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International Journal of Food Microbiology 145 (2011) 379–389

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Simplification of a complex microbial antilisterial consortium to evaluate the contribution of its flora in uncooked pressed cheese

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ARTICLE INFO

Article history:
Received 8 July 2010
Received in revised form 17 December 2010
Accepted 21 December 2010

Keywords:
L. monocytogenes
Raw milk cheese
Inhibition
Microbial communities

ABSTRACT

A complex microbial consortium derived from raw milk and composed of populations classified in 4 groups (lactic acid bacteria (A), Gram positive catalase positive bacteria (B), Gram negative bacteria (C) and yeasts (D)) can contribute to the inhibition of *Listeria monocytogenes* in the core of an uncooked pressed cheese. To identify what groups may be involved in the inhibition, the consortium was simplified by successively omitting one group at a time. Pasteurized milk was inoculated with these more or less complex consortia and their effects on *L. monocytogenes* count, pH, acids and volatile compounds in the core of uncooked pressed cheese were evaluated. The growth of *L. monocytogenes* was the highest in cheeses prepared with pasteurized milk and only *St. thermophilus*. Inhibition in other cheeses was expressed by comparison with growth in these ones. All the consortia containing both lactic acid bacteria (group A) and Gram positive catalase positive bacteria (group B) — ABCD, ABD, ABC, AB — were more inhibitory than those containing lactic acid bacteria on its own (A) or associated only with yeasts (AD) or/and Gram negative (ADC). Consortia without lactic acid bacteria were weakly inhibitory or had no effect. Gram positive catalase positive bacteria alone were not inhibitory although most of the species became established in the cheeses. The *Lactobacillus* population (*Lb. casei*, *Lb. plantarum*, *Lb. curvatus* and *Lb. farciminis*) was predominant in cheeses (9 log CFU/g) with a higher count than *Leuconostoc* (7 log CFU/g) and *Enterococcus* (7 log CFU/g). *Lactobacillus* counts were negatively correlated with those of *L. monocytogenes* ($r = -0.84$ at 18 days) and with the level of D-lactic acid. There was no correlation between *L. monocytogenes* and *Leuconostoc* or *Enterococcus* counts. Complex consortium ABCD and AB not only had a stronger inhibitory power in cheeses than consortium AD, they were also associated with the highest levels of L-lactic and acetic acids. All cheeses inoculated with lactic acid bacteria differed from those without by higher levels of ethyl formiate, pentane and alcohols (2-butanol, 2-pentanol), and lower levels of ketones (2-hexanone, 2,3-butanedione) and aldehydes (2-methyl-butanal). Levels of 2-methyl-butanal, 2-butanol and 2-pentanol were higher in ABCD and AB cheeses than in AD cheeses. Beside their contribution to the inhibition, their effect on cheese flavour must be evaluated.

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1. Introduction

Under current European Community regulations for cheese, at the end of production, *Listeria monocytogenes* must be absent or less than 100 CFU/g if it is demonstrated that there is no development during storage until consumption. This can be achieved by monitoring the *L. monocytogenes* count in milk and applying an appropriate process during manufacturing and ripening. The growth of *L. monocytogenes* during cheese ripening can be reduced by lowering pH (Margolles et al., 1997; Millet et al., 2006), by cooking the curd or by appropriate salting, temperature and ripening time (Pearson and Marth, 1990). These parameters are not independent and their interactions in *Listeria* inhibition must be taken into account (Augustin and Carlier, 2000). Inhibition is also linked to the microbial community in the cheese. Biopreservation in cheeses using bacteriocin-producing bacteria has long been widely studied. Indeed, enterocins produced by *Enterococcus faecalis* and *E. faecium* (Garcia et al., 1997; Nunez et al., 1997), lacticin or nisin from *Lactococcus lactis* (O'Sullivan et al., 2006), linocin from *Brevibacterium linens* (Motta and Brandelli, 2008) and micrococcin from *Staphylococcus equorum* (Carnio et al., 2000) can inhibit *L. monocytogenes* in the core or at the surface of cheeses. Studies dealing with the inhibition of *L. monocytogenes* in cheeses with complex microbial community have shown that pH values and/or production of organic acids may be involved in the inhibition (Millet et al., 2006). They have also suggested that inhibition may be correlated with a high degree of microbial diversity in the community and other unknown factors resulting from microbial interactions (Eppert et al., 1997; Carminati et al., 1999; Maoz et al., 2003; Mayr

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0168-1605/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.ijfoodmicro.2010.12.019

et al., 2004; Roth et al., 2010). None of these studies have clearly demonstrated how microbial populations interact in the inhibition. Is inhibition due to only one species or to a group of microbial species having the same function in the cheese ecosystem (acidification, production of inhibitory compounds)? By comparing the bacterial Single Strand Conformation Polymorphism profiles of St-Nectaire type cheeses with and without antilisterial properties, Saubusse et al. (2007) identified that *Lactococcus* species, *Corynebacterium flavescens* and *Enterococcus saccharominimus* can inhibit *L. monocytogenes*.

The purpose of the present study is to propose another methodology, based on simplifying an inhibitory community according to the method used by Engel et al. (2002). The microbial community from raw milk studied by Saubusse et al. (2007) was simplified by omitting one microbial group at a time. Uncooked pressed cheeses were prepared with pasteurised milk and each of these more or less complex consortia. The growth of *L. monocytogenes* and other microbial populations were monitored in the cheese cores throughout ripening.

2. Materials and methods

2.1. Experimental methodology

The raw milk microbial community with antilisterial properties had been previously identified by Saubusse et al. (2007). Omitting *Lactococcus* because of its strong inhibitory properties and one Gram negative bacteria (*Comamonas testosteroni*), one strain of each species were selected and classed in 4 groups A, B, C and D as described in Table 1.

Cheeses were prepared with pasteurized milk inoculated with a complex consortium including the 4 groups A, B, C and D, or simplified consortia obtained by omitting one or more of these groups. This simplification was achieved in three successive sets of experiments as indicated in Fig. 1. After each set, the consortium with the strongest antilisterial activity was selected for the next experiment and simplified again by omitting a further microbial group.

2.2. Cheese manufacturing

For all experiments, the raw milks were collected from the ENIL farm (Ecole Nationale d'Industrie Laitière) in Aurillac after milking and transported to the cheese experimental plant under refrigerated conditions. It was immediately pasteurized (Pasteurisator Pieralisi PC15-2, Jesy-France) for 30 s at 72 °C, divided between 6 vats containing 40 L of milk each and then warmed to 32 °C.

The 29 selected strains of the complex community, the starter culture and the strain of *L. monocytogenes* were separately pre-cultured for 24 h in 15 mL of broth media as shown in Table 1. After enumeration on appropriate solid medium (Table 1), each preculture was then centrifuged for 15 min at 6000 rpm. The pellets were diluted in 15 mL of sterile reconstituted milk added with 15% of glycerol and stored 1.5 mL tubes at −20 °C. For each experiment, one tube per strain was defrosted and diluted to the desired level for inoculation into milk.

For each experiment, all vats of pasteurized milk were inoculated with the strain of *St. thermophilus* at a concentration of 1.10^7 CFU/mL of milk and with *L. monocytogenes* at 5 to 10 CFU/25 mL of milk. Then each vat was inoculated with a different microbial combination, as described in Fig. 1. The levels of inoculation of each species (Table 1) were defined according to their count in the raw milk from which they were isolated (Saubusse et al., 2007). The composition of the consortia for the 3 experiments was defined as described in Section 2.1.

