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Antagonistic activity of complex cheese surface microbial consortia against food-borne pathogens

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Preface

Chapters 2 to 5 of the present PhD thesis are either published in or submitted to peer-reviewed journals. Each chapter has its own introduction which may overlap to a certain extent since each is part of a separate manuscript. Additionally, a general introduction was added including background information concerning the human pathogen *Listeria monocytogenes*, the production of red smear soft cheeses as well as recent approaches to improve their microbial safety. The results of this work are summarized in a general discussion.

Chapter 2 comprises a comparison of methods for the determination of the anti-listerial potential of complex cheese ripening consortia applying both *in vitro* and *in situ* approaches. This chapter was published 2010 in Food Microbiology 27, 302-310 by Christophe Monnet, Anne Bleicher, Klaus Neuhaus, Anne-Sophie Sarthou, Marie-Noëlle Leclercq-Perlat and Françoise Irlinger. I performed parts of the experimental work (conduction of Method A and flora analysis using FT-IR spectroscopy) and wrote a part of the manuscript.

Chapters 3 and 4, the survey of complex cheese ripening consortia and the characterization of their culture supernatants, constitute the main part of my research. The fractionation of the complex supernatants was conducted by Dr. Timo Stark (Lehrstuhl für Lebensmittelchemie und Molekulare Sensorik, Technical University of Munich). The screening for genetic determinants for inhibition was partially carried out by Tanja Obermajer and Dr. Bojana Bogovič Matijašić (Chair of Dairy Science, University of Ljubljana, Slovenia). Chapter 3 has been submitted to the Annals of Microbiology by Anne Bleicher, Tanja Obermajer, Bojana Bogovič Matijašić, Siegfried Scherer and Klaus Neuhaus. Chapter 4, written by Anne Bleicher, Timo Stark, Thomas Hofmann, Bojana Bogovič Matijašić, Irena Rogelj, Siegfried Scherer and Klaus Neuhaus is a publication under review in the Journal of Dairy Science.

Chapter 5 was published as "*Vibrio casei* sp. nov., isolated from the surface of two French red smear soft cheeses" by Anne Bleicher, Klaus Neuhaus and Siegfried Scherer in the International Journal of Systematic and Evolutionary Microbiology (2009, *in press*). Aside from the main topic, a novel species was discovered during flora analysis using FT-IR spectroscopy. All experimental work was performed by myself and I wrote the major part of the manuscript.

Summary

The antagonistic potential of 14 complex cheese ripening consortia against the food-borne pathogen *Listeria monocytogenes* was assessed using four different methods. Method A comprised the cultivation of the indicator strains in complex cell-free supernatants (CFS), whereas Method B based on a co-inoculation of the microflora and *Listeria* on model cheeses. Method C and D included successive propagations of the microfloras on these model cheeses, with or without the addition of *Listeria*. The anti-listerial activity considerably depended on the microflora used. Significant correlations (Pearson's analysis) were obtained between Methods A and B and Methods C and D, respectively. The lab-scale propagation of the consortia on model cheeses was associated with a substantial microbial succession, in general favouring Gram negative and lactic acid bacteria, concomitant with a loss of diversity. It was therefore not possible to store and to process microfloras from cheese surfaces without modifying important properties such as the balance between strains and their physiological state (Chapter 2).

FT-IR spectroscopy was applied to record the taxonomical composition of selected cheese ripening consortia. Four anti-listerial (as evaluated using Method A) and two non-inhibitory consortia were investigated, each showing a unique character in terms of species composition and diversity (Chapter 3). The initial composition of two anti-listerial consortia changed considerably when propagated in liquid medium: coryneform bacteria did not multiply or even disappeared, while the growth of Gram negative and lactic acid bacteria was favoured (Chapter 4). A deduction of species combinations for *Listeria* reduction either on model cheeses or in CFS is therefore not possible. In the course of flora analysis the novel species *Vibrio casei* was detected and validly published (Chapter 5).

The presence of bacteriocin genes in total DNA extracts of several consortia was demonstrated by PCR (Chapters 3 and 4). However, individual LAB isolates of two anti-listerial consortia displayed no or only weak inhibition of *L. monocytogenes* on solid medium (Chapter 4).

A preliminary characterization of the inhibitory principle was achieved through fractionation of a complex supernatant (Chapter 4). Reverse phase HPLC revealed the presence of small, heat-stable, non-proteinaceous molecules in a complex CFS. Their resistance towards proteinase K and their broad inhibitory spectrum, comprising also Gram negative bacteria, distinguish them from classical bacteriocins.

Zusammenfassung

Das antagonistische Potential von 14 mikrobiellen Rotschmierekäse-Konsortien gegen das Lebensmittelpathogen *Listeria monocytogenes* wurde mit vier Methoden erfaßt, die miteinander verglichen wurden. In Methode A wurde die inhibitorische Aktivität von zellfreien Kulturüberständen der Konsortien ermittelt; für Methode B wurden die Konsortien zusammen mit *Listeria* auf einem Käsesubstrat ko-inokuliert. Methode C und D umfaßten zudem sukzessive Reifungen der Konsortien auf dem Modellkäse, entweder in Anwesenheit oder unter Ausschluß von *Listeria*. Der anti-listerielle Effekt war stark vom jeweiligen eingesetzten Konsortium abhängig. Signifikante Korrelationen (Pearson-Analyse) bestanden zwischen den Methode A und B bzw. zwischen Methode C und D. Eine Erhaltung und Vermehrung der anti-listeriellen Konsortien im Labormaßstab war nicht möglich, da sich Gram negative und Milchsäurebakterien auf dem Modellkäse stärker vermehrten als coryneforme Gattungen und die Diversität der ursprünglichen Flora abnahm.

Die FT-IR-Spektroskopie wurde angewandt, um die taxonomische Zusammensetzung ausgewählter bakterieller Gemeinschaften aufzuklären. Die vier anti-listeriellen und zwei nicht-inhibitorischen Konsortien wiesen jeweils eine einzigartige Zusammensetzung auf (Kapitel 3). Die Propagierung von zwei anti-listeriellen Konsortien in Bouillon war mit einer deutlichen Veränderung der Populationsstruktur zugunsten von Gram negativen und Milchsäurebakterien sowie mit einem Diversitätsverlust verbunden (Kapitel 4). Es ist somit nicht möglich, eine Kombination von Spezies, die zur Reduktion von Listerien auf dem Modellkäse oder im Kulturüberstand führt, abzuleiten. Im Zuge von Florenanalysen wurde die neue Spezies *Vibrio casei* entdeckt und valide beschrieben (Kapitel 5).

In DNA-Extrakten verschiedener Konsortien konnten Bakteriozingene mittels PCR nachgewiesen werden (Kapitel 3 und 4). Einzelne Milchsäurebakterien wiesen jedoch nur eine schwache, unstabile Hemmung gegen Listerien auf Festmedium auf (Kapitel 4).

Das inhibitorische Prinzip eines komplexen Kulturüberstandes wurde teilweise charakterisiert (Kapitel 4). Eine Fraktionierung mittels Umkehrphasen-HPLC läßt eine Beteiligung von Substanzen geringer Größe und großer Hitzestabilität vermuten. Ihre Resistenz gegenüber Proteinase K und ihr breites Hemmspektrum, das auch Gram negative Bakterien umfaßt, unterscheidet sie von klassischen Bakteriozinen.

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Symbols and abbreviations

API	analytical profile index
BHI	brain heart infusion
CFS	cell-free supernatant
DNA	desoxyribonucleic acid
e. g.	exempli gratia
FT-IR	Fourier Transform InfraRed
LAB	lactic acid bacteria
MIC	minimal inhibitory concentration
PCR	polymerase chain reaction
sp. nov.	species nova
ssp.	subspecies
RNA	ribonucleic acid
TSA	tryptic soy agar
YGCA	Yeast extract Glucose Chloramphenicol Agar
YGCB	Yeast extract Glucose Chloramphenicol Bromophenol blue
WCC	Weihenstephaner Culture Collection
WSLC	WeihenStephaner Listeria Collection

1 General introduction

1.1 *Listeria monocytogenes*, a multi-faceted pathogen

Listeria monocytogenes, the causative agent of human listeriosis, is a Gram positive, nonsporeforming, facultatively anaerobic straight rod. It belongs to the phylum firmicutes with a low G+C content, related to genera like *Bacillus, Staphylococcus, Streptococcus, Enterococcus* and *Clostridium*. The organism can multiply at temperatures ranging from 3 to 45 °C, at high salt concentrations (10 % sodium chloride) and pH values as low as 4.5 (Farber & Peterkin, 1991; Low & Donachie 1997). Motility is observed at temperatures ranging from 10 to 25 °C, where listeriae possess peritrichious flagella, but not at 37 °C. The species was first described by Murray *et al*. (1926) as *Bacterium monocytogenes*, isolated from an epidemic disease in a laboratory animal breeding. The species name refers to the observed mononuclear leucocytosis in rabbits. It was the only recognized species of this genus until 1966; today seven other species are accepted: *L. grayi* (1966), *L. ivanovii* (1975), *L. welshimeri* and *L. seeligeri* (1983), *L. innocua* (1984) and the recently published species *L. marthii* (Graves *et al*., 2009) and *L. rocourtiae* (Leclercq *et al*., 2009). Only two of these species are pathogenic: *L. monocytogenes* causes severe illness in humans and animals, whereas infection of *L. ivanovii* is restricted to animals. Early serological studies could demonstrate the presence of somatic “O” and flagellar “H” antigens, which formed the basis for the today’s accepted scheme including 13 different serovars (Seeliger & Langer, 1979). Serotyping does not correlate with the species distinctions since a number of serovars are shared by different species. 98 % of *L. monocytogenes* isolated from patients belong to serovars 1/2a, 1/2b, 1/2c and 4b with serovar 4b being responsible for all major outbreaks and most of the sporadic cases (Jacquet *et al*., 2002).

