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Original article

Is microbial diversity an asset for inhibiting *Listeria monocytogenes* in raw milk cheeses?

Émilie Retureau, Cécile Callon, Robert Didienne, Marie-Christine Montel*

INRA, UR545 Recherches Fromagères, 20 côte de Reyne, 15000 Aurillac, France

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Abstract – This study aimed at determining if microbial diversity can be an asset to guarantee the microbial safety of raw milk cheeses. Our results show that microbial consortia from the surface of raw milk cheeses can self-protect against *Listeria monocytogenes*. Indeed, 10 complex microbial consortia among 34 tested from the surfaces of raw milk Saint-Nectaire cheeses were particularly effective for reducing the growth of *L. monocytogenes* on cheese surfaces in comparison of a commercial ripening culture, despite the high pH values on the surfaces. One of these consortia (TR15) was selected and propagated on cheese surfaces to create a collection of strains belonging to lactic acid bacteria, Gram-positive and catalase-positive bacteria, Gram-negative bacteria and yeasts. On the surfaces of uncooked cheeses, defined consortia consisting of combinations of several isolates from this collection displayed weaker antagonist activity against *L. monocytogenes* than the complex consortium TR15. The results from plate counting and analysis by single strand conformation polymorphism (SSCP) converged to show that microbial dynamics in cheeses TR15 differed from that of the defined consortia. TR15 cheeses had the highest levels of cultivable lactobacilli and leuconostocs. Their SSCP profiles were the richest in peaks and were characterised by the presence of *Marinilactibacillus psychrotolerans*, *Carnobacterium mobile*, *Arthrobacter nicotianae* or *A. arilaitensis*, *Arthrobacter ardleyensis* or *A. bergerei* and *Brachybactrerium* sp. Further investigation will be necessary to gain a better understanding of the microbial interactions involved in inhibiting *L. monocytogenes*.

***Listeria monocytogenes* / microflora / inhibition / cheese / raw milk**

摘要 – 微生物多样性是否可以抑制鲜乳干酪中的 *Listeria monocytogenes*。本研究旨在确定微生物多样性能否作为监测鲜乳干酪的微生物安全性的有效工具。 结果表明来自鲜乳干酪表面的微生物菌群能够自我防护 *Listeria monocytogenes*。 实际上，来自鲜奶干酪 St Nectaire 表面的 34 份供试微生物菌群中，比商业成熟发酵剂相比，有 10 个微生物菌群显著地降低了 *L. monocytogenes* 在干酪表面的生长，尽管在干酪表面具有高 pH 值。 微生物菌群 TR15 被接种于干酪表面，分离得到了乳酸菌、革兰氏阳性和过氧化氢酶阳性细菌以及革兰氏阴性细菌和酵母。 在未加工干酪的表面，由上述分离菌株组成的菌株明确的微生物菌群，比 TR15 相比，对 *L. monocytogenes* 的抗性较弱。 培养基平板计数和单链构象多态性 SSCP 分析结果，结果表明 TR15 在干酪中的微生物动态不同于明确菌株的微生物菌群的动态。 TR15 干酪在琼脂培养基上含有最高水平的乳杆菌和明串珠菌。 SSCP 分析也表明乳杆菌和明串珠菌最为丰富，同时含有 *Marinilactibacillus psychrotolerans*, *Carnobacterium mobile*, *Arthrobacter nicotianae* (或者 *Arthrobacter arilaitensis*), *Arthrobacter ardleyensis* (或者

*Corresponding author (通讯作者): cmontel@clermont.inra.fr

Arthrobacter bergerei) 和 *Brachybactrerium* sp. 菌株。需要进一步研究以更好地理解抑制 *L. monocytogenes* 过程中微生物交互作用。

***Listeria monocytogenes* / 多样性 / 抑制 / 干酪 / 原料奶**

Résumé – La diversité microbienne est-elle un atout pour inhiber *Listeria monocytogenes* dans les fromages au lait cru ? L'objectif de l'étude était de déterminer si la biodiversité microbienne est un atout pour garantir la sécurité sanitaire des fromages au lait cru. Nos résultats montrent que les consortia microbiens naturellement présents à la surface des fromages peuvent auto-protéger les fromages contre *Listeria monocytogenes*. En effet, dix consortia complexes issus de croûtes de fromages de St Nectaire parmi 34 testés réduisaient la croissance de *L. monocytogenes* à la surface des fromages malgré leur pH élevé, en comparaison avec un ferment commercial de surface. Le consortium le plus inhibiteur (TR15) a été sélectionné. Une collection de souches de bactéries lactiques, de bactérie à Gram positif et catalase positive, de bactéries à Gram négatif et de levures, issues de la culture du consortium TR15 sur des fromages, a été constituée. À la surface d'un fromage à pâte pressée non cuite, le consortium complexe TR15 était plus inhibiteur que les communautés reconstituées en combinant les souches de la collection. Les résultats des dénombrements sur milieux gélosés et l'analyse par Single Strand Conformation Polymorphism (SSCP) convergaient pour montrer que la dynamique microbienne sur les fromages obtenus avec le consortium TR15 se distinguait de celle des fromages obtenus avec les consortia reconstitués. Les fromages obtenus avec le consortium TR15 avaient les niveaux de lactobacilles et de leuconostocs cultivables les plus élevés. Leurs profils SSCP à la surface des fromages étaient les plus riches en pics et étaient caractérisés par la présence de *Carnobacterium mobile*, *Marinilactibacillus psychrotolerans*, *Arthrobacter nicotianae* ou *A. arilaitensis*, *Arthrobacter ardleyensis* ou *A. bergerei* et *Brachybactrerium* sp. Des études complémentaires sont nécessaires afin de mieux comprendre les interactions microbiennes impliquées dans l'inhibition.

***Listeria monocytogenes* / microflore / inhibition / fromage / lait cru**

1. INTRODUCTION

Cheese production is an efficient branch of the food industry in France; in 2008, 1.85 million tons of cheeses were produced for a turnover of more than 7 billion euros. The economic importance of raw milk cheeses, which accounted for 14% of French cheese production in 2008, is connected with their gastronomic reputation, their high added value and their role in rural development and land use.

One of the most important features of raw milks is the diversity of their microbial communities. More than 150 microbial species of lactic acid bacteria, non-lactic bacteria, Gram-negative bacteria, yeasts and fungi have been identified in raw milks, with the qualitative and quantitative balances in terms of species and strains varying from one milk to another. The microbes can generate a variety of flavouring compounds, generating all the diversity and richness of sensorial properties of raw milk cheeses, quite different than those of cheeses made from pasteurised or microfiltered milk [2, 6]. It cannot be denied that some pathogenic bacteria can contaminate milk or cheese. Nevertheless, the health risk with raw milk production is low, as witnessed by the low number of toxi-infections associated with the consumption of raw milk cheeses [9, 10]. The microbial safety of these cheeses depends on hygiene measures, rigorous control and application of procedures adapted from Hazard Analysis and Critical Control Point.

Microbial diversity may help to improve the safety of raw milk cheeses. In fact microbial life, through a large number of

interactions between populations, is at the heart of "hurdle technology". This technology involves microbes creating a succession of barriers such as low pH, inhibitory metabolites (organic acids, ethanol, etc.), peptides (bacteriocins) and nutritional competition. Hurdle technology is particularly effective for controlling pathogenic bacteria when combined with environmental barriers (temperature, water content of the cheese, etc.). *Listeria monocytogenes*, for example, may be inhibited on the surface of washed rind cheeses [13, 25]. Some microbial populations from raw milk cheeses (Saint-Nectaire [25] or Camembert [15]) have been shown to inhibit *L. monocytogenes*. The growth of *L. monocytogenes* has been shown to be highly dependent on the raw milk flora in soft cheeses and was also lower in a raw milk than in a sterilised one [5]. Lactic acid bacteria [29], Corynebacteria such as *Brevibacterium linens*, yeasts and fungi can inhibit *L. monocytogenes* [8, 16, 19, 30], mainly by secreting bactericidal and bacteriostatic substances. However, none of these studies clearly demonstrated how microbial populations interact in the inhibition. The aims of the present study were (i) to evaluate the antilisterial activities of complex microbial consortia from the surface of PDO Saint-Nectaire raw milk cheeses and (ii) to try to constitute microbial consortia with similar antilisterial activities to the complex one selected in a first step.