Small cheeses (600 g) were manufactured according to uncooked pressed cheese technology described by Millet et al. (2006). Cheeses were coated at 1 and 8 days of ripening with orange wax containing natamycin (1000 ppm) to prevent the development of moulds and yeasts on the surface. All cheeses were ripened for 28 days in the Laboratory's ripening cellars at 8 °C with 95% relative humidity.

Table 1
Microbial species composition of the different groups A, B, C, and D.

Microbial community	Media for cultivating strains before inoculation	Levels of inoculation in milk (log CFU/g)	Taxonomic groups
Lactobacillus casei	MRS pH 6.5 at 30 °C	4.00	Goup A
Lactobacillus curvatus	(De Man et al., 1960)	3.00	Lactic acid bacteria
Lactobacillus plantarum		4.00	
Lactobacillus farciminis		3.00	
Leuconostoc citreum		3.00	
Ln, pseudomesenteroides	M17 at 30 °C	3.00	
Enterococcus faecalis	(Terzaghi and Sandine, 1975)	2.00	
Enterococcus hirae		2.00	
Staphylococcus saprophyticus	BHI (Broth Heart Infusion) at 37 °C	2.50	Group B
Staphylococcus equorum	(Forney et al., 1985)	2.50	Gram positive Catalase positive bacteri
Arthrobacter nicotianae		3.00	
Corynebacterium flavescens		2.00	
Corynebacterium casei		2.00	
Brevibacterium linens		2.00	
Exiguobacterium sp		3.00	
Staphylococcus haemolyticus		3.00	
Brachybacterium rhamnosum		2.00	
Macrococcus caseolyticus		2.00	
Pseudomonas putida	BHI (Broth Heart Infusion) at 25 °C	2.00	Group C
Enterobacter amnigenus	(Forney et al., 1985)	4.00	Gram negative bacteria
Acinetobacter sp		2.00	
Chryseobacterium sp		2.00	
Stenotrophomonas maltophilia		3.00	
Rhodosporidium babjevae	YPG (Yeast Peptone Glucose) at 25 °C	2.00	Group D
Debaryomyces hansenii	(Barnett et al., 1990)	2.00	Yeasts
Candida pseudointermedia		2.00	
Candida pararugosa		2.00	
Candida deformans		2.00	

The different species were combined in the three sets of experiments as indicated in Fig. 1.
The level of inoculation of each species was defined according to their counts in the inhibitory raw milk (Saubusse et al., 2007).

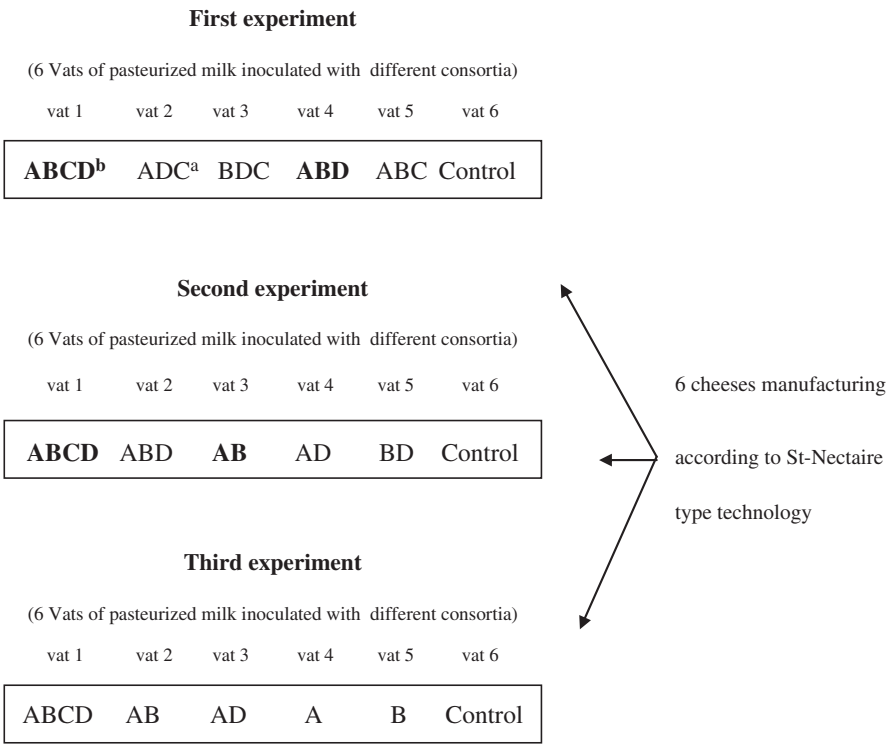

Fig. 1. Experimental design: three sets of experiments. [a]Different consortia ABCD, ABC, ADC, BDC, ABD, AB, AD, BD, A, and B were a combination of the following groups: lactic acid bacteria (A), Gram positive non lactic bacteria (B), Gram negative bacteria (C) and yeasts (D). Control = cheese manufactured with pasteurized milk only inoculated with *St. thermophilus*. [b]Consortia in bold were those selected for the next experiment according to their highest anti-*Listeria* properties.

2.3. Sampling

Samples were taken from the inoculated milks and from the cheeses at 1, 8, 18 and 28 days of ripening. They were stored at −20 °C before microbial and biochemical analysis. *L. monocytogenes*, pH and dry matter (DM) were measured on fresh sample cheeses.

2.4. Physical–chemical analysis

Cheese pH (cores and surfaces) were measured with a 926 VTV pH meter with Ingold 406 MX electrode (Mettler-Toledo S.A., Viroflay, France). Dry matter contents were determined according to reference method NFV 046282 (12/95).

2.5. Acids analysis

D- and L-lactic acid contents were determined by the enzymatic method. Six grams of cheese were blended in 25 mL of distilled water by means of a mixer with turbine (Ultra-Turrax, type 125, Grosseron S.A, Nantes, France) for 1 min at 9500 rpm. After incubation at 40 °C for 1 h and centrifugation at 8800 rpm for 30 min at 4 °C, supernatants were filtered on filters without ash. The contents were determined using the spectrometer method recommended in Diffchamb's EnzyPlus kits (Diffchamb France SARL, Lyon-France). The results were expressed as milligrams per gram of dry matter.

2.6. Determination of volatile compounds and volatile fatty acids

Frozen cheese samples were thawed at room temperature (21 °C) and 10 g of cheese was mixed with 10 g of dehydrated sodium sulphate (Prolabo, France) before extraction. The volatile compounds of the 1, 8 and 28 day old cheeses were extracted by the dynamic headspace method with an automatic Tekmar LSC 3000 system (Cincinnati, Ohio, USA) equipped with a Tenax trap. Ten grams of cheese cut into 12 cubes was placed in a cylindrical glass extractor (diam, 40 mm; height, 120 mm). The volatile compounds were extracted under the following conditions: purge helium flow rate of 40 mL/min; purge of 15 min at 25 °C; dry purge of 4 min; cryo-cooldown at −150 °C; desorb preheat at 225 °C; desorb 2 min at 220 °C; inject 1 min at 250 °C in a gas chromatograph maintained at 40 °C.

The volatile compounds were separated by high resolution gas chromatography (Chromatograph 6890, Hewlett Packard Agilent Technology, Les Ulis, France). Chromatograph conditions were as follows: Supelco capillary column (60 m × 0.32 mm; RTX5, Restek, Evry-France); stationary phase SPB5 (1 μm); carrier gas helium at 2 mL/min; oven temperature 40 °C for 6 min, then heated to 230 °C with a slope of 3 °C/min.