The bacterium is widely present in the environment, being frequently isolated from plants, soil, surface water, silage, sewage, milk of healthy and infected cows, human and animal feces. Infections have been recorded in more than 40 species of wild and domesticated animals in countries over six continents (Low & Donachie, 1997). Listeriosis in animals has

a major veterinary importance in cattle, sheep and goats; in monogastric animals the disease is uncommon.

The bacterium seems to be a normal resident in the human intestinal tract and antibodies are commonly detected in healthy people. The number of carriers of *L. monocytogenes* is estimated to lie between 5 and 10 % of the general population (Farber & Peterkin, 1991). Human listeriosis has a quite low incidence of 2-10 cases per million population per year, but the mortality rate can reach 25-30 % among persons developing the disease (de Valk *et al*., 2005). The majority of cases occurs in individuals whose T-cell-mediated immunity is suppressed (elderly, pregnant, neonates, HIV and diabetes patients). Contrary to most other pathogens targeting the gastrointestinal tract, infections with *L. monocytogenes* may not be self-limiting. The clinical symptoms of adult listeriosis mainly manifest in infections of the central nervous system (meningitis and encephalitis), bacteremia and endocarditis. Pregnant women may contract only a mild, influenza-like illness, but due to the organism's capability of crossing barriers like the placenta, the infection can spread to the fetus, leading to stillbirth, abortion or neonatal diseases with meningitis as the predominant form. Apart from the natural resistance of *Listeria* to cephalosporins, resistance mechanisms are not yet of clinical relevance in antibiotic treatment. There are no precise numbers available concerning the minimal infectious dose, which depends on the host's susceptibility. Feeding experiments indicated that 50 % of pregnancies in non-human primates are affected adversely when animals were challenged with 10^7 bacteria (Smith *et al*., 2008). In another study healthy monkeys became not noticeably ill until receiving 10^9 cells of *L. monocytogenes* (Farber *et al*., 1991).

Pathogenicity of *L. monocytogenes* is characterized by a cytosolic lifestyle and the ability of spreading from cell to cell, thus avoiding the host's humoral immunity response. Membranes of endo- and epithelial cells, the placenta and the blood-brain barrier are breached. After oral uptake, the pathogen triggers its endocytosis into intestinal cells or macrophages by two proteins, internalins A and B (Freitag *et al*., 2009). *Listeria* is able to escape from the phagosome and replicates in the cytoplasm of the host cell. Bacteria mediate their movement through reorganization of the actin filament which finally leads to intercellular spread.

The transmission route for *L. monocytogenes* infections remained unclear for long time. Its food-borne nature was not recognized before 1981, when 41 persons became ill after consumption of refrigerated coleslaw in the Maritime Provinces of Canada. Two of seven adults and fifteen of twenty-seven infants died (Schlech *et al*., 1983). During the 1980s, a

number of listeriosis outbreaks were linked with the consumption of contaminated soft cheeses. In 1985, a Mexican-style soft cheese caused 48 deaths (34 % mortality rate) in California. For the first time in history, the incriminated product was identified and recalled during the epidemic. In the western part of Switzerland, 122 listeriosis cases with 31 deaths were recorded between 1983 and 1987. The organism could be detected on the surface of Vacherin Mont d´Or soft cheese and all products were removed from the market. Today there is a general agreement that food-borne transmissions constitute the predominant infection route; nosocomial infections and person-to-person spread are known but uncommon.

As a result of these outbreaks, the pathogen has received more and more attention by food manufacturers as well as governmental bodies. The cases are mandatory notifiable and are published in annually surveillance reports in several countries of Europe and in the USA. The informations available lead to the conclusion that listeriosis is a disease rather emerging than declining. The pathogen ranges at rank three after *Salmonella* and *Campylobacter* in causing hospitalizations in France (Vaillant *et al*., 2005) and at rank two after *Salmonella* in causing deaths in the USA (Mead *et al*., 1999). In Germany however, the data show a continuous increase of cases since 2001, when the national reporting system was introduced. No common source vehicle could be identified (Koch & Stark, 2006). In recent years, there was a general tendency towards sporadic cases rather than epidemics. One reason may be the surge of susceptible persons (patients surviving organ transplantation, AIDS, cancer, liver or kidney failure), as suggested by Kasper *et al*. (2009) for the situation in Austria. Another explanation could be the increasing popularity of convenience foods. A late nationwide outbreak involving 24 states of the USA could be assigned to contaminated frankfurters. A total of 104 cases were identified with 14 deaths and four miscarriages. The low cell counts of *L. monocytogenes* serovar 4b detected emphasizes the varying virulence of strains and confirm that large epidemics can occur even at low contamination levels (Mead *et al*., 2006).

The European Union Regulation 2073/2005 specify a maximum *L. monocytogenes* titer of 100 cfu per gram allowable in food not dedicated for infants or medical purposes, regardless if the respecting food supports growth of the organism or do not. Surveillance of this food-borne illness is complicated by the long incubation time (weeks to months) before symptoms become manifest. Further, milder cases may not be recognized and the evidence of the causing agent may not be proven, leading to an underreporting and difficult assignment of cases.

1.2 The production of traditional cheeses: the impact of stable in-house floras

Among raw meat, poultry, shrimps, cold-smoked salmon, raw milk, ice cream, fresh vegetables and pre-packed salads, soft cheeses are particular susceptible to a contamination with *L. monocytogenes*. The occurrence of the pathogen in smeared cheeses has not decreased sufficiently during the past years, showing exceedingly high cell counts in some products (Jacquet *et al*., 1993; Loncarevic *et al*., 1995; Rudolf & Scherer 2001). Assessing the pathogenicity of food-associated isolates using both *in vitro* and *in vivo* methods, the prevalence of virulent or highly virulent strains in foods was clearly proven (Neves *et al.,* 2009; Roche *et al*., 2009). The cross-contamination ways of *L. monocytogenes* strains within a dairy and their persistence in a production line over years give cause for concern (Unnerstad *et al*., 1996; Loncarevic *et al*., 1998). Though a main source of contamination was circumvented by pasteurizing the raw milk prior to cheese making, the pathogen may enter the production line post-processing, during storage or even in retail establishments (Sauders *et al*., 2009). The extended or unknown shelf life of smear cheeses at low temperatures further acts as a selective enrichment for *L. monocytogenes*, which is able to grow at temperatures so low that concomitant bacteria either enter the stationary phase or die.

Bacterial surface-ripened cheeses have a long tradition in Germany, Austria, France, Italy and Switzerland. Brands like Munster, Limburger, Tilsiter, Romadur and Appenzeller constitute an important proportion of artisanal produced, high quality foods. The surface flora, consisting of coryneform bacteria, micrococci, staphylococci and yeasts contributes to the ripening of cheeses by the production of proteolytic and lipolytic enzymes and aroma compounds. Ripening is performed at temperatures ranging from 14 to 19 °C and a relative humidity of at least 95 % (Bockelmann & Hoppe-Seyler, 2001). The yeasts develop first, metabolizing the lactic acid produced by the starter and forming alkaline metabolites, followed by an increase of pH on the cheese surface from 5.0 to 6.0. This enables the salt-tolerant, but less acid-tolerant coryneform bacteria to grow. In addition, bacterial development is stimulated by branched-chain amino acids which are either secreted by yeasts during their growth or as a consequence of their accelerated autolysis. *Brevibacterium linens* for instance requires panthothenic acid, produced by certain yeasts, as a growth factor. During ripening, a microbial succession occurs: the yeasts start to

colonize of the cheese surface, followed by the rise of staphylococci in the early stages of ripening while coryneform bacteria predominate the flora in the late stages of ripening (Rea *et al*., 2007). Flora analysis using strain typing methods revealed species being often present as single clones (Brennan *et al*., 2002; Mounier *et al*., 2005). The sources of the adventitious, so-called “house flora” of a certain cheese-making facility may comprise not only the smear bath but also the dairy environment, the wooden shelves in the ripening room as well as the skin of hands and arms of the personnel (Mounier *et al*., 2006; Mariani *et al*., 2007). The smear is applied repeatedly by brushing, thereby beginning with the mature (“old”) cheeses followed by the freshly produced (“young”) cheeses. Old-young-smearing provides an inexpensive technique to propagate a surface flora and ensures the uniqueness of the product. A disadvantage of this approach is the high risk of spreading contaminating microorganisms to the smear bath, fresh batches of cheeses and the ripening facilities. Protective ripening cultures, either single strains or mixed cultures, are commercially available and applied in high titers to direct ripening and reduce undesirable microorganisms.

In recent studies, the fate of these ripening cultures was monitored. Brennan *et al*. (2002) deliberately inoculated farmhouse smear-ripened cheeses with *B. linens* BL2 at the beginning of the ripening and performed random amplified polymorphic DNA (RAPD) PCR and pulsed-field gel electrophoresis (PFGE) for strain typing. *B. linens* BL2 was not recovered from the inoculated cheeses at any time point because it was inhibited by all indigenous *Staphylococcus* isolates and most of the coryneform species, as evaluated in overlay assays. In other studies, the reason for the poor competition of *B. linens* could not be clearly identified (Feurer *et al*. 2004b; Rea *et al*. 2007). Goerges *et al*. (2008) inoculated cheese milk with a multispecies starter from Danisco for Limburger production, containing *Debaryomyces hansenii*, *Galactomyces geotrichum*, *Arthrobacter arilaitensis* and *Brevibacterium aurantiacum.* The indigenous Limburger flora had a comparable low diversity but a high temporal stability: it was able to overcome the relocation to a new ripening room and persisted a weekly disinfection of the cellars. None of the commercial bacterial or yeast starters was able to establish in this consortium in a stable fashion although it contained nonstarter strains belonging to the same species.