2. MATERIALS AND METHODS

2.1. Preparation of undefined complex consortia from cheese surfaces

Thirty-four Saint-Nectaire raw milk cheeses named TR1–TR20 and TR22–TR35, prepared in different farms and ripened in different cellars for at least 18 days, were obtained from ripeners in the Saint-Nectaire area. A quarter of each cheese's rind (cheese surface to a thickness of 3 mm) was sampled then blended in phosphate buffer (20 mmol·L^{-1} KH_2PO_4, 0.01 mol·L^{-1} K_2HPO_4, Gomri, 1946) pH 7.5 (diluted 1 volume of sample to 10 volumes of buffer), two times for 2 min, using a stomacher blender (Interscience, Saint-Nom-la-Bretèche, France). This homogenised rind suspension, referred to below as a "complex consortium", was aliquoted and frozen at −20 °C.

2.2. Bacterial strains and microbial community isolates

2.2.1. Listeria monocytogenes

The purity of *L. monocytogenes* strain S1 [25] was checked on ALOA agar plate medium then precultured in TS-YE broth medium. After 18 h of incubation at 37 °C, the culture was centrifuged for 15 min at 5000 rpm and 4 °C and the supernatant discarded. The pellet was homogenised in sterile reconstituted skim milk supplemented with 15% glycerol and stored at −20 °C until its inoculation onto the cheese surfaces.

2.2.2. Characterisation of microbial isolates from complex cheese surface consortium TR15 cultivated on cheese

2.2.2.1. Collection of bacterial and yeast isolates from TR15 cultivated on cheese

In order to constitute simplified defined consortia that are able to inhibit *L. monocytogenes*, a collection of bacterial and yeast isolates was created from the complex consortium TR15, cultivated on cheese. TR15 was inoculated onto the surfaces of cheeses that were made from pasteurised milk.

Table I. Phylogenetic affiliation of 95 bacterial isolates and 16 yeast isolates from complex consortium TR15 cultivated on cheeses.

Agar plate medium origin	RFLP clusters[1]	Number of isolates	Species-specific PCR amplifications[2]							Number of sequenced isolates	Closest 16S and 26S rDNA sequences > 99% homology
(a) Bacteria											
			paracasei	Lpl	Lc	Lmn1	Lncit1	ddlE1	ddlF1		**Gram+ Catalase−**
FH and CRBM	G1	16	+	−	−	−	−	−	−	2	2 *Lactobacillus casei*
			−	−	+	−	−	−	−	2	2 *Lactobacillus curvatus*
MSE, FH and CRBM (days 11, 18, 21 and 28)	G2	12	−	−	−	+	−	−	−	3	*Leuconostoc pseudomesenteroides*
SB and CRBM (days 21 and 28)	G3	11	−	−	−	−	−	+	−	1	*Enterococcus faecalis*
CRBM (days 21 and 28)	G4	2	−	−	−	−	−	−	−	2	*Carnobacterium mobile*
CRBM (days 21 and 28)	G5	5	−	−	−	−	−	−	−	5	*Marinilactibacillus psychrotolerans*
											Gram+ Catalase+
CRBM (days 21 and 28)	G6	13								7	1 *Arthrobacter nicotianae – arilaitensis*
CRBM (days 21 and 28)	G7	4								3	6 *Arthrobacter ardleyensis – bergerei* 2 *Brevibacterium linens – casei* 1 *Brevibacterium casei – antiquum* (97% homology)
CRBM (days 21 and 28)	G8	1								1	*Brachybacterium* sp.

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Table I. Continued.

Agar plate medium origin	RFLP clusters[1]	Number of isolates	Species-specific PCR amplifications[2]	Number of sequenced isolates	Closest 16S and 26S rDNA sequences > 99% homology
CRBM and RPF (days 18, 21 and 28)	G10	4		4	3 *Staphylococcus pulvereri*
					1 *Staphylococcus xylosus* **Gram−**
PCA + M + CV, CFC and RPF (days 18, 21 and 28)	G11	17		3	*Pseudomonas fluorescens – syringae*
PCA + M + CV (day 18)	G12	2		2	*Serratia proteomaculans – liquefaciens*
RPF (days 18 and 21)	G9	8		4	*Proteus vulgaris*
(b) Yeasts					
			Physiological identification[3]		
OGA (day 18)		11	*Candida sake/tropicalis*	2	*Candida sake*
OGA (day 18)		2	*Yarrowia lipolytica*	2	*Yarrowia lipolytica*
OGA (day 18)		3	*Debaryomyces hansenii*	3	*Debaryomyces hansenii*
Total number of isolates		111		46	

(a) Bacteria: 16S rRNA gene analysis by the RFLP method followed by 16S rRNA gene sequencing, (b) Yeasts: phenotypic identification and 26S rRNA gene sequencing. In brackets () are indicated the time of ripening where the isolate was found. Media details in Section 2.4.

[1] RFLP patterns were analysed with BioNumerics software using UPGMA analysis. Isolates with the same pattern were grouped together and one or several isolates from each group were analysed by 16S rDNA sequencing.

[2] The description of primers is given in Table II.

[3] Phenotypic identification using the morphological, biochemical and physiological characteristics and assignment to species as described by Callon et al. [7].

Table II. PCR primers used for identification of isolates and SSCP analysis.

Primers[1]	Species target	Sequences	PCR conditions	Target
W02 W18	Bacterial 16S rDNA	GNTACCTTGTTACGACTT GAGTTTGATCMTGGCTCAG	25 cycles of: 30 s at 94 °C, 30 s at 50 °C, 90 s at 72 °C	SSU 16S rDNA
W34 W49	Partial bacterial 16S rDNA	TTACCGCGGCGTGCTGGCAC ACGGTCCAGACTCCTACGGG	30 cycles of: 30 s at 96 °C, 30 s at 61 °C, 30 s at 72 °C	SSU 16S rDNA
Gram1F Gram2R	Partial bacterial 16S rDNA	CCTAATACATGCAAGTCG CTCAGTCCCAATGTGGCC	30 cycles of: 30 s at 96 °C, 30 s at 52 °C, 45 s at 72 °C	SSU 16S rDNA
nl1 nl4	Yeast 26S rDNA	GCATATCAATAAGCGGAGGAAAAG GGTCCGTGTTTCAAGACGG	30 cycles of: 30 s at 94 °C, 30 s at 57 °C, 30 s at 72 °C	26S rDNA
Paracasei 16S 16rev	*Lactobacillus* *casei/paracasei*	CACCGAGATTCAACATGG GAAAGGAGGTGATCCAGC	30 cycles of: 60 s at 94 °C, 00 s at 53 °C, 60 s at 72 °C	16S rDNA
16 forward Lpl	*Lactobacillus* *plantarum*	GCTGGATCACCTCCTTTC ATGAGGTATTCAACTTATT	30 cycles of: 60 s at 94 °C, 00 s at 53 °C, 60 s at 72 °C	16S/23S spacer region
16 forward Lc	*Lactobacillus* *curvatus*	GCTGGATCACCTCCTTTC TTGGTACTATTTAATTCTTAG	30 cycles of: 60 s at 94 °C, 00 s at 53 °C, 60 s at 72 °C	16S rRNA gene

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Table II. Continued.