The volatile compounds were detected by mass spectrometry with a 5973N Hewlett Packard mass spectrometer (Agilent Technology, Les Ulis, France), in scan mode at 70 eV. They were then identified by comparing the experimental mass spectra with those contained in the NIST/EPA/MSDC Mass Spectral Database (Royal Society of Chemistry, Milton Road, Cambridge CB4 4WF, UK) and by comparing the experimental retention indices with those of the data bank compiled by Kondjoyan and Berdagué (1996). Each compound was semi-quantified with the arbitrary unit of area of a specific ion (au).

Volatile fatty acids were analyzed by SPME-GC/MS. Five grams of cheese were mixed with 45 mL of UHQ water using an Ultra-turrax. 400 or 1000 μL of this suspension were placed in 10 mL SPME glass flasks (Supelco, L'isle d'Abeau-France) with 700 or 100 μL UHQ water respectively and added with 100 μL H_2SO_4 2 N and 10 μL of 2-ethyl butyric acid 1 ppm as internal standard. The flasks were sealed by aluminium caps with Viton joints. The sample was heated to 60°C and the carboxen-PDMS fibre (0.75 mm) was left in contact with the sample's head-space for 30 min. The desorption of the volatile fatty acids from the fibre into the splitless injector of the GC apparatus lasted 5 min at 240 °C. The next steps were the same as for volatile

compounds. An external calibration was performed for all the volatile fatty acids studied, i.e. acetic, propionic, butyric, 2-methyl-propionic, pentanoic, 3-methyl-butyric, 2-methyl-butyric, hexanoic, 3-methyl-pentanoic acids (Sigma, L'isle d'Abeau-France).

2.7. Microbial analysis

L. monocytogenes count was performed in the milk after pasteurization to check its absence, and in the inoculated milk and cheese cores according to ISO 11290-2.

The microbial flora of the milks and cheeses were counted on different culture media: Facultatively Heterofermentative lactobacilli (FH) agar medium (Isolini et al., 1990) at 37 °C for 3 days under anaerobic conditions, Mayeux Sandine and Elliker (MSE) agar medium (Mayeux et al., 1962) at 30 °C for 48 h, Slanetz and Bartley (SB) (Slanetz and Bartley, 1957) at 42 °C for 48 h, Cheese Ripening Bacteria Medium (CRBM) (Denis et al., 2001) at 25 °C for 10 days, Baird Parker with rabbit plasma (RPF) medium (De Buyser et al., 2003) at 37 °C for 48 h, and Plate Count Agar (FIL-IDF 100B, 1991) with crystal violet (1%) and vancomycin (0.5%) as inhibitor of the Gram positive bacteria (PCAI), Oxytetracyclin Glucose Agar (OGA) medium (Mossel et al., 1962) at 25 °C for 5 days.

Some media are known to select certain genera: e.g. FH medium for *Lactobacillus*, MSE medium for dextran-producing *Leuconostoc* and SB medium for *Enterococcus* (Callon et al., 2007). The selectivity of CRBM, RPF and PCAI counting media were assessed by identifying representative populations growing on them using a combination of molecular methods. 141 isolates picked up from CRBM, RPF and PCAI counting plates were analyzed by RFLP (Restricted Fragment Length Polymorphism) with ECOR1 and HaeIII enzymes as described by Callon et al. (2007). Total genomic DNAs were extracted from the same counting plates. All the colonies were resuspended in 1 mL of 4 M of guanidine thiocyanate–Tris 0.1 M, N-lauroylsarcosine 10% and DNA was extracted with phenol/chloroform DNA method described by Duthoit et al., 2003 in cheese. Total extracted DNA were analyzed by SSCP (Single Strand Conformation Polymorphism) of V2 and V3 regions of DNAr 16S as described by Callon et al. (2007). Finally, total DNAs of the three media were amplified with species- or genus-specific primers indicated in the literature or developed in the present study, as indicated in Table 2. The specific primers for species *C. flavescens*, *Micrococcus caseolyticus* and *Enterobacter amnigenus* and for genera *Acinetobacte*r and *Brachybacterium* were designed after aligning 16S rDNA sequences collected from the GenBank database. The specificity of each primers was verified by a BLAST search in NCBI and in the European Ribosomal RNA Database. It was tested by specific PCR primer on all the strains composing the consortium and 9 reference type strains belonging to *Corynebacterium casei* (DPC 5298^T), *C. mooreparkense* (DPC 5310^T), *C. ammoniagenes* (CIP 101283^T), *A. citreus* (CIP 102363), *B. linens* (ATCC 9175), *S. intermedius* (DSMZ 20373), *S. epidermidis* (DSMZ 20044^T), *S. hyicus* (DSMZ 20459), *S. cohnii* (DSMZ 20260), *S. simulans* (DSMZ 20322) and 8 strains from laboratory collection belonging to *C. flavescens*, *Arthrobacter nicotianae, Brachybacterium* spp., *E. amnigenus*. PCR amplifications were performed in a final volume of 25 μL containing 1× PCR buffer with $MgCl_2$, 0.2 mM each dNTP (Roche Molecular Biomedicals Meylan, France), 0.6 μM each primer (Eurogentec France S.A, Angers, France), 0.625 unit Taq DNA polymerase (Appligene Oncor) and 1 μL of extracted DNA. PCR reactions were carried out in a thermal cycler Gene Amp PCR System 9700 apparatus (PE Applied Biosystems, Courtaboeuf, France) programmed for an initial denaturating of 4 min at 94 °C followed by 25 cycles of denaturation for 1 min at 94 °C, hybridization and elongation 1 min at 72 °C and a final elongation 7 min at 72 °C. Hybridization were performed 45 s at 60 °C (*C. flavescens*), 55 °C (*M. caseolyticus*), 62 °C (*Brachybacterium* sp. and *E. amnigenus*), and 58 °C (*Acinetobacte*r sp.). PCR were verified on 1.5% agarose gel by the presence of expected fragments at 500 pb for

Table 2
Sequences and references of primers from the literature or from this study used in species or genera specific PCR.