In the past years, different cheese surface populations were surveyed and characterized. The analysis of four Irish surface ripened cheeses by Mounier *et al*. (2005) using various strain typing methods for bacteria and yeasts suggested each cheese having a unique flora. All investigated samples had only two bacteria in common (*Corynebacterium casei*

and *A. arilaitensis*). Maoz *et al*. (2003) used Fourier Transform-InfraRed (FT-IR) spectroscopy to analyse the composition of two undefined, industrial ripening consortia. The dominant species of the one consortium was not isolated at all from the second. Larpin *et al*. (2006) monitored the dynamics of the yeast population in Livarot using a quantitative real-time PCR approach in combination with FT-IR spectroscopy. The ripening, performed in different dairies, resulted in a unique yeast community of each of the four batches. Ripening consortia may differ not only regarding their species mixture but also in terms of diversity. After quantitative analysis of two geographically separated surface floras, Wenning *et al*. (2006) came to the conclusion that the microbial composition is determined by the conditions prevailing on the cheese surface which obviously select a limited variety of bacteria. In other studies, a high diversity of species was reported (Feurer *et al*., 2004a; Rea *et al*., 2007). There is no evidence that uncultivable organisms contribute to such ripening consortia (Carnio *et al*., 1999) but the number of subdominant species detected and hence the diversity index may be greater using molecular techniques (Feurer *et al*., 2004a).

1.3 Application of bacteriocins in cheese production and ripening

Bacteriocins can be defined as small, heat stable, cationic peptides, which are ribosomally synthesized by a variety of lactic acid bacteria (LAB) as well as some coryneform bacteria (for review see Jack *et al*., 1995; Cotter *et al*., 2005). They can be distinguished from bacterial toxins since they are not active on eukaryotic cells, even in doses 100-fold higher than required for antimicrobial activity (Jasniewski et al., 2009). Their primary target is the cytoplasmic membrane of other, often closely related Gram positive bacteria, whereas the producing strain possesses a specific immunity mechanism. Monomers of the peptides (19-48 amino acid residues) are thought to aggregate in the cytoplasmic membrane thus forming a water-filled pore of 0.2 to 2 nm, causing dissipation of the proton motive force, leakage of small solutes and subsequent cell lysis. The mode of action is identical for all bacteriocins although pore formation was observed to be voltage-dependent in case of class I or independent of charge in case of class II bacteriocins (Abee 1995). The participation of a specific docking molecule for target cell recognition was revealed by characterization of spontaneous or adapted resistant bacteria. High-level resistance in *L. monocytogenes* and *Enterococcus faecalis* against class IIa bacteriocins was

associated with the loss of a mannose-specific phosphotransferase system (PTS), which seems to serve as a receptor on the cell surface (Gravesen *et al*., 2002; Arous *et al*., 2004). The resistance of *L. monocytogenes* against the class I bacteriocin nisin is only intermediately because an eradication of its docking molecule, lipid II, is lethal (Gravesen *et al*., 2004).

The genetic determinants for bacteriocin production are usually arranged in operons with genes encoding the structural protein, the regulatory machine for expression, transcriptional and posttranslational modification, transport and self-protection. Bacteriocin-associated genes are often plasmid-borne but some are also located on the chromosome. The observation that LAB strains of different origins, species or even genera may produce the same bacteriocin suggests this feature being a quite mobile trait. Multiple class IIa producers are described among *Enterococcus* and *Carnobacterium* species.

Many LAB experience a long history of application in food fermentations and have the GRAS status (generally recognized as safe). Nisin, produced by *Lactococcus lactis* ssp. *lactis* is one of the best characterized bacteriocins and the only one which has been approved by the World Health Organization as a food preservative. The commercial product Nisaplin (Danisco) has entered the food industry of over 50 countries worldwide since 1956. Pediocin AcH, produced by *Pediococcus acidilactici* and *Lactobacillus plantarum* strains, was also commercially exploited for biopreservation. The product ALTA 2431 (Quest) is based on fermentation broth of the organism grown under optimized conditions.

Conferring “innate immunity” to food (Cotter *et al*., 2005) can be achieved by (i) the addition of deliberated strains to the starter culture which produce antimicrobial substances *in situ*, (ii) spraying or dipping procedures using purified bacteriocins or crude extracts of producer cells or (iii) packaging films coated with bacteriocins which are then gradually released into the food matrix. Each technique has been successfully used in pilot-scale productions of cheeses. An initial inoculum of 10^4 cfu/g *L. monocytogenes* Scott A was reduced at least 99.9 % within five days of storage at 4 °C in cottage cheese manufactured using the lacticin 3147-producer *Lc. lactis* DPC4275 as a starter. When the storage temperature was altered to 30 °C, a reduction to zero was achieved within 12 hours (McAuliffe *et al*., 1999). In other studies, bacteriocinogenic strains are used as adjunct cultures since their acidification potential and proteolytic properties are lower than that of conventional starters. A mixed starter culture containing the nisin Z-producing strain *Lc. lactis lactis* biovar *diacetylactis* was used for the production of Cheddar cheese from

milk inoculated with 10^5 to 10^6 cfu/ml *L. innocua*. After six months, 10^4 viable *Listeria* cells per gram cheese could be detected (Benech *et al*., 2002). In the same study, the effect of purified nisin Z encapsulated in liposomes was investigated, which performed much better. Only 10 cfu/g *L. innocua* survived until the end of ripening and the cheeses still contained 90 % of the initial nisin activity. As example for a so-called active packaging, polyethylene films coated with enterocin 416K1 caused a reduction of 1 log unit within 24 h on frankfurters and on soft cheeses contaminated with a 10^8 cfu/ml suspension of *L. monocytogenes* (Iseppi *et al*., 2008).

1.4 Antagonistic acting surface consortia

Although bacteriocin producers have been frequently isolated from cheese ripening consortia, the present data suggest only a minority of the strains in a ripening consortium being able to inhibit *Listeria.* Using different *in vitro* assays, the proportion of inhibitory strains among the coryneform bacteria was estimated to be less than 0.1 % (Ryser *et al*., 1994), approximately 1 % (Hug-Michel *et al*., 1989) or 12 % (Valdés-Stauber *et al*., 1991). The inhibition could sometimes be assigned to the production of bacteriocins (Ryser *et al*., 1994; Valdés-Stauber & Scherer, 1994; Ennahar *et al*., 1998) or other anti-listerial compounds (Dieuleveux *et al.*, 1998; Carnio *et al*., 2000). However, for several antagonistic acting surface consortia the precise mechanism for inhibition is unknown. Stable microbial communities exhibited a growth reduction of *L. monocytogenes* whereas strains isolated from such consortia performed only weak *in vitro* (Carminati *et al*., 1999; Carnio et al., 1999; Maoz *et al*., 2003; Saubusse *et al*., 2007). Inhibitory substances of different characters seem to be involved: Hug-Michel *et al*. (1989) supposed an extracellular but cell-bound compound being produced by *Arthrobacter protophormiae, Arthrobacter uratoxydans* and *Serratia liquefaciens* isolates*.* The inhibitory substance was only present on solid media and showed a poor ability to diffuse into the agar. The effect could be abolished by removing the producing cells with a swab. Martin *et al*. (1995) isolated three strains of *B. linens* from brine which produced a very small (< 1 kDa) substance, acting bactericidal against *Listeria* species on solid and in liquid media. These properties are different from linocin M18 (31 kDa) which is also produced by *B. linens* (Valdés-Stauber & Scherer, 1994). The proteinaceous nature of inhibitory compounds produced by *Enterococcus faecalis, Staphylococcus xylosus, Staphylococcus warneri* and

various coryneform bacteria, isolated from the surface of French smear cheeses, was proven by Ryser *et al*. (1994).

More information was derived from *in situ* applications of such bacteriocin producers: Loessner *et al*. (2003) observed growth suppression of *L. monocytogenes* by a pediocin-producing *L. plantarum* was dependent on contamination levels of the brine. The complete eradication of the pathogen from cheese surfaces could be achieved by addition of *L. plantarum* supernatants; however, the development of spontaneous resistant mutants could not be excluded in long-term ripening experiments. Sulzer & Busse (1991) reported an efficient *Listeria* reduction on Camembert cheeses when the inhibitory strain was used as sole starter and contamination occurs at an early stage of ripening.

Because of *Listeria´*s acid tolerance, the organism holds a growth advantage over the ripening bacteria. The search for anti-listerial yeasts able to inhibit multiplication of the pathogen in the early stages of cheese production therefore is reasonable. Goerges *et al*. (2006) identified three strains of *Candida intermedia* and one *Kluyveromyces marxianus* able to reduce *L. monocytogenes* 3 to 4 log units upon cell-cell-contact. The cheese-associated yeast *Geotrichum candidum* was shown to produce two low-molecular substances, D-3-phenyllactic acid and D-3-indollactic acid, which act anti-listerial. Culture filtrates inhibited growth of *L. monocytogenes* both on solid and in liquid medium (Dieuleveux *et al.*, 1998).

