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# Microbial biodiversity in cheese consortia and comparative *Listeria* growth on surfaces of uncooked pressed cheeses

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ABSTRACT

The study set out to determine how changes in the microbial diversity of a complex antilisterial consortium from the surface of St-Nectaire cheese modify its antilisterial activities. On the basis of the microbial composition of a natural complex consortium named TR15 (Truefood consortium 15), three new consortia of different species and strain compositions were defined: TR15-SC (58 isolates from TR15 collection), TR15-M (pools of isolates from selective counting media) and TR15-BHI (pools of isolates from BHI medium). Their antilisterial activities on the surfaces of uncooked pressed cheese made with pasteurised milk were compared with the activity of complex consortium TR15 and a control cheese inoculated only with starter culture (*Streptococcus thermophilus*, *Lactobacillus delbrueckii*). The natural consortium TR15 was the most inhibitory, followed by reconstituted consortium TR15-BHI. The dynamics of the cheese rind microbial flora were monitored by counting on media and by isolate identification using 16S rDNA sequencing and direct 16S rDNA Single Strand Conformation Polymorphism analysis. The combination of these methods showed that rind with natural consortium TR15 had greater microbial diversity and different microbial dynamics than cheese rinds with reconstituted consortia. Cheese rind with the natural consortium showed higher citrate consumption and the highest concentrations of lactic and acetic acids, connected with high levels of lactic acid bacteria such as *Carnobacterium maltaromaticum*, *Vagococcus fluvialis*, *Enterococcus gilvus*, *Leuconostoc mesenteroides*, *Brochothrix thermosphacta* and *Lactococcus lactis*, ripening bacteria such as *Arthrobacter nicotianae/arilaitensis*, and Gram negative bacteria (*Pseudomonas psychrophila* and *Enterobacter* spp.). The highest *L. monocytogenes* count was on rind with TR15-M and was positively associated with the highest pH value, high succinic and citric acid contents, and the highest levels of *Marinilactibacillus psychrotolerans* and Gram positive catalase positive bacteria represented by *Staphylococcus vitulinus*, *Brevibacterium linens*, *Microbacterium gubbeenense* and *Brachybacterium tyrofermentans*.
The results show that the species composition of consortium is more important than the number of species. It is likely that inhibition mechanisms differ from one consortium to another; investigating gene expression will be an effective way to elucidate microbial interactions in cheese.



## 1. Introduction

Raw milk cheeses are often criticised on safety grounds although they are known to be safe products. During the past decade the prevalence of *Listeria monocytogenes* in raw milk cheese has been relatively low (EFSA, 2013; Jakobsen et al., 2011; Little et al., 2008) owing to good health and hygiene procedures. Dairy products are involved in fewer than 3.45% of European outbreaks (EFSA, 2011). European food safety alerts (EFSA, 2011; Koch et al., 2010) suggest that *Listeria* contamination is more common in soft cheeses made from pasteurised or heat-treated milk than in those made from raw milk. Complex microbial communities can contribute to their self-protection. The importance of the role played by a cheese system's microbial biodiversity in limiting pathogen growth has often been mentioned. Several multispecies microbial consortia with antilisteria activity have been identified on the surfaces of complex red-smear cheeses (Bleicher et al., 2010; Eppert et al., 1997; Imran et al., 2010; Maoz et al., 2003; Roth et al., 2010) and on the surfaces or in the cores of Saint-Nectaire cheeses (Millet et al., 2006; Retureau et al., 2010; Saubusse et al., 2007). All these studies suggest that no single strain was responsible for all the inhibitory activities of a consortium and that quite different microbial consortia in terms of species composition, level of diversity and structure were able to inhibit *L. monocytogenes*. Maoz et al. (2003) described a red-smear cheese consortium whose inhibitory activity was associated with Gram positive non-lactic acid bacteria (*Corynebacterium*, *Brevibacterium* etc.). Marine lactic acid bacteria (*Vagococcus*, *Facklamia*, *Alkalibacterium*, *Marinilactibacillus*) may be involved in inhibition on the surfaces of red smear cheeses (Bleicher et al., 2010; Roth et al., 2010, 2011).