Genera or species	Primers	Sequences	References
Lactobacillus casei	16rev	5′GAAAGGAGGTGATCCAGC-3′	Berthier et al., 2001
	paracasei 16S	5′CACCGAGATTCAACATGG-3′	
Lactobacillus plantarum	16 forward	5′GCTGGATCACCTCCTTTC-3′	Berthier and Ehrlich, 1998
	Lpapl	5′ATGAGGTATTCAACTTATG-3′	
Enterococcus faecalis	ddlE1	5′ ATC AAG TAC AGT TAG TCT 3′	Dukta-malen et al., 1995
	ddlE1	5′ACG ATT CAA AGC TAA CTG 3′	
Enterococcus hirae	DuHIF	5′ TTA TGT CCC WGT WTT GAA AAA TCA A 3′	Knijff et al., 2001
	HIR	5′TTT TGT TAG ACC TCT TCC GGA3′	
Leuconostoc mesenteroïdes	Lnm1	5′ TGTCGCATGACACAAAAGTTA 3′	Cibick et al., 2000
	Lnm2	5′ ATCATTTCCTATTCTAGCTG 3′	
Leuconostoc citreum	Lncit1	5′ ACTTAGTATCGCATGATATC 3′	Cibick et al., 2000
	Lncit2	5′ AGTCGAGTTGCAGACTGCAG 3′	
Corynebacterium casei	Fs15	5′ CCG CAA GGC TAA AAC TCA AAG GAA T 3′	Monnet et al., 2006
	Fs17	5′ ACC GAC CAC AAG GGA AAG ACT 3′	
Pseudomonas putida	put f	5′ ATG CTG GTT CGY CGT GGC 3′	Ercolini et al., 2007
	put r	5′ TGA TGR CCS AGG CAG ATR CC 3′	
Stenotrophomonas maltophila	SM1	5′ CAG CCT GCG AAA AGT A 3′	Whitby et al., 2000
	SM4	5′ TTA AGC TTG CCA CGA ACA G 3′	
Staphycoccus saprophyticus	sap1	5′ TCA AAA AGT TTT CTA AAA AAT TTA C 3′	Corbiere Morot-Bizot et al., 2004
	sap2	5′ ACG GGC GTC CAC AAA ATC AAT AGG A 3′	
Brevibacterium spp.	Brevifor	5′ CGG TAC CTS CAG AAG AAG T 3′	Gelsomino et al., 2004
	Brevirev	5′ GTC AGT HAC AGC CCA GAG T 3′	
Acinetobacter spp.	AC1	5′ ACT TTA AGC GAG GAG GAG GCT 3′	In this study
	AC2	5′ GTC AGT ATT AGG CCA GAT GGC T 3′	
Enterobacter amnigenus	Ea for	5′ GAC TTG GAG GTT GTT CCC TTG 3′	In this study
	Ea rev	5′ GCA TGA ATC ACA AAG TGG TAA AGC 3′	
Brachybacterium spp.	Brachyfor	5′ TCG GGA TAA CCT CGG GAA ATC 3′	In this study
	Brachyre	5′ CGC ACG CCC GAG GTT G 3′	
Micrococcus caseolyticus	Macfor	5′-TAG CTT CGC ATG AAG CAA TA-3′	In this study
	Macrev	5′-TTA CGA TCC GAA AAC CTT CTT-3′	
Corynebacterium flavescens	CF1	5′-GCC TTT TTT AAG GTG ACG GTA CCT-3′	In this study
	CF2	5′-ACA AGC CAT CTC TGA CCC AAT C-3′	

C. flavescens, 253 pb for *M. caseolyticus*, 450 pb for *Brachybacterium* sp., 300 pb for *Acinetobacte*r sp. and 650 pb for *E. amnigenus*.

2.8. Data analysis

Microbial counting results were expressed as log of Colony Forming Unit (CFU) per mL in milk and per g in cheese cores.

In order to quantify the differences in *L. monocytogenes* population between treatments and control throughout ripening, the area of inhibition (AI) between two days (t1 and t2) of ripening was calculated using the formula of Wenzel and Marth (1990): AI = (t2 − t1)/2 × [(Ct2 + Ct1) − (Tt2 + Tt1)] where C = count of *L. monocytogenes* in control cheese inoculated with *St. thermophilus* and T = count of *L. monocytogenes* in trial cheese inoculated with *St. thermophilus* and other microbial species. The higher the AI values, the stronger the inhibition of *L. monocytogenes*.

For AI, pH, dry matter (DM), microbial counts, acid contents and volatile compounds data at different ripening times, standard analysis of variance (main-effects ANOVA) was performed to compare the effect of microbial milk inoculation (Statistica software).

Correlations between *L. monocytogenes* growth and variables such as pH, DM, microbial flora, acid contents and volatile compounds in the cheeses were calculated using Pearson's correlation coefficient.

3. Results

3.1. Inhibition of L. monocytogenes

The ability of each consortium to inhibit *L. monocytogenes* was evaluated by calculating Area of Inhibition (AI) values as described in the Materials and methods section. The cheeses prepared with milk only inoculated with *St. thermophilus* were taken as positive controls due the extensive growth of *L. monocytogenes* in the cores. The higher the AI values, the stronger the inhibition of *L. monocytogenes*. To identify the groups most involved in the inhibition, after the first set of experiments, the consortium ABD with the strongest inhibitory activity was selected for further simplification. ABD was chosen rather than ABC also inhibitory because of the technological interest of yeasts. After the second experiment, as AB was the most inhibitory, the consortiums A and B were separately tested as indicated in Fig. 1. The cheeses can be classed according to their AI values as indicated in Table 3i. Inhibition was strongest in experiment 3 for all inoculations, as illustrated by comparing ABCD consortia in the three experiments. All the consortia containing both lactic acid bacteria (group A) and non lactic acid bacteria (group B) — ABCD, ABD, ABC, AB — were more inhibitory than those containing lactic acid bacteria alone (A) or associated with yeasts only (AD) or/and Gram negative (ADC). Consortia without lactic acid bacteria were either weakly inhibitory or had no effect (Table 3i and ii). The Newman–Keuls tests taking into account the results from cheeses prepared twice with the same consortia indicated that the inhibition of *L. monocytogenes* was significantly lower in AD cheeses than in ABCD and AB cheeses throughout ripening (Table 3iii). AB cheese was more inhibitory than ABCD between 8 and 18 days of ripening. Between 18 and 28 days, ABCD and AB cheeses showed the same degree of inhibition. *L. monocytogenes* grew better in BD and B cheeses than in those prepared with only *St. thermophilus* as indicated by the negative values of their SAI (BD-SAI = −13.3, B-SAI = −4.4).

3.2. Listeria-pH and Listeria-DM correlations

In the first and second experiments, cheese pH varied in a similar way during ripening, regardless of the composition of the microbial consortia inoculated into the milk. The differences in cheese pH values according to inoculum were below 0.10 until 28 days and was 0.12 at 28 days.

In the third experiment, the pH value was the same in all cheeses at 1 day, but at 8 and 28 days the pH of cheeses inoculated only with

Table 3
Classification of cheeses according to the inhibition of *L. monocytogenes* expressed as AI in reference with cheese with only *St. thermophilus*.

	Trials	Experiment	AI(8-1)	AI(18-8)	AI(28-18)	SAI
i)	ABCD	3	3.6	11.9	31.3	46.7
	AB	3	−0.8	15	29.9	44
	ABCD	1	4.7	17.2	13.5	35.4
	AB	2	1.1	7.8	23.6	32.4
	ABD	1	0.6	12.8	16.3	29.6
	ABC	1	1.4	11.4	16.6	29.4
	AD	3	−2.2	5.7	24.3	27.7
	ABCD	2	3.2	6.9	16.7	26.7
	ABD	2	1.8	2.7	18.1	22.5
	A	3	−4.2	5.8	19.6	21.2
	ACD	1	0.2	8.6	8.9	17.6
	BDC	1	0	7.5	5.1	12.6
	AD	2	−0.1	0.5	8.4	8.8
	B	3	0.2	−2.5	−2.2	−4.4
	BD	2	−2.7	−6.8	−3.9	−13.3
ii)	A+	1 + 2 + 3	0.78 ± 2.36	8.9 ± 4.9	18.9 ± 7.3	28.5 ± 10.6
	A−	1 + 2 + 3	−0.83 ± 1.6	−0. 6 ± 0.2	−0.33 ± 0.1	1.7 ± 0.5
iii)	ABCD	2 + 3	**3.4 ± 0.32 b**	**9.4 ± 3.5 b**	**24.0 ± 10.3 b**	**36.7 ± 14.2 b**
	AB	2 + 3	**0.1 ± 1.34 a**	**11.4 ± 5.06 c**	**26.7 ± 4.5 b**	**38.2 ± 8.21 b**
	AD	2 + 3	**−1.2 ± 1.48 a**	**3.1 ± 3.64 a**	**16.3 ± 11.2 a**	**18.3 ± 13.4 b**