1.5 Unspecific antagonism between microorganisms

Fermentation of foods results in an increased shelf life and a higher microbial safety of the product. The unique metabolic traits of the lactic acid bacteria exploited in biopreservation include the production of organic acids, hydrogen peroxide, diacetyl, acetaldehyde, ethanol and carbon dioxide which serve to control spoilage microorganisms (Caplice & Fitzgerald, 1999). Lactic, acetic or propionic acid lower the cytoplasmic pH and interfere with the maintenance of the membrane potential, thereby inhibiting active transport. The antimicrobial concentrations and the inhibitory spectra of these organic acids differ with acetic and sorbic acid being more toxic and targeting also yeasts and moulds (Stratford *et al*., 2009). Hydrogen peroxide is generated in the presence of oxygen since LAB lack catalase for its breakdown. The inhibitory effect of H_2O_2 is mediated through its strong oxidizing effect on membrane lipids and cell proteins. Carbon dioxide is toxic to some aerobic bacteria by creating an anaerobic environment and by its ability to reduce the internal and external pH. Diacetyl is believed to interfere with the utilization of arginine especially in Gram negative bacteria. The contribution of acetaldehyde and ethanol to biopreservation is minor since the levels produced by LAB are lower than the respective minimal inhibitory concentrations (MICs, for review see Caplice & Fitzgerald, 1999).

Raw milk contains the enzyme lactoperoxidase which mediates the oxidation of thiocyanate to hypothiocyanite. The oxidation product in turn reacts with the amine and thiol groups of bacterial enzymes essential for metabolism. The lactoperoxidase system (LPS) of raw milk acts as a natural inhibitor against Gram positive bacteria but is inactivated during pasteurization. When Camembert cheeses were produced from raw and pasteurized milk artificially contaminated with 5×10^{-1} cfu/ml *L. monocytogenes*, the lag phase of the pathogen was 26 d and 15 d of ripening, respectively (Gay & Amgar, 2005). Principal component analysis further revealed an inhibitory influence of the microbial composition of the raw milk against *L. monocytogenes.*

Guillier *et al*. (2008) performed co-inoculation experiments on soft cheeses to model the growth of *L. monocytogenes* together with a microbial community obtained from wooden shelves as used in ripening cellars. The authors could report that the pathogen stopped growing, not reaching the exponential growth phase, as soon as the biofilm population entered the stationary phase. This reduction of the maximum population density is referred to as the “Jameson” effect after Jameson (1962) who observed that in co-culture the

growth of *E. coli* is stopped at the time *Salmonella* reached the stationary phase. The authors suggested this inhibition resulting from a non-specific competition for nutrients. A reduced growth rate of *L. monocytogenes* was also observed by Nilsson *et al*. (2005) when the pathogen was grown together with a non-bacteriocinogenic *Carnobacterium piscicola*. The effect could be abolished by the addition of glucose. Also Mellefont *et al*. (2008) concluded that specific inhibitors are not needed to achieve growth suppression in co-cultures when both organisms rely on the same nutrients. The Jameson effect is often seen in foods where lactic acid bacteria predominate the population. They possess a growth rate advantage because they are adapted to these environments and are present in high cell numbers when used as starter culture.

As recently shown by Millet *et al*. (2006) and Saubusse *et al*. (2007), the antagonism may result from a synergistic action of different strains. Research has mainly focused on the identification of single inhibitory compounds and determination of their MICs in simple *in vitro* systems, but little is known about the complex, synergistic interactions of these substances in a food environment. For this purpose, the inhibitory potential of complex cheese surface consortia against *Listeria* ssp. was evaluated using different experimental approaches which were compared with each other.

2 Assessment of the anti-listerial activity of microfloras from the surface of smear-ripened cheeses

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2.1 Abstract

The anti-listerial activity of microfloras from the surface of various smear-ripened cheeses was evaluated using four methods that were then compared. Method A measured the anti-listerial potential of supernatants from short-time liquid cultures, whereas in Method B, a model cheese was co-inoculated with the microflora and *Listeria innocua* test strains. Method C was based on successive propagations of the microfloras on this model cheese, and Method D on successive propagations of the microfloras together with *Listeria* test strains. Anti-listerial activity considerably depended on the microflora used. Significant correlations were obtained between Methods A and B and Methods C and D. With Methods C and D, the highest anti-listerial activity was obtained with the microflora from a Livarot-type cheese (FC12). To investigate the cause of the anti-listerial activity of FC12, Fourier Transform InfraRed (FT-IR) analyses of microbial populations were performed on the original microflora as well as on the microflora after propagations on the model cheese. The composition of FC12 had changed considerably upon propagation, and in the propagated microflora, the population of yeasts was dominated by *Yarrowia lipolytica* strains, whereas the population of bacteria was dominated by *Vagococcus* species.

2.2 Introduction

Listeria monocytogenes is an important food-borne pathogen that contaminates various categories of foods such as meat, egg products, vegetables, seafood and dairy products (Donnelly, 2001; Farber & Peterkin, 1991; Ryser & Marth, 1999). In the case of dairy products, a high occurrence of *L. monocytogenes* was observed in smear-ripened cheeses (Rudolf & Scherer, 2001). The surface of these types of cheeses is characterised by the development of a viscous, red-orange smear during ripening, composed of bacteria and yeasts. The ripening process of fresh cheese curd starts with the development of yeasts that consume lactose and lactate and produce ammonia (Corsetti *et al*., 2001; Vilioen, 2001. This increases the pH from approximately 5 to 7, which enables the growth of salt-tolerant but less acid-tolerant bacteria, including *L. monocytogenes*. It has been reported that in soft cheeses, *L. monocytogenes* contamination is almost exclusively localised on the surface of the rind (Liu *et al*., 2007; Michard *et al*., 1989; Ryser & Marth, 1987). This

phenomenon appears to be due to a pH effect since a wide pH gradient develops during ripening, with a more acidic pH inside the cheese (Liu *et al*., 2007). In addition, *L. monocytogenes* is more frequently found in high-moisture cheeses (Rudolf & Scherer, 2001; Ryser, 1999).

Numerous studies have revealed the great complexity of microbial populations found on the surface of smear-ripened cheeses (Bockelmann *et al*., 2006; Brennan *et al*., 2002; Corsetti *et al*., 2001; Feurer *et al*., 2004a; Goerges *et al*., 2008; Hoppe-Seyler *et al*., 2000; Larpin *et al*., 2006; Maoz *et al*., 2003; Rea *et al*., 2007). Bacteria that are able to inhibit *L. monocytogenes* have been isolated from various smear-ripened cheeses. They include strains of *Brevibacterium linens* (Eppert *et al*., 1997; Maisnier-Patin & Richard, 1995; Valdés-Stauber & Scherer, 1994), *Enterococcus faecium* (Izquierdo *et al*., 2009), *Microbacterium lacticum* (Carminati *et al*., 1999), *Enterococcus faecalis*, *Arthrobacter ilicis*, *Brevibacterium iodinum* (Carnio *et al*., 1999), as well as *Staphylococcus* species (Bockelmann *et al*., 2006; Ryser *et al*., 1994). The inhibitory potential of yeast strains isolated from soft cheeses was recently studied by Goerges *et al*. (2006) who found *Candida intermedia* and *Kluyveromyces marxianus* strains displaying an antagonistic effect against *L. monocytogenes* in a co-cultivation assay. In some cases, the anti-listerial effect has been attributed to bacteriocins or bacteriocin-like substances (Grattepanche *et al.*, 2008; Maisnier-Patin & Richard, 1995; Valdés-Stauber & Scherer, 1994). However, it is also likely that the competition for nutrients and the production of organic acids and H_2O_2 by the ripening microflora act as antagonistic factors. The activity of single strains may not accurately reflect the anti-listerial activity of the complex microflora from which they were isolated. Crosstalk and quorum sensing of the different bacterial species present on a single cheese might also play an important role, enabling (or disabling) bacteriocin production. Maoz *et al*. (2003) underlined the considerable anti-listerial potential of complex microbial consortia from smear-ripened cheeses. Studies concerning the anti-listerial activity of complex microfloras are therefore of great interest for the dairy industry. However, there is no simple procedure to evaluate the anti-listerial activity of complex microfloras. Mayr *et al*. (2004) evaluated the protective potential of microfloras by monitoring the growth of *Listeria monocytogenes* during a cheese production trial. However, as such experiments are fastidious and time-consuming, they are only appropriate when a limited number of samples are studied. Furthermore, it is not possible to store and to process microfloras from cheese samples without modifying important properties such as the balance between the strains and their physiological state. This

implies that, regardless of the procedure, anti-listerial activity measurements may not fully reflect the activity of the microflora in the cheese from which it originated. However, anti-listerial activity measurements of microfloras may help to identify combinations of strains that limit the growth of *Listeria* strains and, subsequently, to devise new anti-listerial cultures. For that purpose, we compared different procedures to determine the anti-listerial activity of complex microfloras.

2.3 Materials and methods

2.3.1 Strains and growth conditions

Listeria innocua strains WSLC 2340 and WSLC 2543 are from the Weihenstephan *Listeria* Strain Collection (Zentralinstitut für Ernährungs- und Lebensmittelforschung, Freising, Germany), strains NL8 and NL19 are from the LMILA (Laboratoire des Micro-organismes d'Intérêt Laitier et Alimentaire, Caen, France), and strain CIP 107775 is from the CIP (Collection de l'Institut Pasteur, Paris, France). All these strains were originally isolated from cheeses. The yeasts, *Geotrichum candidum* 3E17, *Debaryomyces hansenii* 1L25 and *Yarrowia lipolytica* 1E07, from Livarot cheese, are from the LMILA. These yeasts correspond to the predominant species found in Livarot cheeses. In the present study, they were only used for their pH-increasing activity to determine whether or not contaminations had occurred during curd production (see description in section 2.3.11). Bacterial strains were grown in 50-ml conical flasks containing 10 ml of brain heart infusion broth (BHI; Biokar Diagnostics, Beauvais, France); yeasts were grown in potato dextrose broth (Biokar Diagnostics). Strains were incubated for 48 h at 25 °C on a rotary shaker at 150 rpm. Two successive cultures inoculated at 1:20 were performed before inoculating the cheese curd. All strains were stored at -80 °C after mixing one volume of a 48-h culture with two volumes of a solution containing lactose (10 g/l), sodium glutamate (10 g/l) and glycerol (120 g/l).