Primers[1]	Species target	Sequences	PCR conditions	Target
Lnm1 Lnm2	*Leuconostoc mesenteroides/ pseudomesenteroides*	TGTCGCATGACACAAAAGTTA ATCATTTCCTATTCTAGCTG	25 cycles of: 30 s at 94 °C, 30 s at 62 °C, 90 s at 72 °C	16S rDNA position 185 forward 16S rDNA position 470 reverse
Lncit1 Lncit2	*Leuconostoc citreum*	ACTTAGTATCGCATGATATC AGTCGAGTTGCAGACTGCAG	30 cycles of: 30 s at 94 °C, 30 s at 58 °C, 90 s at 72 °C	16S rDNA position 183 forward 16S rDNA position 1326 reverse
ddlE1 ddlE2	*Enterococcus faecalis*	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	30 cycles of: 60 s at 94 °C, 60 s at 54 °C, 60 s at 72 °C	glycopeptide resistance gene
ddlF1 ddlF2	*Enterococcus faecium*	TAGAGACATTGAATATGCC TCGAATGTGCTACAATC	30 cycles of: 60 s at 94 °C, 60 s at 54 °C, 60 s at 72 °C	

[1] References [7, 11, 28].

The inoculated cheeses were ripened for 28 days at 9 °C and 95% relative humidity (RH) in the ripening room at INRA. The micoorganisms able to establish themselves on the cheese surface were enumerated on various agar media at seven different ripening times (1, 8, 11, 18, 21, 25 and 28 days). The agar media presenting the highest number of colonies with different morphotypes were observed for samples taken at 11, 18, 21 and 28 days of ripening. The media presenting the highest diversity in terms of morphotypes were selected from these ripening times. From these agar media, 111 colonies, representative of each of the morphotypes observed, were isolated and purified before identification at species level (Tab. I).

2.2.2.2. *Total DNA extraction*

Total DNA from each isolate cultivated in an appropriate broth medium was extracted using Easy-DNA kit with phenol/chloroform (Invitrogen, Cergy-Pontoise, France) according to the manufacturer's instructions.

The ribosomal 16S rRNA genes (1450 bp) from all isolates were amplified by polymerase chain reaction (PCR) using the universal primers W02 and W18 (complete gene for bacterial strains – Tab. II) as described by Callon et al. [7].

2.2.2.3. *Identification of bacterial isolates*

The 16S rRNA genes of the isolates were screened by restriction fragment length polymorphism (RFLP) as described by Callon et al. [7]. The 16S rRNA RFLP profiles of the 111 isolates were compared using the BioNumerics software and the unweighted pair group method with arithmetic averages to obtain a dendrogram. Patterns showing > 90% similarity (calculated by Pearson's correlation) were grouped in the same cluster (G1–G12, see Tab. I). Each cluster was assigned to a bacterial genus by comparing the profiles with those of a reference database. The reference database was composed of 109 different species from the laboratory collection, including Gram-positive lactic acid bacteria (27 strains), non-lactic Gram-positive bacteria (56 bacteria including 19 Staphylococci) and Gram-negative bacteria (26). The 16S rDNA of 1–7 isolates representing each restriction pattern (G1–G12) was sequenced or amplified with specific primers.

Specific sequences of 16S rRNA genes from isolates which were identified as lactic acid bacteria according to their RFLP profiles were amplified using specific PCR primers as described in Table II.

Twenty-five microlitres of bacterial 16S rRNA gene PCR amplification products were sequenced using W34 primer by GeneCust (Évry, France). The sequences were compared to the sequences available in the GenBank database using the Blast program. In this study, a 99% similarity was taken to assign an isolate to a species.

2.2.2.4. *Identification of yeast isolates*

Yeasts were identified by a combination of phenotypic tests and sequencing of the D1/D2 domain of the 26S rRNA gene as described by Callon et al. [7].

2.2.2.5. *Antilisterial effect of microbial isolates*

The antilisterial activity of each isolate in the collection was tested using the agar-well diffusion method as described by Saubusse et al. [28].

2.2.2.6. *Creating defined microbial consortia*

From the collection of strains from complex consortium TR15 that were able to grow on cheese surfaces, four different defined

consortia were made up. The most complex, called ABCD, was composed of 19 strains randomly selected for each microbial species identified (Tab. I): six species of Gram-positive lactic acid bacteria (Group A), seven species of Gram-positive and catalase-positive bacteria (Group B), three species of Gram-negative bacteria (Group C) and three species of yeasts (Group D). Each group was composed of one strain of each species as follows: Group A, *Lactobacillus casei/paracasei*, *Lactobacillus curvatus*, *Leuconostoc mesenteroides*, *Enterococcus faecalis*, *Marinilactibacillus psychrotolerans* and *Carnobacterium mobile*; Group B, *Arthrobacter ardleyensis* or *bergerei*, *Arthrobacter nicotianae* or *arilaitensis*, *Staphylococcus xylosus*, *Staphylococcus pulvereri*, *Brevibacterium antiquum* or *casei*, *Brevibacterium linens* or *casei* and *Brachybacterium* sp.; Group C, *Pseudomonas fluorescens* or *syringae*, *Serratia proteomaculans* or *liquefaciens* and *Proteus vulgaris*; Group D, *Candida sake*, *Debaryomyces hansenii* and *Yarrowia lipolytica*. The strains could not be selected for their antilisterial properties as none of the isolates identified was able to inhibit *L. monocytogenes* by agar-well diffusion test in vitro. The three other defined consortia, ABC, ABD and ACD, were defined by omitting one group: either B, C or D.

Each isolate was purified on the appropriate agar plate medium and cultivated in 10 mL of the appropriate broth medium. The culture was centrifuged for 15 min at 8500 rpm and 4 °C. The pellet was homogenised in sterile reconstituted milk supplemented with 15% glycerol and with or without 5% ascorbate. Suspensions were transferred to 2 mL microtubes and stored at −20 °C until their inoculation onto the cheese surfaces. Before inoculation, suspensions were thawed in a water bath for 5 min at 25 °C, counted on agar media and diluted to inoculate the appropriate concentrations on the cheese surfaces.

2.3. Challenge test with *L. monocytogenes* on the surface of an uncooked pressed type cheese

2.3.1. Cheese manufacturing

Small cheeses (600 g) were made from pasteurised milk collected at a school farm (ENILV, Aurillac, France), using an uncooked pressed cheese method similar to that of Saint-Nectaire. Milk was pasteurised at 72 °C for 30 s, cooled to 33 °C then inoculated with 0.6% of a commercial starter culture (MY800, *Streptococcus thermophilus*; *Lactobacillus delbrueckii* spp. *bulgaricus*, Danisco, Sassenage, France) and with a commercial mould culture of *Penicillium nalgiovensis* (2.5 mL·200 L^{-1}, Laboratoire Interprofessionnel de Production – LIP, Aurillac, France). Forty-five minutes after adding 40 mL of calf rennet (Beaugel 520-Ets Coquard, Villefranche-sur-Saône, France), the curd was cut and gently stirred to eliminate whey. The curds were moulded and pressed for 24 h at 2.1 bar and 22 °C. After pressing, the cheeses (pH 5.14) were vacuum-packed and frozen at −20 °C until inoculation.

2.3.2. Inoculation of experimental cheese surfaces

The experimental cheeses made from pasteurised milk were thawed for 5 h at room temperature under a laminar hood and turned over each hour to remove excess water. In all trials, their surfaces were inoculated by depositing and brushing with a sterile toothbrush 1 mL of different microbial consortia containing strain 167 of *L. monocytogenes*. *L. monocytogenes* was inoculated at 2–5 colony-forming units (CFU)·cm^{-2}. The microbial consortia were inoculated in three sets of experiments.

In the first set of experiments, 34 different microbial consortia were inoculated;

corresponding to each of the complex cheese microbial consortia named TR1–TR20 and TR22–TR35, prepared as described in Section 2.1 and thawed in a 25 °C water bath before inoculation.

In the second set of experiments, consortium TR15, the most inhibitory of the 34, was inoculated after 3 and 24 months of storage at −20 °C in order to verify the stability of the antilisterial activity.

In the third set of experiments, five different microbial consortia were inoculated: one TR15 consortium (selected in the first set of experiments and stored at −20 °C) and four defined microbial consortia (ABCD, ABC, ACD and ABD). The inoculum level of each species was based on counting results obtained after growing TR15 on cheese. Each species of Groups A, B and D was inoculated at 2.7 log CFU·cm^{-2}. The Gram-negative bacteria (Group C) were inoculated at only 1 log CFU·cm^{-2}.