AI = (t2 − t1)/2 × [(Ct2 + Ct1) − (Tt2 + Tt1)].
C = count of *L. monocytogenes* in control cheese inoculated with *St. thermophilus* and T = count of *L. monocytogenes* in trial cheese, *i.e.* prepared with *St. thermophilus* and different microbial combinations.
t1 and t2 = two days of ripening.
AI (t2 − t1): area of inhibition between two days of ripening.
SAI = ∑ AI at all times of ripening.
Trials = cheeses prepared with different consortia ABCD, ABC, ADC, BDC, ABD, AB, AD, BD, A, and B with the following groups: A: lactic acid bacteria, B: Gram positive non lactic acid bacteria, C: Gram negative bacteria, and D: yeasts.
i) AI results for the different trials for the 3 experiments, without statistical treatment.
ii) Statistical analysis main-effects ANOVA with AI in cheeses inoculated with group A (A+) or not (A−).
iii) Statistical analysis ANOVA main-effects with AI in cheeses ABCD, AB, AD of the both experiments 2 and 3 (2 replicates).
Means within column with different (in bold in the table) are significantly different ($P<0.2$) with a<b<c by statistical test of Newman–Keuls.

Table 4
Statistical analysis with counts of flora (log CFU/) on different media (SB, MSE, FH, PCAI, CRBM and OGA described in Materials and methods) at 1, 8 18 and 28 days of ripening in cheeses inoculated with different groups: A: lactic acid bacteria, B: Gram positive non lactic acid bacteria, C: Gram negative bacteria and D: yeasts.

Counts of lactic acid bacteria on selective media (log CFU/g)

Combinations		SB 1 day	SB 8 days	SB 18 days	SB 28 days	FH 1 day	FH 8 days	FH 18 days	FH 28 days	MSE 1 day	MSE 8 days	MSE 18 days	MSE 28 days
i)	A+	6.4 ± 0.2	7.0 ± 0.7	6.9 ± 01	6.8 ± 02	5.8 ± 0.2	7.7 ± 0.1	8.3 ± 0.2	8.7 ± 0.2	6.9 ± 0.4	7.5 ± 0.	7.4 ± 0.3	7.7 ± 0.3
	A−	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
ii)	ABCD	6.0 ± 0.9	7.2 ± 0.1	7.0 ± 0.7	**6.9 ± 0.3 a**	5.4 ± 0.6	7.7 ± 0.4	8.4 ± 0.4	9.4 ± 05	6.4 ± 1.1	7.2 ± 1.1	7.1 ± 1.3	6.5 ± 1.5
	AB	6.7 ± 0.3	6.9 ± 0.3	7.1 ± 0.3	**7.2 ± 0.2 b**	5.6 ± 1.0	7.7 ± 0.4	8.9 ± 0.2	8.9 ± 0.3	6.5 ± 1.8	7.1 ± 1.4	6.5 ± 0.5	8.0 ± .0.1
	AD	6.7 ± 0.4	7.3 ± 0.3	7.2 ± 0.5	**7.3 ± 0.3 b**	5.8 ± 1.3	7.6 ± 0.4	8.4 ± 0.3	8.8 ± 0.2	6.3 ± 1.2	7.2 ± 1.3	6.6 ± 0.6	8.0 ± 0.2

		Counts of Gram + catalase + (log CFU/g)[a]						Counts of yeasts (log CFU/g)			
		CRBM 1 day	CRMB 8 days	CRBM 18 days	CRBM 28 days			OGA 1 day	OGA 8 days	OGA 18 days	OGA 28 days
iii)	B+	6.2 ± 0.3	6.8 ± 0.3	6.8 ± 0.3	6.4 ± 0.3	vii)	D+	3.9 ± 0.09	4.1 ± 0.1	4.8 ± 0.2	4.3 ± 0.2
	B−	<2	<2	<2	<2		D−	<2	<2	<2	<2
iv)	ABCD	5. 7 ± 0.5	6.6 ± 0.5	6.8 ± 0.7	6.7 ± 0.3	viii)	ABCD	3.9 ± 0.4	4.01 ± 0.3	4.6 ± 0.3	4.4 ± 0.7
	AB	6.7 ± 0.5	6.6 ± 0.6	6.8 ± 0.7	6.5 ± 0.2		AB	<2	<2	<2	<2
	AD	<2	<2	<2	<2		AD	4.2 ± 0.2	4.5 ± 0.2	4.9 ± 0.1	4.8 ± 0.1

Counts of Gram − (log CFU/g)

Gram−		PCAI 1 day	PCAI 8 days	PCAI 18 days	PCAI 28 days
v)	C+	5.6 ± 0.5	6.1 ± 0.3	5.7 ± 0.3	4.8 ± 0.2
	C−	<2	<2	<2	<2
vi)	ABCD	4.7 ± 0.9	5.5 ± 0.2	5.5 ± 0.2	4.6 ± 0.6
	AB	<2	<2	<2	<2
	AD	<2	<2	<2	<2

Statistical analysis main-effects ANOVA with counts of flora:
i) in all cheeses inoculated with group A (A+) or not (A−), iii) with group B (B+) or not (B−), v) with group C (C+) or not (C−), vii) with group D (D+) or not (D−).
ii) ii), iv), vi), viii) in cheeses ABCD, AB, AD of the both experiments 2 and 3 (2 repetitions).
Means within column with different letters are significantly different ($P<0.05$) with a<b.
ns: non significant by statistical test of Newman–Keuls ($p<0.05$).
[a] Level on CRBM have been estimated according to the selectivity and the ratio of *Enterococcus* identified on this medium (i.e. paragraph 3.3).

Gram positive catalase positive bacteria had the highest pH values. At 28 days this was 0.3 units higher than in those with only lactic acid bacteria, which had the lowest pH values (5.25).

The *L. monocytogenes* count at 28 days was positively correlated with pH values at 8 ($r = 0.52$, $n = 15$) and 18 days ($r = 0.6$, $n = 15$).

The dry mater content of the cheeses varied greatly from 45.7 to 51.0, according to ripening time and milk inoculation, but there was no correlation with *L. monocytogenes* count ($r = 0.18$, $n = 15$).

3.3. Microbial analysis

Lactic acid bacteria can be counted at genus level on media recognized for their selectivity (Callon et al., 2007); their growth during ripening was then evaluated. No lactic acid bacteria were found in cheeses not inoculated with group A (BDC, BD and B, results not shown). First, statistical treatments were applied to microbial data considering all combinations and experiments (Table 4i, iii, v, vii). Then, on the basis of *L. monocytogenes* results, it was decided to present only the microbial results from cheeses prepared twice with the same consortium (ABCD, AB and AD in the experiments 2 and 3 (Table 4ii, iv, vi, viii)). After 28 days of ripening *Lactobacillus* (counted on FH medium) was the dominant population in the core of cheeses prepared with group A (Table 4i); its level was over 8.7 log CFU/g. There was no significant difference in the dynamics of *Lactobacillus* between cheeses ABCD, AB and AD (Table 4ii). In all the 3 cheeses, *Lb. curvatus* was detected by SSCP analyses (Table 5), and *Lb. casei* and *Lb. plantarum* by PCR primers, but *Lb. farciminis* was not found. Considering all cheeses prepared with group A, *L. monocytogenes* count at 8 days was negatively correlated with that of *Lactobacillus* at 1 day ($r = -0.67$, $n = 12$) and 8 days ($r = -0.75$, $n = 12$). A negative correlation was observed between the count of *L. monocytogenes* at 28 days and that of *Lactobacillus* at 18 days ($r = -0.84$, $n = 12$). *L. monocytogenes* counts at 1 and 18 days were also negatively correlated with the initial level of *Lactobacillus* ($r = -0.74$, $n = 12$ at 1 day and $r = -0.6$, $n = 12$ at 8 days). Inhibition of *L. monocytogenes* was strongest in the third experiment cheeses with the highest initial levels of *Lactobacillus*.