Adding a microbial species to a consortium, or omitting one, can increase or decrease its inhibitory properties against *Listeria* (Callon et al., 2011; Imran et al., 2010). On a cheese agar, the association of

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five individual strains of yeast, Gram positive and Gram negative bacteria had inhibiting effects similar to the initial complex consortium (Imran et al., 2010, 2013). In uncooked cheese cores, *Listeria* was inhibited by an association of four species of lactic and non-lactic acid bacteria (Callon et al., 2011). By successive propagation on cheese surfaces, Monnet et al. (2010) selected a consortium with strong antilisterial activity, composed of *Yarrowia lipolytica* and the *Vagococcus–Carnobacterium–Enterococcus* group. The microbial interactions governing inhibition are still unknown. The production of antagonistic substances in supernatant (lactate, acetate (Callon et al., 2011), bacteriocins and heat stable non-proteinaceous molecules (Bleicher et al., 2010)) was found to be associated with inhibition, but no causal effect was demonstrated. Maoz et al. (2003) found *Listeria* to be in a stressed state when in contact with antilisteria consortia. Elsewhere, the transcriptome of *Listeria*, monitored by microarray analysis, changed with the induction of genes involved in energy supply, stress response and cell wall synthesis (Hain et al., 2007).

Most of the literature deals with consortia from the surfaces of red smear cheeses; studies of the surfaces of other cheese types are scarce. The purpose of this study was to determine how changes in the microbial composition and diversity of a complex antilisterial consortium from the surface of St-Nectaire, an uncooked pressed cheese, modify its antilisterial activities. On the basis of the microbial composition of a previously studied consortium named TR15 (Retureau et al., 2010), several new consortia, differing in their species and strain compositions, were defined. The growth of *Listeria* on the surfaces of uncooked pressed cheeses made with these consortia was compared with growth on cheeses made with the initial complex consortium, TR15. The microbial population dynamics were compared by culture-dependent analysis and Single Strand Conformation Polymorphism analysis. Production of lactic, acetic, succinic, citric and formic acids was measured by HPLC.

## 2. Materials and methods

### 2.1. Preparation of cheeses

#### 2.1.1. Cheesemaking

Cheeses (600 g) were manufactured from pasteurised milk collected at an agricultural school farm (ENILV, Aurillac, France), using an uncooked pressed cheese technology (Callon et al., 2011). Milk was pasteurised at 72 °C for 20 s, cooled to 33 °C, then inoculated with 0.6% of a commercial starter culture (MY800, *Streptococcus thermophilus*; *Lactobacillus delbrueckii* spp. *bulgaricus*, Danisco, Paris La Défense, France) and with a commercial mould culture of *Penicillium commune* (2.5 ml/200 l, Laboratoire Interprofessionnel de Production — LIP, Aurillac, France). Forty-five minutes after adding 40 ml/100 l 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 one-day-old cheeses were vacuum-packed and frozen at −20 °C until surface inoculation.

#### 2.1.2. Preparation of the consortia

The complete experimental design of the study is shown in Fig. 1. Different consortia named TR15, TR15-M, TR15-SC and TR15-BHI were prepared for inoculating the cheese surface (Section 2.1.3).

The natural microbial consortium (TR15), taken from 1/4 of the rind (3 mm thick) of a raw milk St-Nectaire cheese after 28 days of ripening, was diluted 1/10 in phosphate buffer at pH 7.5 and blended for 4 min using a Stomacher blender (Retureau et al., 2010). The suspension was aliquoted into sterile tubes and frozen at −20 °C until use. Before inoculation, the suspension was thawed at 25 °C.

Three different consortia were reconstituted from this natural microbial consortium TR15.