2.3.2 Recovery of the microfloras from the surface of smear cheeses

Fourteen French smear-ripened cheeses (FC1 to FC14, from various manufacturers) sold on the retail market, were investigated (Table 1). The surface was scraped off with a sterile knife. The material was homogenised in 100 ml of saline (5 % NaCl) using a Stomacher

Lab-Blender. The cell suspension was centrifuged for 25 min at 8000 × *g* and 4 ℃, and the pellet was washed twice with saline (5 % NaCl) to remove excessive cheese fat. The cells were resuspended in 15 ml of saline (5 % NaCl) and glycerol was added at a final concentration of 15 %. Aliquots were stored at -80 ℃. Cell counts after freezing were determined on plate count agar plates supplemented with 3 % NaCl (PC3+ agar).

Table 1: Overview of the commercial smear-ripened cheeses investigated and antagonistic activities of the supernatants from liquid cultures of the 14 microfloras towards *Listeria* (Method A, see description in Fig. 1). Each experiment was independently replicated three times.

Microflora	Cheese variety	Days before expiry date	Mean *Listeria* concentration after 24 h (log_{10} cfu/ml) ± standard deviation (method A)
FC1	Munster	3	7.40 ± 0.061
FC2	Munster	9	7.13 ± 0.043
FC3	Epoisses	7	9.38 ± 0.031
FC4	Livarot	4	8.19 ± 0.048
FC5	Munster	3	2.44 ± 0.541
FC6	Maroilles	45	7.05 ± 0.514
FC7	Langres	5	9.48 ± 0.018
FC8	Livarot	0	9.52 ± 0.033
FC9	Epoisses	14	9.42 ± 0.014
FC10	Munster	6	8.67 ± 0.401
FC11	Munster	18	7.76 ± 0.006
FC12	Livarot	8	7.41 ± 0.047
FC13	Munster	15	9.54 ± 0.027
FC14	Livarot	14	9.54 ± 0.034

2.3.3 Supernatant measurements (Method A)

In this method (Fig. 1), 500 µl of the microflora glycerol stock cultures were used to inoculate 15 ml of plate count broth (Difco Laboratories, Detroit, MI, USA), which were supplemented with 3 % NaCl (PC3+ broth) to better mimic the conditions of the cheese brines (Valdés-Stauber *et al*., 1991). The flasks, containing an initial cell density of

approximately 3×10^8 cfu/ml, were incubated for 8 h at 30 ℃ on a rotary shaker at 180 rpm. The cells were subsequently removed by centrifugation for 10 min at 4 ℃ and 12.000 × *g*. The supernatants were filter-sterilised (Millex GP 0.2 µm filter units, Millipore, Schwalbach, Germany) and stored at -20 ℃ until use. A cultivation assay was performed to determine the anti-listerial effects of the supernatants. As indicators we used the five *L. innocua* strains. Overnight cultures of the five *L. innocua* strains in BHI broth were diluted to an optical density of 0.1 at 600 nm (OD_{600}) with PC3+ broth and grown to a final OD_{600} of 0.3. The five *Listeria* cultures were then mixed in the same proportions. Five ml of the filter-sterilised supernatant were inoculated with 0.5 ml of the *Listeria* cell suspension (the inoculation level was equivalent to 5×10^7 cfu/ml) and incubated for 24 h at 30 ℃ and 180 rpm. Samples of 100 µl were serially diluted and plated onto PC3+ agar in duplicate. Viable *Listeria* cells were counted after two days of incubation at 30 ℃. The preparation and testing of supernatants was independently replicated three times.

2.3.4 Cheese curd production

The curd used for the model cheese was produced in a single batch. This makes it possible to use the same raw material for all experiments and enables better comparisons (Mounier *et al*., 2008). Curd production (coagulation, cutting, moulding and draining of the curd) was carried out according to a process used for Livarot cheese, under aseptic conditions in a sterilised, 2-m^3 chamber (Leclercq-Perlat *et al*., 1999) The milk used (~100 litres) was standardised at 29 g fat per litre by mixing raw milk and skim milk. After pasteurisation (2.5 min at 74 ℃), it was cooled to 37 ℃, pumped into the coagulation tank and inoculated with the starter culture (Flora Danica CHN13 and CHN15; Chr. Hansen, Arpajon, France). One hundred ml of a filter-sterilised 10 % $CaCl_2$ solution and 30 ml of filter-sterilised coagulant containing 520 mg/l of chymosin were then added. The coagulation time was 20 min; the curds were cut after 30 min of hardening. The curd was then manually stirred for 5 min at a rate of 10 stirs/min. After the curd had set for 15 min, 70 litres of whey were removed prior to moulding. The cheeses were shaped in circular polyurethane moulds with a diameter of 9 cm and a height of 11 cm. They weighed approximately 350 g. The moulds were inverted four times, after 10 min, 2 h, 5 h, and 15.5 h, with a temperature of 20 ℃ in the chamber, and a relative humidity close to 100 %. After 17 h, the cheeses were unmoulded, transferred to sterile bags and the curd was stored at -80 ℃ until use.

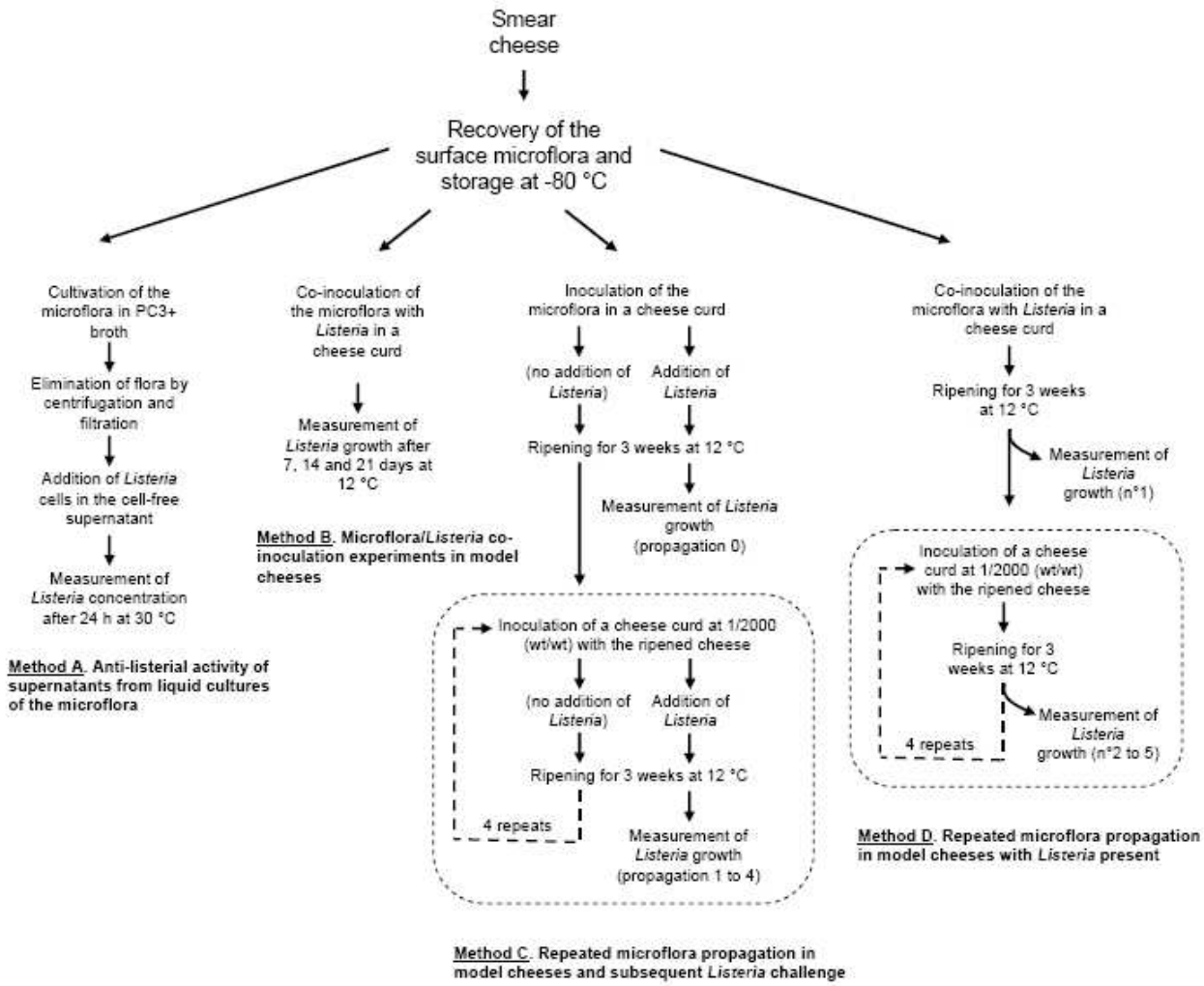

Figure 1. Flow chart of the methods used to measure the anti-listerial activity of microfloras from smear-ripened cheeses. The stages surrounded by a dashed box are replicated four times.

2.3.5 Model cheeses challenged with Listeria *(Method B)*

The curds were thawed by incubation at 4 ℃ for 24 h. Fifty-seven ml of a saline solution (92 g/l NaCl) were added to 246 g of unsalted curd under sterile conditions and mixed four times for 10 s at maximum speed using a Waring blender (Fisher Scientific, Elancourt, France). The microflora stored at -80 ℃ was thawed on ice and added to the cheese curd at a final microbial concentration of 5×10^6 cfu/g. The cultures of the five *Listeria* strains in BHI broth were centrifuged for 10 min at 4 ℃ and 3 000 × *g*, and the cell pellets were resuspended in physiological saline and subsequently added at a final concentration of 1×10^4 cfu/g (2×10^3 cfu/g of each of the five strains). Seventeen grams of inoculated curd

were then transferred onto a grid (5.3 cm in diameter) in sterile crystallising basins (5.6 cm in diameter). These model cheeses were ripened at 12 ℃ and 93 % relative humidity. Samples were taken at 7, 14 and 21 days. All these experiments were done in triplicate.