The level of dextran-producing *Leuconostoc* on MSE medium reached 7.68 log CFU/g at 28 days in cheeses prepared with lactic acid bacteria (Table 4i). There were no significant differences in their dynamics between cheeses ABCD, AB and AD (Table 4ii). *Ln. pseudomesenteroides* and *Leuconostoc citreum* were distinguished by their morphology on MSE medium and by specific PCR for *Ln. citreum*. They were both detected in three cheeses, with higher levels for *Ln. pseudomesenteroides* (7.7 log CFU/g) than *Ln. citreum* (5 to 6 log CFU/g). There was no correlation between *L. monocytogenes* and *Leuconostoc* count.

Enterococcus on SB medium reached a peak of 6.92 log CFU/g at 18 days of ripening (Table 4i). Irrespective of the inoculum used, the *Enterococcus* counts on SB medium (Table 4ii) were not significantly different in cheeses ABCD, AB and AD until 18 days of ripening. At 28 days of ripening, their level was higher in the AB and AD cheeses than in ABCD. It was shown by SSCP, specific PCR, RFLP and counts on SB medium that species *E. faecalis* and *Enterococcus hirae* were both present in all the cheeses (Table 5). Whatever the ripening time, there was no correlation between their levels and those of *L. monocytogenes*.

Gram positive non lactic acid bacteria populations were evaluated on CRBM medium after checking the medium's selectivity. Microbial populations growing on CRBM medium were identified by combining different molecular methods (SSCP, RFLP and specific PCR analysis as described in Table 5). The genus *Enterococcus* represented 62 to 75% (ratio of *Enterococcus* peak in SSCP profiles) of colonies on CRBM medium from cheeses inoculated with lactic acid bacteria (results not shown). *L. monocytogenes* was also found occasionally. Taking this into account, Gram positive catalase positive count was then estimated. It was highest (6.8 log CFU/g) at 8 and 18 days of ripening (Table 4iii) and was similar in ABCD and AB cheeses at all ripening times (Table 4iv). Combining the results obtained by SSCP, RFLP and specific PCR (Table 5), bacteria of group B inoculated in milk — *C. casei*, *A. nicotianae, Exiguobacterium*, *M. caseolyticus, Staphycoccus saprophyticus* and *S. equorum* — were all detected in cheeses ABCD and AB whereas *C. flavescens*, *Brachybacterium rhamnosum* and *Staphylococcus haemolyticus* were only detected in cheese ABCD and *B. linens* was never detected. There was no correlation between the overall level of all Gram positive catalase positive bacteria and that of

Table 5
Implantation of species of the consortium in cheeses ABCD, AB, AD(inoculated with the following groups: A: lactic acid bacteria, B: Gram positive non lactic acid bacteria, C: Gram negative bacteria and D: yeasts) analysed by combining SSCP, RFLP and specific PCR analysis on isolates and DNA from the counting plates of media, all times at once as described in Materials and methods.

Cheeses	Methods	Strains of group A							Strains of group B										Strains of group C				Strain of group D				
		E. faecalis	*E. hirae*	*Ln. pseudomesenteroides*	*Ln citreum*	*Lb. plantarum*	*Lb. casei*	*Lb. curvatus*	*S. saprophyticus*	*S. equorum*	*S. haemolyticus*	*M. caseolyticus*	*A. nicotianae*	*Brachy. rhamnosum*	*B. linens*	*C. casei*	*C. flavescens*	*Exiguobacterium spp*	*Ps. putida*	*Acinetobacter sp*	*E. amnigenus*	*Stenotro. maltophilia*	*Rht ; babjevae*	*D. hansenii*	*C. deformans*	*C. pseudointermedia*	*C. pararugosa*
ABCD	RFLP	x	x											x					x		x							
	SSCP	x	x		x	x	x	x	x	x	x	x	x			x	x											
	PCR	x	x		x	x	x		x							x	x		x		x	x						
	Count	x	x	x	x													x					x		x	x	x	
AB	RFLP	x	x																									
	SSCP	x	x					x	x	x		x	x			x		x										
	PCR	x	x		x	x	x		x							x	x											
	Count	x	x	x	x													x										
AD	RFLP	x	x																									
	SSCP	x	x					x																				
	PCR	x	x		x	x	x																					
	Count	x	x	x	x																		x		x	x	x	

x: species detected, □: non detected, ▩: non researched.

L. monocytogenes. Nevertheless, they may play a part in inhibition as the inhibition was greater in AB and ABCD cheeses than in AD ones.

In cheeses inoculated with Gram negative bacteria (C group), the bacterial level on PCAI was 4.8 log CFU/g (Table 4v) but this medium was not selective enough to quantify Gram negative bacteria well. Indeed, *Brachybacterium* spp. and *Staphylococcus* (representing up to 24% of the total population on some counting plates) or *E. faecalis* and *Lactobacillus* (40% of population on some counting plates) were detected by molecular methods on this medium. In ABCD cheese, among Gram negative bacteria inoculated, only *Pseudomonas putida*, *Stenotrophomonas maltophilia, E. amnigenus* were present but not *Acinetobacter* sp. and *Chryseobacterium*.

The yeast population was only counted in cheeses prepared with this group (Table 4vii) and highest level was 4.8 log CFU/g at 18 days. Regarding the differences in their morphology on OGA medium, it can be concluded that the 4 species *Rhodosporidium babjevae*, *Candida deformans*, *Candida pararugosa* and *Candida pseudointermedia* were present in cheeses ADCB and AD (Table 5) whereas the species *Debaryomyces hansenii* was not detected. Yeast levels were similar in ABCD and AD cheeses (Table 4viii). Considering all cheeses inoculated with group D ($n=10$), their level was not correlated with that of *L. monocytogenes*. However, between 18 and 28 days of ripening, the presence of yeast reduced the inhibitory effect of lactic acid bacteria: inhibition of *Listeria* was weaker in ABD cheese than in AB cheese in the experiment 2, and weaker in AD cheese than in A cheese in experiment 3, as shown in Table 3iii.

3.4. Acid contents

The effect on cheese acid content of the presence or absence of lactic acid bacteria in cheeses was analyzed (Table 6i). The D-lactic acid contents at 8, 18 and 28 days were significantly higher in cheeses prepared with lactic acid bacteria (group A) than without, whereas the L-lactic acid contents at 8 and 18 days were similar in the two kinds of cheese. Moreover, L-lactic acid was present at the same level in the control cheese prepared with only *St. thermophilus*, so it may be produced by this species. Butyric and hexanoic acid contents were similar at 1 day in cheese with or without the group A but at 28 days, they were significantly higher in cheeses with A than in those without (Table 6i). This was also true of acetic acid at both 8 and 28 days. These different levels of a number of acids may explain the lack of inhibition in cheeses without lactic acid bacteria in the consortia. The levels of 3-methyl-butyric acid at 28 days were higher in cheeses without A.