Each set included a control, inoculated with 1 mL of a commercial ripening culture frequently used for manufacturing Saint-Nectaire cheese (*Penicillium nalgiovensis* 3.10^4 CFU·cm^{-2}, LIP, Aurillac, France).

The inoculated cheeses were ripened for 28 days in sterile stainless steel boxes in INRA's cellars at 8–9 °C, 98% RH (in the cellar). After 8 and 18 days of ripening, the cheeses were washed with sterile salt water (20% NaCl).

2.4. Microbial analysis

Rind samples 3 mm thick were taken from inoculated cheeses after 1, 8, 18 and 28 days of ripening, for microbial analysis.

L. monocytogenes was counted as prescribed in ISO 11 2090-2, by an accredited laboratory (LIAL, Aurillac, France).

The rind samples were blended in phosphate buffer (20 mmol·L^{-1} KH_2PO_4, 0.01 mol·L^{-1} K_2HPO_4) pH 7.5 using a stomacher blender (Interscience, Saint-Nom-la-Bretèche, France). Each suspension was diluted with Ringer's solution and appropriate dilutions were spread on agar plate media using a spiral system (DS+, Interscience, Saint-Nom-la-Bretèche, France).

As described by Millet et al. [25], the microbial populations were enumerated on the following agar plate media, according to morphotype: coagulase positive and negative Staphylococci on rabbit plasma fibrinogen agar (RPF); facultatively heterofermentative lactobacilli on facultatively heterofermentative agar (FH); dextran-producing leuconostocs on Mayeux Sandine and Elliker agar medium (MSE); enterococci on Slanetz and Bartley agar (SB); *Pseudomonas* on cephalosporin fucidin cetrimide (CFC) agar; Gram-positive and catalase-positive bacteria on cheese ripening bacteria medium (CRBM); Gram-negative bacteria on plate count agar (PCA) supplemented with milk and crystal violet (PCA + M + CV); yeasts and moulds on oxytetracycline glucose agar (OGA) medium. All media were purchased from Biokar.

On CRBM, white colonies corresponding to *S. pulvereri*, yellow or cream colonies corresponding to *A. ardleyensis* or *bergerei*, *A. nicotianae* or *arilaitensis* and *Brachybacterium* sp. and orange colonies corresponding to *S. xylosus* or *B. antiquum* or *casei* and *B. linens* or *casei* were counted specifically. On OGA medium, white colonies corresponding to *D. hansenii* or *C. sake* and cream colonies corresponding to *Y. lipolytica*.

Colony counts were expressed as log CFU·cm^{-2} of cheese rind. Counts < 10 CFU·g^{-1} were shown as < 1 log·cm^{-2}.

2.4.1. Calculation of area of inhibition

In order to easily compare the *L. monocytogenes* populations of the different cheeses during ripening (second set of experiments), the area of inhibition (AI) between two ripening days ($t1$ and $t2$) was calculated using the following formula as

described by Saubusse et al. [28]

$$AI = (t2 - t1)/2 \times [(Ct1 + Ct2) - (Tt1 + Tt2)],$$

where C is the count of *L. monocytogenes* in control cheeses whose surfaces were inoculated with a commercial ripening culture and T is the count of *L. monocytogenes* in cheeses whose surfaces were inoculated with other microbial consortia.

2.5. Single strand conformation polymorphism analysis of the cheese rinds

Total genomic DNA was extracted from 1 g cheese rind sample at 1, 8, 18 and 28 days of ripening, using the phenol-chloroform method described by Delbès et al. [11]. Partial 16S rRNA gene sequences were amplified using Gram1F–Gram2R primers with Gram1F labelled with hexachloro derivative of fluorescein HEX and Gram2R labelled with fluorescein phosphoramidite NED [28]. The PCR-single strand conformation polymorphism (PCR-SSCP) products were analysed by SSCP capillary electrophoresis on an ABI Prism 310 genetic analyser. The fluorescence signal was analysed using the Genescan analysis software. The SSCP peak patterns were aligned using internal standard genescan 400 Rox. The dominant peaks in the SSCP patterns obtained were assigned by comparing their migration with that of the 16 bacterial strains used to make up the microbial community. To analyse the different profiles, relative peak proportion P_i is determined as $P_i = a_i \times 100/\Sigma a_i$, where P_i is the proportion and Σa_i is the sum of the peak areas of the whole SSCP patterns.

2.6. Chemical analysis

2.6.1. pH measurements

The pH of cheese surface was measured at 1, 8, 18 and 28 days of ripening at three locations on each cheese with a 926 VTV pH-meter with Ingold electrode 406 MX (Mettler-Toledo S.A., Viroflay, France). The results are the means of the three measurements.

2.6.2. Analysis of sugar and organic acid contents of cheese surfaces by high pressure liquid chromatography

Suspensions of finely ground rind were prepared (10 g for organic acids and 10 g for sugar compounds) in 20 mL distilled water. After incubating for 1 h at 50 °C, the rind suspensions were homogenised using an Ultra-Turrax device at 9500 rpm for 2 min. Organic acids and sugar compounds were extracted as described by Leclercq-Perlat et al. [20]. Analysis was performed by high-performance liquid chromatography (HPLC). The HPLC system (Waters, Guyancourt, France) consisted of an automatic injector, two serial detectors (a UV detector 210 nm for organic acids and a differential refractometer for sugar compounds) and two integrators. The cation-exchange column (Aminex HPX-87H – Bio-Rad, Marnes-la-Coquette, France) was maintained at 35 °C. Sulphuric acid (0.1 $mol \cdot L^{-1}$) was used as the mobile phase at a flow rate of 0.6 $mL \cdot min^{-1}$. Propionic acid (1%) was used as an internal standard.

The results were expressed in grams per kilogram of dry rind content.

3. RESULTS

3.1. Inhibition of *L. monocytogenes* on the surfaces of uncooked pressed type cheeses

The *L. monocytogenes* counts on the surfaces of cheeses inoculated with the 34 complex microbial consortia from Saint-Nectaire cheeses (TR1–TR20 and

TR22–TR34) and with a commercial ripening culture as a control, after different ripening times at 9 °C, are given in Table III. Results obtained for TR15 after several months of storage at −20 °C are also included.

The level of *L. monocytogenes* was below the detection threshold of 1 log CFU·cm^{-2} after 1 and 8 days of ripening in most cheeses, whichever inoculation was used (TR1–TR20 and TR22–TR34). *L. monocytogenes* grew mainly between day 8 and day 18 and the *L. monocytogenes* count at days 8, 18 and 28 varied according to the consortium inoculated, allowing us to arbitrarily classify the cheeses in three groups. Group 1 (10 consortia) corresponded to cheeses with the lowest *L. monocytogenes* counts (below 5 log CFU·cm^{-2}). Interestingly, one microbial consortium (TR15) from this group, tested several times, can be stored at −20 °C in phosphate buffer for at least 24 months without losing its capacity to inhibit *Listeria* compared to a control. In Group 2 (15 consortia), *L. monocytogenes* populations reached between 5.26 and 5.96 log CFU·cm^{-2}. Group 3 (9 consortia) had the highest *L. monocytogenes* counts – 6 log CFU·cm^{-2} or more. The control cheeses inoculated with only the commercial ripening culture were in this group.

There was no correlation between the count of *L. monocytogenes* at a given ripening time and the pH values; $r = 0.10$ at day 18 (pH varying between 6.20 and 7.52, $n = 35$) and $r = -0.14$ at day 28 (pH varying between 5.80 and 8.40, $n = 35$). However, the increase in pH during ripening was associated with an increase in *L. monocytogenes.*

Due to its high antilisterial properties and stability in storage at −20 °C, microbial consortium TR15 was selected for the subsequent studies of antilisterial activity.