2.3.6 Repeated microflora propagation in model cheeses and subsequent Listeria *challenge (Method C)*

Curds were inoculated with each microflora as in Method B, but in the absence of *Listeria.* The model cheeses were incubated for three weeks at 12 ℃ and 93 % relative humidity. One gram of ripened cheese was then homogenised in physiological saline and fresh curd was inoculated at a concentration equivalent to 1:2000 (ripened cheese weight/fresh curd weight). This procedure was repeated four times. The anti-listerial activity was measured at the end of each propagation step by performing microflora/*Listeria* co-inoculation experiments in model cheeses in triplicate, as described above for Method B. However, instead of using the original cheese microflora, it consisted of the propagated microflora, inoculated at a concentration equivalent to 1:2000 (ripened cheese weight/fresh curd weight).

2.3.7 Repeated microflora propagation in model cheeses with Listeria *present (Method D)*

The microfloras and the *Listeria* strains were co-inoculated as in Method B, but this time the contaminated cheeses (21 days incubation at 12 ℃) were used to further inoculate subsequent curd batches at a concentration equivalent to 1:2000 (ripened cheese weight/fresh curd weight). No new addition of *Listeria* was performed in the subsequent batches. *Listeria* counts were determined in triplicate after each ripening.

2.3.8 Microbiological analyses

One gram of cheese was thoroughly homogenised using a rolling pin with 22.5 ml of physiological saline in a polyethylene bag. Ten-fold serial dilutions were prepared in physiological saline and plated in triplicate on agar plates. The bacteria were counted on BHI agar supplemented with 50 mg/l amphotericin (Biokar Diagnostics), which inhibits the growth of fungi, after three days of incubation at 25 °C. The lactic acid bacteria from the starter culture (lactococci and leuconostocs) were enumerated on deMan-Rogosa-Sharpe agar (MRS, Biokar Diagnostics) supplemented with 50 mg/l amphotericin after two days of incubation at 30 °C. *Listeria* strains were selectively enumerated on Palcam agar (Biokar Diagnostics) after two days of incubation at 37 °C. Yeasts were counted on yeast extract-glucose-chloramphenicol agar (YGCA, Biokar Diagnostics) supplemented with 0.01 g/l tetrazolium chloride (TTC) after two days of incubation at 25 °C.

2.3.9 Analysis of the microbial composition by Fourier Transform InfraRed (FT-IR) spectroscopy

The composition of the microflora FC12 was studied in detail using FT-IR spectroscopy. This is a spectroscopic technique able to distinguish microorganisms at the strain level (Büchl *et al*., 2008; Oberreuter *et al*., 2002). It is based on infrared absorption of all cellular compounds in the mid-infrared range 4000-500 cm^{-1}, providing a specific fingerprint for each strain when incubated under standardised conditions. A database including the spectra of well-characterised strains is used for identification of an unknown isolate. Colonies from PC3+ agar plates for bacteria or YGCA supplemented with 10 mg/l bromophenol blue (YGCB) for yeasts were selected at random after 3-5 days of aerobic incubation and were streaked twice to confirm their homogeneity. The cells were subsequently transferred to FT-IR agar plates, a tryptic soy agar (TSA, Oxoid, Wesel, Germany) for bacteria, and YGCA (Merck, Darmstadt, Germany) for yeasts. The sample preparation to obtain reliable infrared spectra was carried out as described by Kümmerle *et al*. (1998). Measurements were made with a HTS-XT FT-IR spectrometer (Bruker, Karlsruhe, Germany). For data processing, OPUS version 6 software (Bruker) was used. All FT-IR spectra recorded from bacterial isolates underwent calculation of their second derivative and vector normalisation. A database comprising 3287 spectra of the most important genera was used for a primary identification. When identification results pointed

towards the group of lactic acid bacteria, a sublibrary containing 650 spectra of lactic acid bacteria was used for further identification. When the results indicated the presence of coryneform bacteria, a sublibrary comprising 1577 different spectra of this group was used for further identification. For the identification of yeast isolates, the second derivative, normalised FT-IR spectra were compared with a library containing 3250 yeast spectra. The spectral distances of the isolates, reflecting the similarity of two spectra by comparing the size of non-overlapping areas, were used to discriminate the isolates at the strain level.

2.3.10 Sequencing of the 16S rRNA gene from selected isolates

16S rDNA from selected isolates was amplified using primers pA and pH, as previously described (Monnet *et al*., 2006), and the resulting amplicons were sequenced by Cogenics (Meylan, France). The sequences were then assembled using the CAP2 programme (Huang 1996) and compared to the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) to determine the closest known relatives of the 16S rDNA sequences.

2.3.11 Absence of cheese contamination

Possible contaminants may be introduced during the preparation or inoculation of the cheese curds. Two types of controls were conducted during each trial in order to check for the absence of contamination. In the first type, a curd aliquot was neither inoculated with a microflora nor with *Listeria*. In the second type of control, the curd was only inoculated with a mixture of three de-acidifying yeasts (*Geotrichum candidum* 3E17, *Debaryomyces hansenii* 1L25 and *Yarrowia lipolytica* 1E07). Microorganism growth was checked using BHI-amphotericin, Palcam, MRS-amphotericin and YGCA plates as described above.

2.4 Results

2.4.1 Cheese samples, cheese model selection, contamination control

The microfloras from the surface of 14 different smear-ripened cheeses (Table 1), representing different types of cheeses and produced at different locations, were collected, and their anti-listerial activity was measured according to four different methods. Method A uses microflora supernatants and Methods B, C and D involve a model cheese. We developed a model cheese instead of performing trials with real cheeses in order to be able to analyse large numbers of samples. In preliminary experiments (data not shown) an appropriate model cheese system was established. The optimised model cheese is mainly representative of the surface of cheeses due to the low thickness of the loaf (approximately 1 cm). The increased surface-to-volume ratio favours the development of the surface microflora, which we wanted to examine here.

The control experiments revealed no contamination of the curds. No growth was observed on BHI-amphotericin, palcam and on yeast extract-glucose-chloramphenicol agar. Lactic acid bacteria were detected on MRS-amphotericin. Their presence was due to the addition of the lactic starter during the preparation of the curd. However, their concentration continuously decreased, from approximately 1×10^8 cfu/g on day 0 to 2×10^7 cfu/g at the end of ripening (day 21) of the uninoculated control cheeses. In addition, there was no change in the pH of the cheeses (pH value of the cheese curd was approximately 4.8).When the control curds were inoculated with the three yeast strains to achieve de-acidification, no growth of contaminants was observed using BHI-amphotericin, palcam and MRS-amphotericin agar, although the pH of the curds had increased, as expected, to approx. pH 7.6 after 21 days of ripening.

2.4.2 Supernatant measurements (Method A)

Method A is based on the evaluation of the anti-listerial activity of supernatants from short-time liquid cultures with high cell densities (Fig. 1). The anti-listerial activity varied considerably as a function of the microflora (Table 1). The highest activity corresponded to the microflora FC5, for which the *Listeria* concentration dropped from 5×10^7 cfu/ml (inoculation level) to 2.7×10^2 cfu/ml (2.44 $\log_{10}$ cfu/ml). However, the *Listeria* strains grew to a high concentration in the supernatants from several other microfloras, especially FC3, FC7, FC8, FC9, FC13 and FC14, reaching up to 3.4×10^9 cfu/ml (9.54 $\log_{10}$ cfu/ml) after 24 hours of incubation.

2.4.3 Model cheeses challenged with Listeria *(Method B)*

In Method B, the model cheeses were co-inoculated with a microflora that was directly obtained from the cheeses and a mixture of five *Listeria* strains (Fig. 1). The corresponding pH values and *Listeria* concentrations at day 7, 14 and 21 are reported in Table 2. The pH of the curd before inoculation was close to 4.8 and increased in all experiments due to the activity of the yeasts and bacteria. In all cases, the pH values at day 21 were higher than 7.5. Growth of the *Listeria* strains, which had been inoculated at 1×10^4 cfu/g, was observed in all cases. However, the extent of growth varied considerably as a function of the type of microflora. In fact, at day 21, the *Listeria* concentration varied from 1.5×10^6 for FC1 to 1.9×10^9 cfu/g for FC7, which represents a 1000-fold difference. No additional growth of the *Listeria* strains was observed when the incubation of the model cheeses was extended after 21 days (results not shown), indicating that the concentrations around day 21 represent the maximal growth level in these experiments. The correlation coefficient between the *Listeria* concentration at day 7 (expressed as $\log_{10}$ of cfu/g) and the concentration at day 14 was 0.43, which represents a low correlation. The correlation coefficient between the *Listeria* concentration at day 14 and the concentration at day 21 was 0.90. There was a slight correlation between the pH values and the *Listeria* concentration at day 7 (correlation coefficient of 0.64) and day 14 (correlation coefficient 0.66). However, no significant correlation could be established at day 21 (correlation coefficient: 0.20). In addition, there was no correlation between the pH values at day 7 (or 14) and the *Listeria* concentration at day 21. A second trial using Method B was performed in triplicate after the microfloras FC1, FC3 and FC6 were stored for five months at -80 °C.

There was no major difference in *Listeria* concentration at day 21 compared to the experiments conducted before (differences were less than 40 %), showing that the storage of the microfloras at -80 ℃ did not substantially affect their activity.

Table 2: Overview of the results obtained with Method B. The pH values and growth of *Listeria* at day 7, 14 and 21 in model cheeses co-inoculated with the respective microflora and a mixture of five *Listeria* strains (see Fig. 1) are given. Each number corresponds to the mean of three replications [1].