The acid contents in ABCD, AB and AD cheeses were compared by main-effects ANOVA for cheeses manufactured twice with the same microbial consortia (Table 6ii). At 8 and 28 days the D-lactic acid content was significantly higher in cheeses AB than in cheeses ABCD or AD. In all cheeses it increased throughout the ripening. D-lactic acid contents were strongly negatively correlated with the *L. monocytogenes* count (at 1 day, $r=-0.89$, $n=15$ and at 18 days $r=-0.75$, $n=15$). Moreover, D-lactic acid at 18 and 28 days was strongly correlated with *Lactobacillus* levels at every ripening time ($r=0.80$ to 0.90). Similarly, there was a strong positive correlation between D-lactic acid and *Leuconostoc* levels at 28 days ($r=0.77$, $n=15$).

At 28 days of ripening the L-lactic acid content was similar in cheeses ABCD and AB (19.4 and 17.6 mg/g) but lowest in cheese AD (14.6 mg/g) due to a decrease between 18 and 28 days of ripening. Whatever the ripening time, there was no correlation between *Listeria* count and L-lactic acid content. No correlation was found between L-lactic acid contents and microflora counted on different media.

Acetic acid content increased in the 3 cheeses ABCD, AB and AD until 28 days. It was similar in the 3 cheeses at 8 days but at 28 days, it was lower in AD cheese than in ABCD and AB (Table 6ii). The correlation between acetic acid content and *L. monocytogenes* counts during ripening ($r<0.5$, $n=15$) was weak. There was a positive correlation between acetic acid content at 28 days and *Leuconostoc* levels at 1 to 28 days ($r=0.65$ at 1 day and $r=0.78$ at 28 days, $n=15$).

Butyric and hexanoic acid contents were significantly higher in AB cheese than in ABCD or AD at 28 days (Table 6ii).

3.5. Volatile compounds

136 volatile compounds were identified by GC/MS analysis. They were aldehydes (14 compounds), alcohols (15), ketones (18), esters (10), sulphur compounds (5), furans (6), ethers (1), alcanes (20), alcenes (7), benzene derivatives (12), terpenes (8), chloride derivatives (4) and nitrogen derivatives (2). But the levels of only 7 volatile compounds varied according to the consortia inoculated.

All cheeses inoculated with lactic acid bacteria were distinguished from those without by higher levels (arbitrary units of area) of ethyl formiate, pentane and alcohols (2-butanol, 2-pentanol), and lower levels of ketones (2-hexanone, 2,3-butanedione) and aldehydes (2-methyl-butanal) (Table 7i).

Levels of 2-methyl-butanal, 2-butanol and 2-pentanol were higher in ABCD and AB cheeses than in AD (Table 7ii). Levels of other compounds were similar between these 3 consortia.

Counts of *L. monocytogenes* at 28 days were negatively correlated with 2-butanol and 2-pentanol at 28 days ($r=-0.8$ and $r=-0.7$, $n=15$). Both compounds were positively correlated with lactic acid bacteria (same value of $r=0.9$ with counts of *Lactobacillus* on FH medium, *Enterococcus* on SB medium and *Leuconostoc* on MSE medium, $n=15$).

4. Discussion

The simplification methodology was effective for identifying microbial groups of a complex microbial consortium involved in the inhibition of *L. monocytogenes* compared to its growth in cheeses

Table 6
Statistical analysis with D- and L-lactate (mg/g DM), acetic acid (mg/kg DM), butyric and hexanoic acids (mg/kg DM) contents at 1, 8, 18 and 28 days of ripening in cheeses inoculated with different groups: A: lactic acid bacteria, B: Gram positive non lactic acid bacteria, C: Gram negative bacteria and D: yeasts.

	Cheeses	D-lactic acid			L-lactic acid			Acetic acid		Butyric acid		Hexanoic acid	
		8 days	18 days	28 days	8 days	18 days	28 days	8 days	28 days	1 day	28 days	1 day	28 days
i)	A+	**2.6 ± 0.4 b**	**5.5 ± 0.5 b**	**7.0 ± 0.5 b**	17.7 ± 0.7	19.1 ± 0.6	18.4 ± 1.0	**612 ± 223 b**	**1490 ± 236 b**	1.6 ± 0.8	**46.4 ± 23 b**	1.2 ± 0.5	**18.6 ± 13.6 b**
	A−	**1.3 ± 0.6 a**	**0.8 ± 0.7 a**	**0.7 ± 0.6 a**	19.0 ± 0.6	20.9 ± 2.8	18.2 ± 4.4	**74 ± 34 a**	**178 ± 95 a**	1.5 ± 1.5	**20.1 ± 10.5 a**	1.2 ± 1.2	**3.9 ± 3.9 a**
ii)	*ABCD	**3.0 ± 1.5 a**	4.8 ± 2.3	6.6 ± 1.9	18.0 ± 2.0	18.1 ± 1.9	**19.4 ± 1.3 b**	630 ± 194	1167 ± 263	**3.0 b**	**23.3 ± 9.3 a**	2.5	**5.5 a**
	AB	**3.5 ± 1.6 b**	5.7 ± 2.2	**7.1 ± 1.4**	18.3 ± 3.3	17.2 ± 0.6	**17.6 ± 0.6 b**	664 ± 35	**1305 ± 735**	**3.8 b**	**55.1 ± 34 b**	2.0	**17.2 b**
	AD	**3.3 ± 1.5 ab**	5.9 ± 1.5	6.2 ± 0.7	16.8 ± 1.8	18.5 ± 1.5	**14.6 ± 1.1 a**	613 ± 237	903 ± 228	**0.0 a**	**15.1 ± 14 a**	1.1	**8.2 a**

D- and L-lactic acids have been analyzed by enzymatic kits. Acetic and volatile fatty acids have been analyzed by CPG.
Statistical analysis main-effects ANOVA of acid contents:
i) in cheeses inoculated with group A (A+) or not (A−).
ii) Statistical analysis main-effects ANOVA with D- and L-lactate, acetic, butyric and hexanoic contents in cheeses ABCD. AB. AD of the both experiments 2 and 3 (2 repetitions). Means within column with different letters are significantly different ($P<0.05$) with a<b according to statistical test of Newman–Keuls.

Table 7
Statistical analysis with volatile compounds (log arbitrary unit of area) analyzed by PT-GC/MS at 1 and 28 days of ripening in cheeses inoculated with different groups: A: lactic acid bacteria, B: Gram positive non lactic acid bacteria, C: Gram negative bacteria and D: yeasts.