3.2. Identification of strains from culture of cheese complex consortium TR15

Ninety-five bacterial isolates were distinguished in 12 RFLP patterns (clusters G1–G12). First, by reference to a database of reference strains most commonly isolated from milks or cheeses, nine RFLP profiles (G1–G3, G6–G8 and G10–G12) were first assigned to different genera. Profiles of three clusters (G4, G5 and G9) presented no similarity with those present in the database. The 46 isolates belonging to clusters G1–G5 corresponding to lactic acid bacteria were identified according to their amplification with specific primers and the identifications were confirmed by the closest match of their 16S RNA in the GenBank database (Tab. I). Thus, 14 isolates of G1 were assigned to *Lb. casei* or *paracasei*, the other two isolates in this group to *Lb. curvatus*, the 12 isolates of G2 to *Ln. mesenteroides* or *pseudomesenteroides* and the 11 isolates of G3 to *E. faecalis*. Some representative isolates of the other groups were identified only by 16S rDNA sequencing (Tab. I). Their 16S rDNA sequences presented more than 99% homology with those of the following species: *Cb. mobile* (G4, two isolates), *M. psychrotolerans* (G5, five isolates), *A. nicotianae* or *arilaitensis* (G6, one isolate), *A. ardleyensis* or *bergerei* (G6, six isolates), *B. linens* or *casei*, *B. antiquum* or *casei* (G7, three isolates), *Brachybacterium* sp. (G8, one isolate), *S. pulvereri*, *S. xylosus* (G10, four isolates), *P. fluorescens* or *syringae* (G11, three isolates), *Ser. proteomaculans* or *liquefaciens* (G12, two isolates) and *Prot. vulgaris* (G9, four isolates).

The 16 yeast isolates were identified as *C. sake* (11 isolates), *Y. lipolytica* (three isolates) and *D. hansenii* (three isolates) on the basis of their phenotypic characteristics and 26S DNA sequencing (Tab. I).

Table III. pH and *L. monocytogenes* growth during ripening on the surfaces of trial cheeses inoculated with 34 complex consortia collected from cheese surfaces in the Saint-Nectaire area.

Group	Trial cheese	*L. monocytogenes* log CFU·cm^{-2}				pH	
		Day 1[1]	Day 8[1]	Day 18	Day 28	Day 18	Day 28
G1	TR15.1	1.48	< 1	3.33	4.05	6.42	7.12
	TR15.2	< 1	1.48	3.34	4.11	7.17	7.83
	TR15.3	< 1	1.18	2.01	3.32	7.27	7.71
	TR11	< 1	< 1	3.18	4.00	7.15	8.04
	TR26	< 1	< 1	3.26	4.13	6.72	6.75
	TR19	< 1	< 1	4.21	4.84	7.11	7.21
	TR13	< 1	1.78	4.01	4.86	7.10	6.84
	TR9	< 1	1.48	3.48	4.91	6.41	6.20
	TR22	< 1	1.96	3.74	4.93	6.74	6.73
	TR30	1.78	1.78	3.82	4.97	6.60	7.15
	TR24	1.48	< 1	3.18	5.04	6.63	6.93
	TR25	< 1	1.48	4.23	5.05	7.41	8.47
G2	TR23	< 1	1.96	4.33	5.26	7.10	5.94
	TR12	< 1	1.48	4.36	5.27	7.47	8.00
	TR29	< 1	1.48	3.52	5.27	6.35	7.00
	TR6	< 1	< 1	4.38	5.34	7.17	8.18
	TR8	< 1	< 1	4.17	5.39	7.14	8.06
	TR34	< 1	1.48	4.03	5.44	6.98	6.70
	TR20	1.48	1.96	4.47	5.45	6.92	6.10
	TR28	1.96	< 1	4.24	5.52	7.34	8.26
	TR16	< 1	2.44	3.94	5.63	7.05	8.21
	TR32	1.48	2.08	4.66	5.66	6.28	6.85
	TR2	< 1	< 1	4.48	5.69	7.34	7.85
	TR17	1.48	1.78	3.76	5.74	6.48	6.87
	TR1	1.78	< 1	4.09	5.93	6.21	7.03
	TR31	2.52	2.88	4.52	5.96	7.05	7.96
	TR35	< 1	< 1	4.27	5.96	6.97	7.06
G3	TR27	< 1	< 1	4.08	6.03	6.31	6.95
	TR3	1.48	< 1	4.29	6.08	7.14	6.82
	TR14	< 1	2.08	4.01	6.14	6.93	5.80
	Control 2	< 1	1.48	4.15	6.13	6.67	7.61
	Control 1	1.48	< 1	4.41	6.21	7.52	6.70
	TR33	< 1	< 1	4.23	6.23	7.14	5.96
	TR18	< 1	< 1	4.90	6.25	7.07	7.66
	TR10	1.96	2.33	5.45	6.34	6.42	6.97
	TR5	1.48	< 1	4.26	6.36	6.45	6.94
	TR4	< 1	< 1	4.71	6.40	6.67	5.91
	TR7	< 1	1.78	5.34	6.42	6.35	6.95
	Control 3	1.48	3.33	4.63	7.66	7.29	7.70

[1] At days 1 and 8, the level of *L. monocytogenes* was below the detection limit of 1 log CFU·cm^{-2}.
Trial cheese, TRx = complex cheese surface consortium prepared as described in Section 2.1; Control = commercial ripening culture. Groups = Trial cheeses according to *L. monocytogenes* counts at 28 days of ripening: G1 = < 5 log CFU·cm^{-2}, G2 = < 6 log CFU·cm^{-2} and G3 = > 6 log CFU·cm^{-2}. TR15.1 = 1st challenge test using complex cheese surface consortium TR15. Control 2 and Control 3 = commercial ripening culture from the 2nd and 3rd challenge tests, TR15.2 = 2nd challenge test using TR15 after 3 months of storage at −20 °C, TR15.3 = 3rd challenge test using TR15 after 24 months of storage at −20 °C.

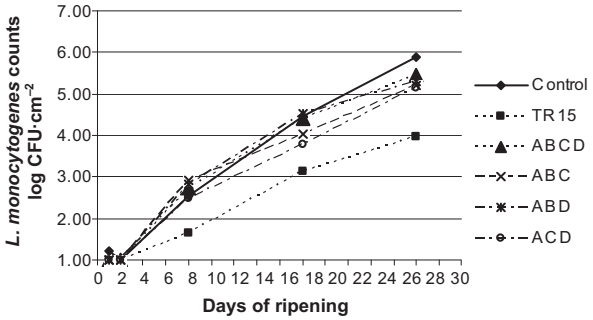

Figure 1. *L. monocytogenes* growth on the surfaces of cheeses inoculated with complex consortium TR15 and defined consortia. *L. monocytogenes* counts expressed in log CFU·cm^{-2}; cheese surfaces inoculated with control = commercial ripening culture, TR15 = complex consortium (rind suspension), ABCD, ABC, ABD and ACD = defined consortia from identified isolates (A = six lactic acid bacteria, B = seven Gram-positive and catalase-positive bacteria, C = three Gram-negative bacteria and D = three yeasts).

Table IV. Areas of inhibition of *L. monocytogenes* growth on the surfaces of cheeses inoculated with complex consortium TR15 and defined microbial consortia.

	AI (8–2)	AI (18–8)	AI (28–18)
TR15	**2.66**	**11.20**	**16.33**
ABCD	−0.60	−0.66	2.48
ABC	−1.11	0.39	5.65
ABD	−0.84	−1.70	2.71
ACD	0.12	**3.69**	**7.28**

AI = area of inhibition calculated between 2 days of ripening ($t2-t1$) according to the formula, AI = $(t2 - t1)/2 \times [(Ct2 + Ct1) - (Tt2 + Tt1)]$, with C = *L. monocytogenes* counts in the control cheese and T = *L. monocytogenes counts* in cheeses inoculated with consortia. Cheese surfaces inoculated with control = commercial ripening culture; TR15 = complex consortium (rind suspension) and ABCD, ABC, ABD and ACD = defined consortia from identified isolates (A = six lactic acid bacteria, B = seven Gram-positive and catalase-positive bacteria, C = three Gram-negative and D = three yeasts). Numbers in bold fonts indicate the highest inhibition.