Microflora	pH			*Listeria* concentration (cfu/g)		
	7 days	14 days	21 days	7 days	14 days	21 days
FC1	6.23	7.64	7.68	1.6×10^4	2.5×10^6	1.5×10^6
FC2	6.05	7.77	7.75	7.0×10^3	2.0×10^7	1.7×10^7
FC3	5.33	7.28	7.80	1.0×10^4	3.3×10^7	1.7×10^8
FC4	6.20	7.56	7.76	1.6×10^4	4.7×10^7	8.7×10^7
FC5	5.93	6.70	7.54	9.5×10^4	5.2×10^5	2.0×10^6
FC6	5.76	6.80	8.00	8.0×10^3	5.7×10^6	6.0×10^6
FC7	6.75	7.47	7.72	1.4×10^6	1.3×10^8	1.9×10^9
FC8	6.20	7.36	7.72	5.3×10^5	4.5×10^7	6.3×10^7
FC9	6.21	7.62	8.03	2.5×10^5	2.1×10^7	5.7×10^7
FC10	5.91	6.33	7.61	2.0×10^3	9.2×10^5	4.9×10^6
FC11	6.09	7.40	7.81	1.4×10^4	9.9×10^6	1.6×10^7
FC12	6.26	7.68	7.74	1.2×10^4	2.4×10^7	4.5×10^7
FC13	5.42	7.12	7.80	5.0×10^3	1.7×10^7	1.6×10^7
FC14	6.46	7.33	7.73	7.6×10^4	4.2×10^7	1.6×10^8

[1] standard deviations for the pH values were lower than 0.1 units, and standard deviations for $\log_{10}$ of *Listeria* concentrations were lower than 0.25 $\log_{10}$ units

2.4.4 Repeated microflora propagation on model cheeses and subsequent Listeria *challenge (Method C)*

In Method C, competition experiments between the microflora and *Listeria* were conducted, similar to Method B, but the microflora was previously sequentially propagated several times on the model cheeses (Fig. 1). There were two objectives for this experiment: firstly, we wanted to know if anti-listerial microfloras can be propagated to conserve their important properties; secondly, we wanted to determine whether such propagation steps improve the adaptation of the microfloras to the model cheese and their anti-listerial activity. Except for FC5, FC6 and FC1, the anti-listerial activities were, in fact, higher after propagation in the model cheeses than in the absence of propagation (Fig. 2). In addition, the anti-listerial activities appeared to be constant from the second propagation on. The correlation coefficient between the growth of *Listeria* (expressed as $\log_{10}$ of cfu/g) at the second and the fourth propagations was 0.92 (Fig. 3), which indicates that only two rounds of adaptation are necessary to achieve stable activity. After the fourth propagation, the highest anti-listerial activity was obtained for the microflora FC12. This high anti-listerial activity was already present after the second propagation (Fig. 2). This propagated microflora was able to limit the growth of *Listeria* to approximately 1.8×10^5 cfu/g of cheese, which corresponds to a growth of about four generations (*Listeria* had been inoculated at 1×10^4 cfu/g).

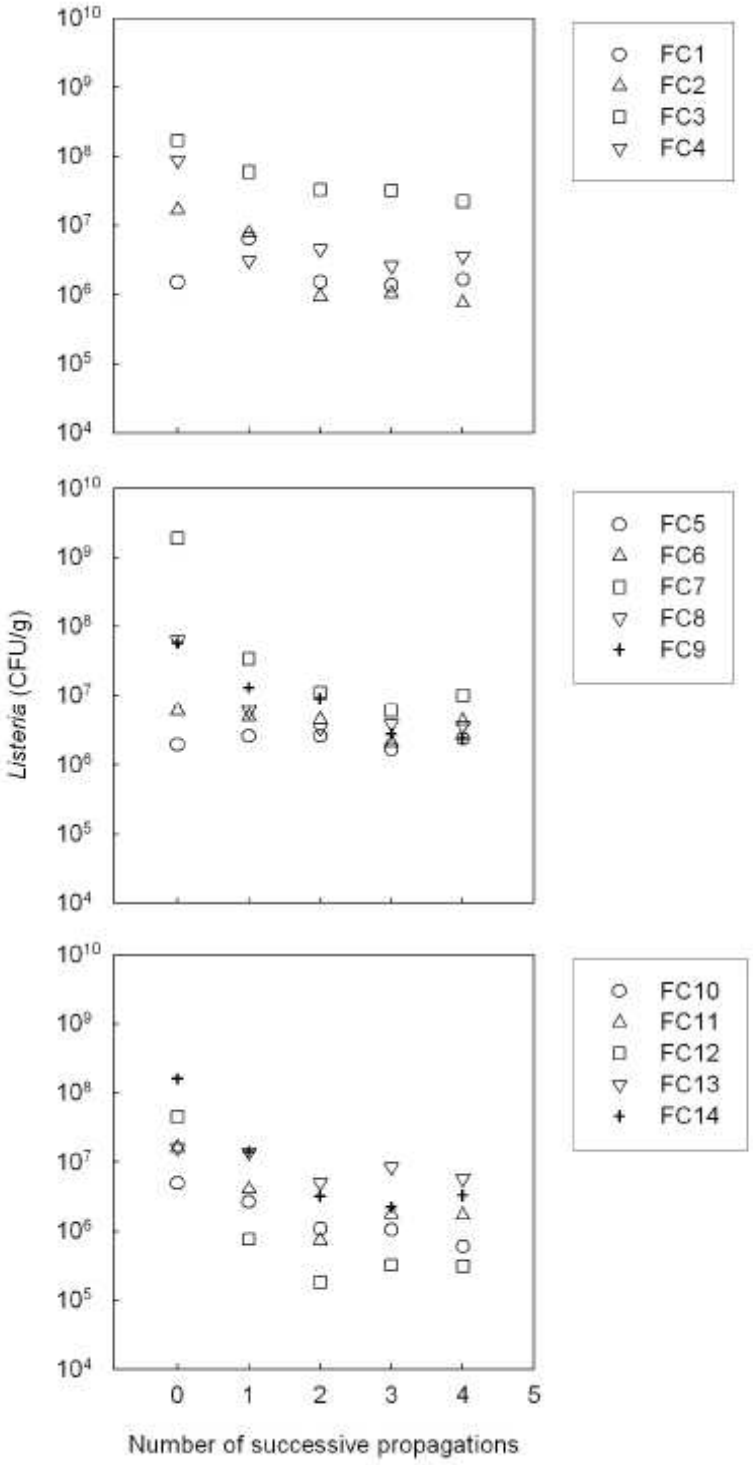

Figure 2. Populations of *Listeria* at day 21 in model cheeses prepared with microfloras from successive propagations according to Method C (see depiction in Fig. 1). The microfloras used were either directly taken from cheese (0) or recovered after performing one to four successive propagations (1-4) in the model cheese. This figure shows the data of one batch of successive propagations for each microflora, but the competition experiments were performed in triplicate. The standard deviations of the triplicates for $\log_{10}$ of *Listeria* concentrations were lower than 0.25 $\log_{10}$ units.

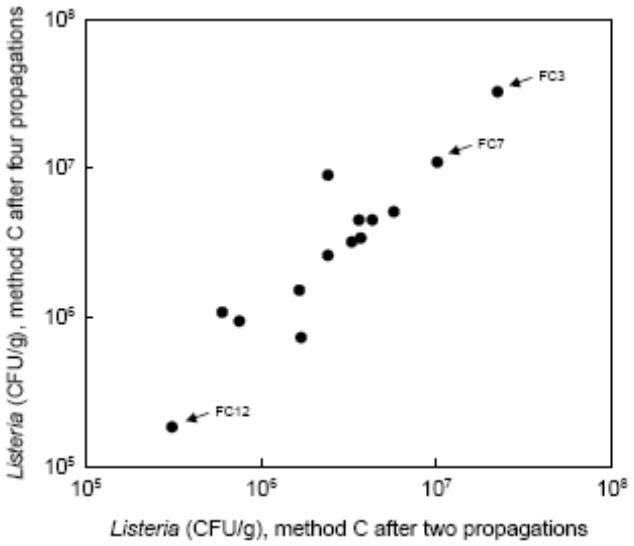

Figure 3. Correlation of the populations of *Listeria* at day 21 in competition experiments according to Method C after two and four propagations. Each point represents one microflora. Some points have been labelled.

2.4.5 Repeated microflora propagation on model cheeses with Listeria *present (Method D)*

Method D, similar to Method C, also involved several rounds of propagations on the model cheeses. However, this time, *Listeria* strains were introduced at the first inoculation and the microflora contaminated with *Listeria* was then propagated. *Listeria* counts were determined after each propagation (Fig. 1). In all cases, a linear decrease of $\log_{10}$ of *Listeria* concentration from the second to the fifth propagation could be observed (Fig. 4). The highest absolute value of the corresponding slope was obtained for FC12 and the lowest for FC13 (Table 3). The steepness of the slope is an indicator of the ability of the microflora to outcompete the *Listeria* strains. The decrease in *Listeria* concentration was 1.42 $\log_{10}$ units per propagation for FC12, whereas it was only 0.14 $\log_{10}$ units for FC13. For FC10 and FC12, no viable *Listeria* cells could be detected after the fifth propagation.