		2-methyl-butanal		2-butanol		2-pentanol		2-hexanone		3-methyl-2-butanone		Ethyl formiate		Pentane	
		1 day	28 days	1 day	28 days	1 day	28 days	1 day	28 days	1 day	28 days	1 day	28 days	1 day	28 days
i)	A+	4.1	3.9	**4.7 b**	**5.9 b**	3.5	**6.3 b**	3.1	**3.4 a**	1.7	2.1	**2.9**	**4.5 b**	4.5	**4.4 b**
	A−	4.2	3.7	**4.0 a**	**4.5 a**	2.5	**2.9 a**	3.3	**4.5 b**	1.8	3.0	**2.6**	**3.3 a**	4.4	**3.1 a**
ii)	ABCD	**4.1 b**	4.1	**5.1 b**	5.9	**4.4 c**	6.3	4.0	3.1	3.1	3.4	3.0	4.5	4.7	4.7
	AB	**4.2 b**	3.7	**5.0 b**	5.8	**4.2 b**	6.4	3.7	2.9	2.9	3.8	3.1	4.5	4.9	4.8
	AD	**3.7 a**	4.5	**4.7 a**	6.1	**3.7 a**	6.5	4.1	2.0	2.0	3.2	2.8	4.7	4.7	4.9

Statistical analysis main-effects ANOVA with volatile compound contents:
i) in cheeses inoculated with group A (A+) or not (A−).
ii) in cheeses ABCD, AB, AD of the both experiments 2 and 3 (2 repetitions).
Means within column with different letters are significantly different ($P<0.2$) with a<b<c according to statistical test of Newman–Keuls.

inoculated only with *St. thermophilus*. The omission of Gram negative bacteria (group C) from ABCD did not change the inhibition of *L. monocytogenes*, whereas the omission of yeasts (group D) decreased it. It was concluded that, during ripening, lactic acid bacteria had the most significant effect on *Listeria* inhibition but that the addition of the ripening bacteria (group B) increased the inhibition. Inhibition was correlated mainly with the level of *Lactobacillus*, suggesting the role of this genus as mentioned by Gay and Amgar (2005) in Camembert cheeses.

The strongest interaction for the inhibition was found when lactic acid bacteria (*Lb. casei, Lb. plantarum, Lb. farciminis, Lb. curvatus, Ln. citreum, Ln. pseudomesenteroides, E. faecalis, E. hirae*) were associated with Gram + catalase + bacteria detected in AB cheeses (*C. casei*, *C. flavescens, A. nicotianae, S. saprophyticus, S. equorum, M. caseolyticus, Exiguobacterium* spp.). Saubusse et al. (2007) have pointed out that *C. flavescens* inoculated into milk can inhibit *L. monocytogenes*. Low pH can contribute to the inhibition as the growth of *L. monocytogenes* in the experiment 3 was the highest when the pH was the highest.

Inhibition was associated with levels of D-lactic acid, L-lactic acid and acetic acid. These acids may act in synergy, especially at the end of ripening. Le Marc et al. (2002) modeled the growth kinetics of *Listeria innocua* as a function of pH values from 4.5 to 9.4, the undissociated forms of lactic acid, acetic and propionic contents and temperature values. The minimum inhibitory concentrations of undissociated acids were 8 mM for lactic acid, 20.3 mM acetic acid and 8.8 mM for propionic acid. *L. monocytogenes* may also be more sensitive to D-lactic acid than L-lactic acid (Gravesen et al., 2004). Millet et al. (2006) also pointed out that the inhibition of *L. monocytogenes* may be due to lactic acid content at concentrations above 14 mg/g in cheese. The correlations suggest that, in our consortia, bacterial populations able to produce D-lactic acid may have been *Lb. plantarum, Lb. curvatus, Ln. pseudomesenteroides, Ln. citreum* but not Gram + catalase + bacteria. This may explain why the latter on their own were not inhibitory. The decrease in L-lactic acid at 28 days of ripening in cheeses inoculated with lactic acid bacteria and yeast is not surprising, as yeasts *C. deformans* and *C. pararugosa* are able to consume it.

The highest level of acetic acid was produced when lactic acid bacteria and Gram + catalase + bacteria were associated. Then, these both groups can be involved in this production. Heavin et al. (2009) showed that the growth of *L. monocytogenes* in a synthetic medium was reduced by 50% by 3000 mg/kg of acetic acid, but this concentration is higher than those found in our cheeses mesophilic lactobacilli such as *Lb. plantarum* or *Lb. casei* can produce acetic acid from lactose, citrate or amino acid metabolism (Palles et al., 1998; Skeie et al., 2008; Hussain et al., 2009; Ong and Shah, 2009).

Butyric and hexanoic acids were only detected in the cheeses in experiment 3 with the highest levels of *Lactobacillus*, that were most strongly inhibitory. *Lb. casei*, *Lb. acidophilus, Lb. rhamnosus* were able to produce these acids by lipolysis of milk fat (Yadav et al., 2007; Ong and Shah, 2009), or by amino acid catabolism (Ganesan et al., 2004). Butyric acid was detected in ewe's milk cheeses prepared with *Lb. casei* and *Lb. plantarum* (Irigoyen et al., 2007). Hexanoic acids at 530 mg/kg, at upper concentration than in our study, inhibited *L. monocytogenes* in a soft blue cheese with *Pen. roqueforti* (Kinderlerer and Lund, 1992).

Among volatile compounds only 2-3-butanedione, acetoine and 2-3 butanediol has been described as bactericide against *Listeria* at concentration upper than 100 ppm (Lanciotti et al., 2003). In our study these compounds were not in highest concentration in inhibitory cheeses. In spite of higher concentrations of some alcohols, aldehydes, ketones, esters in inhibitory cheeses with lactic acid bacteria, their contribution in *L. monocytogenes* inhibition is still questionnable because their causal effect was not demonstrated. They can be only an indicator of the good establishment of some flora. Production of volatile compounds by the consortium AB associating lactic acid bacteria and Gram + catalase + bacteria could have a positive effect on the flavour of cheese. Indeed, this consortium was tested in a farm producing raw milk PDO St-Nectaire cheese (cf deliverable D61.1.2 of Truefood project www.truefood.eu). The cheeses with the consortium were significantly more elastic by finger touching and more sticky and melting in mouth than the control cheese prepared without the consortium. It had more intense butter and pungent odor and higher intensity and salted, acid taste and mould aroma scores but less "hard cooked cheese" and dried fruit aroma than control cheeses.

The inhibition may be due to other metabolites or to nutrient competition by these flora. Bacteriocins were probably not involved as by agar plate method they were not detected in any strain of the consortium (Saubusse et al., 2007). But their absence in cheese remains to be checked.

Our omission methodology can help to identify microbial groups of complex consortium with promising anti-listerial effects. It could constitute an alternative to the various interesting methods described by Monnet et al. (2009) for screening anti-listerial activity. It could contribute to the understanding of microbial interactions responsible of inhibition by complex consortiums associating several coryneform bacteria (*C. casei, C. variabile, B. linens, M. gubbeenense* and *Arthrobacter*) (Eppert et al., 1997, Maoz et al., 2003; Mayr et al., 2004; Roth et al., 2010). For defining simplified consortium our approach should also be based on the evaluation of strains of consortium to produce or consume different acids (lactic, acetic, hexanoic acids…). This will help to define simplified consortium.

The "Jameson effect" described by Mellefont et al. (2008) and pointed out by Guillier et al. (2008) to explain inhibition of *Listeria* on wood may not have occurred in our cheeses. In our study, lactic acid bacteria were at high levels in all cheeses inoculated with A, but the inhibition varied nevertheless (weaker in AD cheeses than in AB).

Our results also suggest that the inhibition can be linked to functional microbial groups producing inhibitory substances that can be counteracted by other species consuming them. They also argue for

the antilisterial properties of all these species isolated from raw milk. Studies are in progress to evaluate these mechanisms.

Acknowledgments

This work received funding from the TRUEFOOD, "Traditional United Europe Food," an integrated project financed by the European Commission under the 6th Framework Programme for RTD (contract no. FOOD-CT-2006-016264).

The authors also sincerely thank R. Lavigne and B. Desserre for their technical assistance concerning cheesemaking and microbial analysis.

English revision was done by Harriet Coleman.

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