3.3. Antilisterial properties on cheese surfaces of complex consortium TR15 and defined consortia

L. monocytogenes developed differently during ripening depending on the microbial consortium inoculated − complex consortium TR15 and defined consortia as shown in Figure 1. The lowest count was observed on cheese surfaces inoculated with complex consortium TR15 and the highest on those with only the commercial ripening culture.

The inhibition of *L. monocytogenes* was then evaluated by calculating the AI as described in the Section 2. The AI data and pH values are shown in Table IV. The higher the AI values, the stronger the inhibition of *L. monocytogenes*. Throughout the ripening, AI values were highest in cheeses inoculated with complex consortium TR15 and lowest in those inoculated with ABCD and ABD. Between days 18 and 28, *L. monocytogenes* was also inhibited (positive values of AI) in the

Table V. pH of cheese rinds inoculated with complex consortium TR15 or defined consortia.

	Day 1	Day 2	Day 8	Day 18	Day 28
Control	5.88	5.62	5.76	7.50	7.04
TR15	5.95	6.07	5.72	7.54	7.04
ABCD	5.77	5.52	5.56	7.46	7.45
ABC	5.93	6.06	5.55	7.58	7.33
ABD	5.90	6.09	5.92	7.58	7.29
ACD	6.17	5.81	5.73	7.58	7.32

pH values are means of three measurements for each time of ripening; cheese surfaces inoculated with control = commercial ripening culture; TR15 = complex consortium (rind suspension) and ABCD, ABC, ABD and ACD = defined consortia from identified isolates (A = six lactic acid bacteria, B = seven Gram-positive and catalase-positive bacteria, C = three Gram-negative bacteria and D = three yeasts).

presence of ACD and to a lesser extent with ABC.

3.4. Evolution of pH

At days 1 and 2, pH values ranged from 5.52 to 6.17 (Tab. V), but nevertheless *L. monocytogenes* counts were below the detection limit on the surfaces of all cheeses. In all cheeses, pH dropped between days 1 and 8, ranging between 5.56 and 5.92 at day 8. The lowest pH, at day 8 in cheese ABCD (pH 5.56) was not associated with the lowest count of *L. monocytogenes*. Between days 8 and 18, the pH increased dramatically, reaching 7.04–7.58 at day 18 in all cheeses (Tab. V). This was matched by an increase of close to 2 log CFU·cm^{-2} in *L. monocytogenes* counts. At day 28, the control cheese had the lowest pH value (7.04), but the highest count of *L. monocytogenes*.

3.5. Dynamics of microbial populations by culture-dependent methods

At all stage of ripening, it was difficult to control Gram-negative bacteria. They were present in abundance on the surfaces of all the cheeses (9.1 log CFU·cm^{-2} in TR15 to 9.7 log CFU·cm^{-2} in ABCD), even the ABD cheeses (8.9 log CFU·cm^{-2} at 28 days) on which they had not been inoculated (Tab. VI).

At days 1 and 8, the highest lactobacilli count was on the surface of cheeses inoculated with consortium TR15, at least 1 log CFU·cm^{-2} higher than in the other cheeses, except for the ACD cheeses where the counts at day 8 were similar to those for TR15 (5.6 log CFU·cm^{-2}; Tab. VI).

At day 1, dextran-producing leuconostocs were detected in all cheeses inoculated with any of the five microbial consortia. As with the lactobacilli, leuconostoc counts were at least 1 log CFU·cm^{-2} lower in the defined consortia than in complex consortium TR15. However, leuconostocs were counted at every ripening time on the surfaces of cheeses inoculated with TR15, where they reached 7.7 log CFU·cm^{-2} at day 28. In cheeses prepared with defined consortia, they were not found on MSE medium up to day 8.

Until day 18, the trend in *E. faecalis* counts was similar in cheeses inoculated with complex consortium TR15 and defined consortia ABD and ACD. From day 18 to 28, they increased to 6.9 log CFU·cm^{-2} in cheeses inoculated with ABD and ACD, but remained stable at 5.6 log CFU·cm^{-2} in cheeses inoculated with complex consortium TR15. There was no growth of *E. faecalis* in cheeses inoculated with ABC (consortium without yeasts).

Table VI. Microbial population counts during ripening on the surfaces of cheeses inoculated with complex consortium TR15 and defined consortia.

	TR15	ABCD	ABC	ABD	ACD
Lactobacillus sp.					
Day 1	4.9	3.6	3.6	3.8	2.8
Day 8	**5.4**	4.5	4.7	3.5	**5.6**
Day 18	6.1	6.6	6.8	**7.1**	**7.5**
Day 28	6.2	6.6	6.4	**7.8**	**7.4**
Leuconostoc mesenteroides					
Day 1	**5.1**	3.5	3.6	4.0	4.4
Day 8	**6.1**	< 2	< 2	< 2	< 2
Day 18	**7.7**	< 3	< 3	< 3	< 3
Day 28	**7.5**	< 3	< 3	< 3	< 3
Enterococcus faecalis					
Day 1	4.0	3.2	**2.8**	3.8	3.8
Day 8	4.5	2.9	**2.4**	4.3	4.4
Day 18	5.6	4.2	**4.0**	5.7	5.5
Day 28	5.3	5.7	**2.9**	6.9	6.9
Arthrobacter nicotianae; *Arthrobacter ardleyensis* or *bergerei*; *Brachybacterium* sp.					
Day 1	3.5	3.9	3.6[a]	4.2[a]	< 2
Day 8	8.2[a]	6.0	4.4	6.4	< 2
Day 18	8.5	8.4	8.1	7.8	< 3
Day 28	9.3[a]	8.7	9.0	8.1	< 3
Staphylococcus pulvereri					
Day 1	< 3	3.2	3.6[a]	4.2[a]	< 2
Day 8	6.8	7.4	7.3	6.6	< 2
Day 18	7.1	8.4	8.5	7.9	< 3
Day 28	9.3[a]	8.4	8.0	8.0	< 3
Gram-negative bacteria					
Day 1	4.9	4.5	3.3	4.5	6.3
Day 8	7.8	6.9	6.9	7.2	7.6
Day 18	8.7	7.8	8.9	7.8	8.8
Day 28	9.1	9.7	9.5	8.9	9.6
Candida sake					
Day 1	4.2[a]	3.1[a]	< 2	4.6[a]	4.5[a]
Day 8	7.0	6.9	< 2	7.7	7.4
Day 18	8.4	7.9	< 3	8.0[b]	8.3[b]
Day 28	8.0	7.5	< 3	8.1[a]	8.2[a]

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Table VI. Continued.

	TR15	ABCD	ABC	ABD	ACD
Debaryomyces hansenii					
Day 1	4.2[a]	3.1[a]	< 2	4.6[a]	4.5[a]
Day 8	7.1	7.4	< 2	6.8	7.5
Day 18	7.3	7.2	< 3	8.0[b]	8.3[b]
Day 28	7.9	8.0	< 3	8.1[a]	8.2[a]
Yarrowia lipolytica					
Day 1	4.2[a]	3.1[a]	< 2	4.6[a]	4.5[a]
Day 8	6.1	6.4	< 2	7.1	6.9
Day 18	6.9	6.0	< 3	7.5	7.4
Day 28	7.3	6.9	< 3	8.1[a]	8.2[a]
Total yeasts					
Day 1	4.2	3.1	< 2	4.6	4.5
Day 8	7.3	7.5	< 2	7.8	7.8
Day 18	8.4	7.9	< 3	8.1	8.3
Day 28	8.3	8.1	< 3	8.1	8.2

Cheese surfaces inoculated with control = commercial ripening culture; TR15 = complex consortium (rind suspension) and ABCD, ABC, ABD and ACD = defined consortia from identified isolates (A = six lactic acid bacteria, B = seven Gram-positive and catalase-positive bacteria, C = three Gram-negative bacteria and D = three yeasts). Results expressed in log CFU·cm^{-2}.
[a] Indicate the total count of species on media.
[b] Counting value including *C. sake* and *D. hansenii*.