Consortium TR15-SC consisted of 58 strains isolated after inoculating TR15 onto selective media (Fig. 2). For this purpose, 200 microbial isolates were taken from media for lactic acid bacteria (FH, MSE and SB), ripening bacteria (RPF, CRBM), Gram negative bacteria (PCAI) and yeasts (OGA), in the conditions described in Section 2.3.1. The isolates were purified before being identified at species level by DNA extraction (using Easy DNA kit with phenol/chloroform Invitrogen, Cergy Pontoise, France), ribosomal 16S rRNA gene amplification (1450 bp) and sequencing, as described by Retureau et al. (2010). The Blast programme was used to compare the sequences with those available in the GenBank database. In this study, a 99% similarity was taken as the criterion to assign an isolate to a species or group of species. Yeasts were identified by a combination of phenotype tests and sequencing of the D1/D2 domain of the 26S rRNA gene, as described by Callon et al. (2006). Then 58 strains (Fig. 2) were chosen according to their frequency and establishment on the rind, as described by Retureau et al. (2010). Each strain was cultured in 10 ml of broth medium (MRS medium for lactobacilli and leuconostocs, M17 medium for enterococci, BHI for other lactic acid bacteria, Gram-positive catalase-positive bacteria and Gram-negative bacteria, and YPG medium for yeasts). Cultures were centrifuged for 15 min at 5000 rpm and 4 °C. Pellets were then resuspended in sterile milk with 15% glycerol, with 0.5% ascorbate added for Gram positive bacteria but not for Gram negative bacteria or yeasts. They were then frozen at −20 °C. Each tube used for inoculation was thawed for 5 min in a 30 °C bath, enumerated, and diluted in a pH 7.5 phosphate buffer in order to inoculate the cheese surfaces at the appropriate concentrations (Fig. 2).

In order to take better account of strain diversity, a pooled consortium TR15-M was composed as follows: all colonies growing on each of the selective media inoculated with consortium TR15 (FH dilution plate −5, MSE dilution −4, SB dilution −4, RPF dilution −2, CRBM dilution −3, PCAI dilution −3 and OGA dilution −4 (see Section 2.3.1)) were scraped off, making one pool per medium. Each pool was then resuspended in 2 ml of phosphate buffer. Suspensions were washed twice by centrifuging for 15′ at 5000 rpm and pellets were then frozen at −20 °C. Before use, the pellets were thawed and the suspensions enumerated on their respective isolation media, then diluted in a pH 7.5 phosphate buffer to obtain the appropriate concentrations on the cheese surfaces. The diluted suspensions were pooled together to form consortium TR15-M.

A further consortium, TR15-BHI, was composed by pooling all colonies growing on non-selective BHI medium inoculated with TR15 (see Section 2.3.1). BHI medium was selected because numerous bacterial species with antilisterial properties can grow on it, as shown by Monnet et al. (2010). The suspensions were prepared and processed as described above.

#### 2.1.3. Inoculation of experimental cheese surfaces and ripening

The one-day-old experimental cheeses made from pasteurised milk were thawed for 5 h at room temperature under a laminar hood and turned over each hour to dry them. The surfaces of all cheeses were inoculated by depositing and spreading 1 ml of the culture of *L. monocytogenes* (strain 167) with a sterile tooth-brush (Millet et al., 2006). The strain was precultured for 18 h at 37 °C in casein soya broth supplemented with 0.6% yeast extract and then processed as described in Section 2.1.2 for the TR15-SC strains. It was inoculated at 2 to 5 CFU/cm$^2$.

The cheese surfaces were then inoculated with either the natural microbial consortium (TR15) or one of the reconstituted consortia (TR15-BHI, TR15-SC and TR15-M). Each cheese surface (area = 132 cm$^2$) was inoculated with 1 ml of reconstituted consortium prepared as described in Section 2.1.2. The inoculation levels of microorganism strains or pools were based on mean counts obtained by different microbial enumerations of the natural microbial consortium TR15 on the corresponding medium (Fig. 2).

The experiment included a control cheese whose the rind was inoculated only with 1 ml *L. monocytogenes* strain (167), used as control for *Listeria* growth.