the different Gram-negative species and the activity of proteolytic and decarboxylating enzymes, and may be as important as precursor availability (Buňková et al., 2010; Novella-Rodrígues et al., 2003). Since such parameters can change from the cheese core to the surface, it cannot be excluded that larger amounts of BA may be produced by the tested strains in the cheese rind layer. Buňková et al. (2010) found 30–50% more cadaverine in the edge layer than in the core of Edam cheese. Jørgensen et al. (2000) also showed that the combination of an arginine deiminase-positive lactic acid bacteria with an ornithine decarboxylase-positive *H. alvei* strain enhanced, by a 10–15 fold factor, the production of putrescine in cold-smoked salmon. Hence, the chances of vicinity and synergistic interactions between proteolytic micro-organisms and BA producers may not be as high with the technological consortium used in our experiments than within the complex microbial community of raw milk cheese.

5. Conclusion

The Gram-negative bacteria studied in this work only slightly affected the microbial growth and sensory characteristics of the model cheese, although the texture of some assay cheeses was considered firmer than that of the corresponding control cheeses. Actual BA-production by Gram-negative bacterial strains has only rarely been studied *in situ* using real and usually aseptic food matrixes (Jørgensen et al., 2000; Veciana-Nogues et al., 2004). The present study showed that BA- production in an uncooked pressed model cheese by *in vitro*-producing Gram negative strains is a complex phenomenon which is only partly explained by the availability of precursor amino acids. The growth of the tested *Enterobacteriaceae* in cheese with a complex microbial consortium was similar to that observed in pressed cheeses made from raw milk (Tornadijo et al., 2001). However, their impact on microbial biodiversity and dynamics, as well as their potentialities and risks in terms of sensory characteristics and BA content should be further investigated within the complex microbial community of raw milk cheese for different cheese technologies.

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