At day 8, *S. pulvereri* was the dominant population within the Gram-positive and catalase positive population in cheeses prepared with defined microbial consortia. Its level increased until day 18 and then stabilised around 8 log CFU·cm^{-2}. In these cheeses, the growth of *Arthrobacter* sp. was similar. In cheeses inoculated with complex consortium TR15 the increase in *Arthrobacter* sp. was > 1–1.5 log CFU·cm^{-2} higher than for *S. pulvereri* throughout ripening. At day 28 it was difficult to count each species due to the great diversity of morphotypes on the plate (Tab. VI).

The yeast counts were similar in all cheeses and increased mainly before day 8, stabilising at 28 days at 8 log units (CFU·cm^{-2}) with dominance of *C. sake* and *D. hansenii*. These two species were still the dominant populations at the end of ripening and their levels with the TR15 and ABCD consortia were similar. At 8 and 18 days counts of *Y. lipolytica* were higher in consortia ABD and ACD than in TR15 and ABCD.

3.6. Evaluation of bacterial balance on cheese rinds by a culture-independent method (direct SSCP) and by plate counting

Table VII shows the percentage of each bacterial population at 18 and 28 days of ripening, as assessed by the culture-independent method SSCP analysis targeting the V2 region of 16S rDNA and the culture-dependent plate counting method. Gram-negative bacteria have not been taken into account as they could not be detected by PCR-SSCP analysis of the V2 region of 16S rDNA. Moreover, data from days 1 and 8 of ripening are not considered because the SSCP V2 profiles were dominated by a single peak corresponding to

Table VII. Microbial populations' establishment at 18 and 28 days of ripening on the surfaces of cheeses inoculated with complex consortium TR15 and defined consortia, analysed by SSCP and counting on agar media.

	TR15				ABCD				ABC				ABD				ACD			
	Day 18		Day 28		Day 18		Day 28		Day 18		Day 28		Day 18		Day 28		Day 18		Day 28	
	SSCP	Plate	SSCP	Plate	SSCP	Plate	SSCP	Plate	SSCP	Plate	SSCP	Plate	SSCP	Plate	SSCP	Plate	SSCP	Plate	SSCP	Plate
Lactobacillus casei/paracasei	–	0.4	2	0.1	–		–		1		1		1	7.8	*d*	19.8	5	99	3	74.4
Lactobacillus curvatus	–		5		–		–		1		–		5		*d*		1		*d*	
Lactobacillus delbrueckii (MY800)*	3		4		–		–		–		1		–		2		–		–	
Leuconostoc mesenteroides	22	15	3	1.6	7		17		71		30		50		3		73		78	
Enterococcus faecalis	1		1		10		11		1		6		4		10	2.6	*d*	1	–	25.2
Carnobacterium mobile	–		4		–		–		–		–		–		–		–		–	
Marinilactibacillus psychrotolerans	5		3		–		–		–		–		–		–		*d*		–	
Arthrobacter nicotianae or *arilaitensis*	10	81	29	98.3	–	51.9	2	67.7	4	26.3	*d*	90.3	5	39.1	*d*	41.4	–		–	
Arthrobacter ardleyensis or *bergerei*	11		6		67		49		9		47		27		77		–		1	
Brachybacterium sp.	6		*d*		3		9		–		–		–		–		–		–	
Staphylococcus pulvereri	30	3.5	28		6	47.3	4	31.7	–	72.4	–	9.5	–	52.8	–	36.1	–		–	
Staphylococcus xylosus	–		–		1		4		–		–		–		–		–		–	
Brevibacterium linens or *casei*	–		–		–		–		–		–		–		–		–		–	
Brevibacterium antiquum or *casei*	–		–		–		–		–		–		–		–		–		–	

continued on next page

Table VII. Continued.

	TR15				ABCD				ABC				ABD				ACD			
	Day 18		Day 28		Day 18		Day 28		Day 18		Day 28		Day 18		Day 28		Day 18		Day 28	
	SSCP	Plate	SSCP	Plate	SSCP	Plate	SSCP	Plate	SSCP	Plate	SSCP	Plate	SSCP	Plate	SSCP	Plate	SSCP	Plate	SSCP	Plate
Streptococcus thermophilus (MY800)*	6		10		6		4		7		3		6		–		14		12	
Listeria monocytogenes	6		5		–		–		5		2		2		–		7		4	
Unknown 1	–		–		–		–		–		9		–		8		–		–	

Proportion of species on cheese rinds from TR15 = complex consortium (rind suspension) and ABCD, ABC, ABD and ACD = defined consortia from identified isolates (A = six lactic acid bacteria, B = seven Gram-positive and catalase-positive bacteria, C = three Gram-negative bacteria and D = three yeasts).

**L. delbrueckii* and *S. thermophilus* are strains from the commercial starter culture (My800) used for cheese manufacturing.

| straight line indicates that the proportion included the different species of the lines.

Plate = results expressed as a proportion of counted species on agar media. SSCP = results expressed as proportion of the peak (P_i) in the pattern with $P_i = a_i \times 100/\Sigma a_i$, where a_i = peak area and Σa_i = total peak area of the pattern. Species names on the same line indicate coelution in the same peak; n-dash (–) indicates no detection and *d* indicates detection of a peak < 1% of total pattern.

St. thermophilus. Both methods, PCR-SSCP and counting, led to the conclusion that *S. xylosus*, *B. linens* or *casei* and *B. antiquum* or *casei* were subdominant populations as they were not detected on the cheese surfaces by either of the two methods.

PCR-SSCP analysis of V2 region was more accurate than plate counting for detecting the diversity of the bacterial populations present on the cheese surfaces. Leuconostocs were detected in all SSCP cheese profiles from day 8 to 28, but were only quantified in TR15 cheeses by the culture-dependent method. This discrepancy may be due to the leuconostoc strain selected losing its ability to produce dextrans on MSE medium, whereas strains from the natural flora were still able to produce them. Then, the two methods gave quite different pictures of bacterial community. The leuconostoc peak can represent > 70% of the peaks on SSCP profiles for cheeses inoculated with defined consortia ABC at 18 days and ACD at 18 and 28 days. With plate counting the dominant populations in cheeses with defined consortium ACD were lactobacilli (99%) at 18 days and lactobacilli (74.4%) and *E. faecalis* (25.2%) at 28 days. The SSCP patterns of the cheese surfaces prepared with consortium TR15 were the most diverse, with 10 peaks in the profile at 18 days and 12 peaks in the profile at 28 days. They were characterised by the presence of *Cb. mobile*, *M. psychrotolerans*, *A. nicotianae* or *arilaitensis*, *A. ardleyensis* or *bergerei* and *Brachybacterium* sp. These species were not specifically quantifiable by plate counting. *S. pulvereri* represented 30% of the profiles of complex consortium TR15, whereas it was subdominant in cheeses with defined consortia (ABC and ABD). The plate counting method gave a quite different picture, as *S. pulvereri* was the dominant population in these cheeses. According to the PCR-SSCP analysis, *A. ardleyensis* or *bergerei* was dominant in cheeses with defined consortia (ABCD, ABC and ABD) at day 28. Such discrepancies may either be due to *S. pulvereri* growing better than *A. ardleyensis* or *bergerei* on CRBM, or to better extraction and amplification of *A. ardleyensis* or *A. bergerei*-DNA for the SSCP analysis. Moreover, the SSCP method detects both dead and live cells.

3.7. Sugar and organic acid contents on cheese surfaces

The dynamics of lactate consumption was quite similar in all cheeses, with a decrease from day 8 to 28 (Fig. 2). In the same way, galactose content decreased rapidly from day 2 to 28 in all cheeses. A peak eluting with formate was not produced in the control and ABC cheeses. This molecule increased until day 18 in TR15, ABD and ACD cheeses and decreased after in cheeses TR15, ACD, whereas it remained stable in ABD cheeses. Maximum production was reached at 8 days for ABCD cheeses and was not detected at day 18. The acetate production curves also differed from one cheese to another. Acetate increased more or less rapidly between days 8 and 18 in all cheeses (Fig. 2). After days 18, acetate content decreased slightly in ABCD cheeses but tended to stabilise in ABC, ABD and ACD cheeses. In the control and TR15 cheeses, acetate content increased sharply between days 18 and 28.

4. DISCUSSION

Complex microbial consortia from the surface of raw milk Saint-Nectaire cheeses showed wide diversity in their ability to inhibit *L. monocytogenes*. The growth of *L. monocytogenes* was reduced for 10 out of the 34 cheeses inoculated with complex microbial consortia. This result shows that microbial consortia from raw milk cheeses can self-protect against some pathogens despite high pH values on their surfaces,

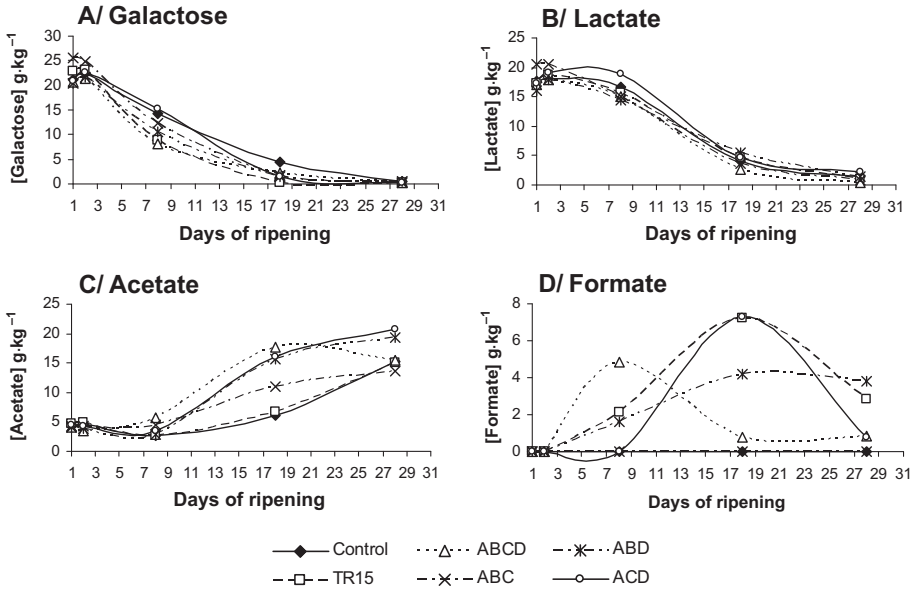

Figure 2. Galactose and organic acid contents on the surfaces of cheeses inoculated with complex consortium TR15 or defined consortia. All contents measured by HPLC at 1, 2, 18 and 28 days of ripening and expressed as $g \cdot kg^{-1}$ of dry matter content of rind. Control = commercial ripening culture; TR15 = complex consortium (rind suspension); ABCD, ABC, ABD and ACD = defined consortia from identified isolates (A = six lactic acid bacteria, B = seven Gram-positive and catalase-positive bacteria, C = three Gram-negative bacteria and D = three yeasts).

which favours *L. monocytogenes* growth. This provides an argument for the safety of raw milk cheeses, in agreement with other published studies. For example, Eppert et al. [13] have described complex consortia from red smear cheeses that inhibited *L. monocytogenes*. Similarly, according to Guillier et al. [17], biofilm recovered from wooden shelves during ripening of soft and smear cheeses was able to inhibit *L. monocytogenes* growth.

Our study confirms the conclusions of studies [11, 14] showing that the picture of microbial community of cheeses varies according to the method used to assess them. The plate counting method and a culture-independent method (SSCP) provided complementary information about the dynamics of microbial populations on cheese surfaces inoculated with different microbial consortia. By the plate counting method, the most inhibitory consortium, TR15, was distinguished from the others by its high level of lactic acid bacteria, including lactobacilli and leuconostocs, at the beginning of ripening, which may play a role in the inhibition. During ripening, on MSE medium a leuconostoc population was detected in complex consortium TR15 but not in the defined consortia, whereas PCR-SSCP analysis detected this population in all cheeses. This may mean that consortium TR15 had a different leuconostoc population to that in the defined consortia. The leuconostoc populations in the defined consortia may have lost their ability to produce dextran, and the SSCP analysis may have detected dead cells as well as live. The SSCP profiles of cheese surfaces with the complex cheese consortium TR15 were more diverse than the others. They were

characterised by the presence of *Cb. mobile*, *M. psychrotolerans*, *A. nicotianae* or *A. arilaitensis*, *A. ardleyensis* or *bergerei* and *Brachybacterium* sp., whereas the profiles of defined consortia were often dominated by only two or three species (*A. ardleyensis* or *bergerei*, *S. pulvereri* and *Ln. mesenteroides*). *M. psychrotolerans* and *Cb. mobile* are halophilic, alkalophilic lactic acid bacteria that have rarely been described in cheeses. They have been found in Saint-Nectaire cheeses [11], in red smear cheeses [14, 24] and Egyptian soft Domiati cheeses [12]. In smear cheeses, *Corynebacterium casei*, *A. nicotianae* and *B. linens* constituted more than 70% of the microbial population [3, 8, 24]. *S. pulvereri* has not often been described in cheeses other than Saint-Nectaire [11]. The population of Gram-negative bacteria present in our cheese was commonly described on the surfaces of cheeses [11, 14] not manufactured and ripened in sterile conditions. And in spite of the difficulty of controlling the proliferation of Gram-negative bacteria, differences in *L. monocytogenes* growth were still observed.

It can be hypothesised that these differences in the balance between groups of microorganisms may have a preponderant role in the inhibition, but it was not possible to identify one population as being responsible.

In the literature, inhibition in cheeses has been usually attributed to bacteriocin-producing strains even in the cases where the molecules have not been quantified. For example, Eppert et al. [13] reported that the inhibition of *L. monocytogenes* in red smear cheeses was due to a strain of *B. linens* producing linocin. Loessner et al. [22] showed that a pediocin produced by *Lactobacillus plantarum* could have an efficient antilisterial activity. In our study, none of the isolates used in the defined consortia were able to inhibit *L. monocytogenes* in the conditions of the agar-well diffusion method, suggesting that bacteriocins were not involved in the inhibition.

A possible link between the inhibition of *L. monocytogenes* with galactose consumption and acid production has not yet been established. *L. monocytogenes* can be inhibited by formate and by acetate [26, 28]. A wide range of lactic acid bacteria including *Lactobacillus* sp., *Enterococcus* sp. or *Ln. mesenteroides* [18] are able to produce acetate from lactose or galactose catabolism by the Leloir pathway or the T-tagatose pathway [1]. Moreover, some strains of lactic acid bacteria such as *E. faecalis* were shown to be able to co-metabolise lactate and citrate to produce acetate, formate and ethanol in a milk medium [27]. If most lactic acid bacteria may have the potentialities to catabolise lactate into acetate or formate [21], further investigations will be needed to characterise the capacity of production by the strains of our inhibitory complex consortium.

Lactate, an antilisterial substance, was consumed at the cheese surfaces. The catabolism of lactate is frequently attributed to yeast strains such as *D. hansenii* [4, 20] and *Y. lipolytica* [23]. In this study, the results did not enable us to link a specific microbial population and organic acid output to the inhibition of *L. monocytogenes*.

5. CONCLUSION

A natural cheese surface consortium having an antagonistic effect against *L. monocytogenes* was identified, but the strategy applied to reconstitute it failed to obtain a defined consortium with similar properties. New strategies need to be established to reconstitute this microbial consortium, taking better account of diversity at strain level, cell preparation and the physiological state of the cells. Further investigations will be needed to gain a better understanding of the microbial interactions involved in *L. monocytogenes* inhibition.

Some hypotheses made about the role of leuconostocs and lactobacilli should be checked.

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