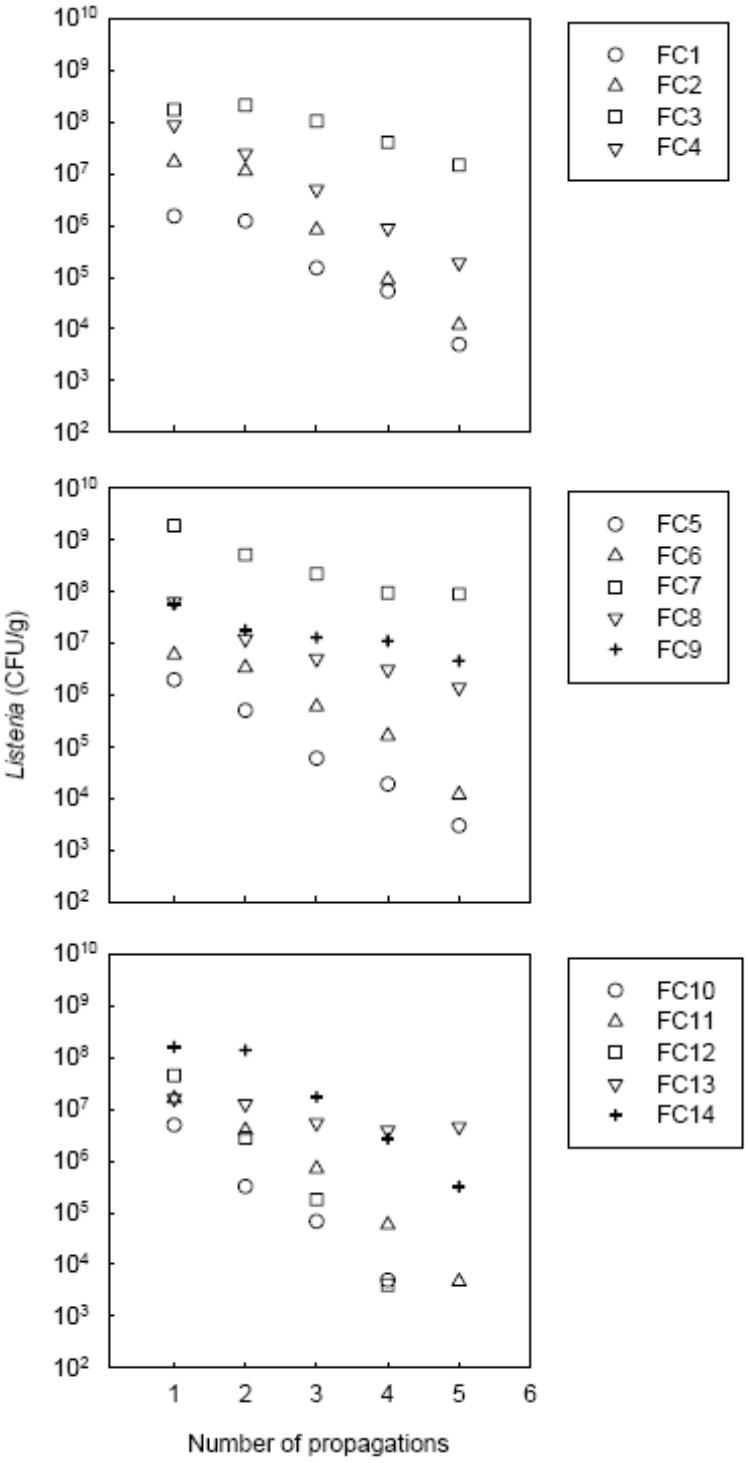

Figure 4. Populations of *Listeria* at day 21 in surface microflora/*Listeria* competition experiments according to Method D after several propagations (see depiction in Fig. 1). The surface microflora was co-inoculated with *Listeria* in model cheeses and propagations were then performed without new inoculation of *Listeria*. The data presented in this figure correspond to one batch of successive propagations per microflora, but the *Listeria* concentration measurements were performed in triplicate. The standard deviations for $\log_{10}$ of *Listeria* concentrations were lower than 0.25 $\log_{10}$ units. Data points for FC10 and FC12 were not presented after five successive propagations because *Listeria* concentrations were below the detection level (<2 × 10^2 cfu/g).

Table 3: Overview of the results for Method D. The values of the slopes of the lines corresponding to log_{10} of *Listeria* concentration *vs.* the number of propagations are given. The slopes were calculated using the data from the second to the fifth propagation (data for the calculation taken from Fig. 4).

Microflora	Slope (log_{10} unit / propagation)	Correlation coefficient	Rank of inhibitory activity
FC1	-0.76	-0.990	7
FC2	-0.99	-0.998	2
FC3	-0.39	-0.997	10
FC4	-0.71	-0.999	9
FC5	-0.72	-0.994	8
FC6	-0.79	-0.990	6
FC7	-0.26	-0.953	12
FC8	-0.30	-0.994	11
FC9	-0.19	-0.944	13
FC10	-0.90[1]	-0.989	4
FC11	-0.98	-0.996	3
FC12	-1.42[1]	-0.996	1
FC13	-0.14	-0.829	14
FC14	-0.87	-0.999	5

[1] slope calculated using the data from the second to the fourth propagation, since no *Listeria* were detected after the fifth propagation.

2.4.6 Comparisons between the four methods

Correlation coefficients between the results of the different methods were calculated. A high value of a correlation coefficient basically indicates that two methods show the same result. The correlation coefficient between the results of Method A (log_{10} of *Listeria* concentration after 24 h incubation in a cell-free supernatant) and Method B (log_{10} of *Listeria* concentration after 21 days of growth in the model cheese) was 0.63. However, it was only 0.35 between Methods A and C (log_{10} of *Listeria* concentration after two successive propagations), and 0.42 between Methods A and D (slope of the line relating log_{10} of *Listeria* concentration to the number of propagations). The correlation coefficient was 0.45 between Methods B and C, 0.34 between Methods B and D, and 0.84 between

Methods C and D. Of those, only correlations A *vs*. B, and C *vs*. D were significant according to a Pearson's correlation analysis ($p<0.05$). Methods A and B did not involve any microflora propagation, whereas Methods C and D, which showed the highest level of correlation, included propagation steps on model cheeses. This correlation is illustrated in Figure 5.

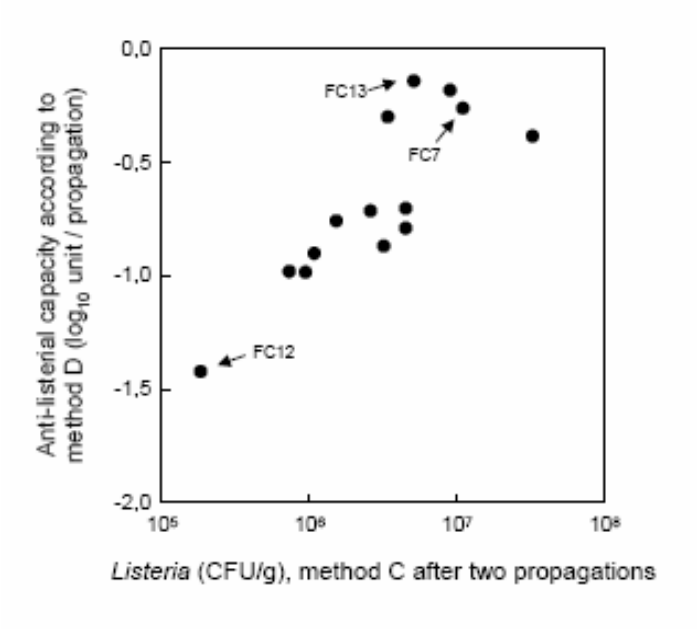

Figure 5. Correlation between results of Methods C and D (see Fig. 1). Slope of the lines relating $\log_{10}$ of *Listeria* concentration to the number of propagations (Method D, data from Fig. 4) as a function of the *Listeria* concentration in competition experiments after performing two propagations of the microfloras (Method C, data from Fig. 2). Each point represents one surface microflora. Some data points have been individually labelled.

2.4.7 Changes of species composition of the anti-listerial microflora FC12 during propagation

The FC12 microflora showed the highest anti-listerial activity in Experiments C and D, but not in Experiments A and B (ranks of 5/14 and of 7/14, respectively). Hence, for the FC12 microflora, the adaptation to the model cheese by successive propagation resulted in a large increase in anti-listerial activity. This was, at first, a promising result, from which we concluded that anti-listerial microfloras can be maintained in the laboratory. To determine a possible reason for the increased anti-listerial activity after propagation, FT-IR analysis was applied on the initial and the propagated FC12 microflora. The initial FC12 microflora

contained three yeast species: *Debaryomyces hansenii*, *Yarrowia lipolytica* and *Geotrichum candidum* (Table 4). Ten groups of bacteria were present, the most numerous being *Corynebacterium*, *Leucobacter* and *Brachybacterium*. After four propagations on the model cheese, the proportion of *Yarrowia lipolytica* among the yeast isolates increased from 49 % to 80 %. The proportion of bacteria from the *Vagococcus-Carnobacterium-Enterococcus* group, which was 3.3 % in the initial microflora, increased to 78.6 % after the fourth propagation. In addition, six of the bacterial groups initially found were no longer detected. To further determine the bacteria from the prominent group, *Vagococcus-Carnobacterium-Enteroccus* (after propagation), 20 isolates were selected at random and their 16S rRNA was sequenced. Fifteen isolates corresponded to *Vagococcus* (98 % identity with GenBank Accession no. AY179329.1), four to *Carnobacterium* (99 % identity with GenBank Accession no. DQ337521.1), and one to *Enterococcus* (99 % identity with GenBank Accession no. EF535229.1).

2.5 Discussion

There is no reference method for the measurement of the anti-listerial activity of complex microfloras at this time for the following reasons. First, it is not possible to recover, to store and to process microfloras from cheese samples without modifying important properties such as the balance between the strains or their physiological state. In addition, the standardised procedures of an anti-listerial activity measurement may not fully reflect the conditions that occurred during the production of the cheeses on which the microfloras developed, especially when diverse samples are investigated. However, despite these limitations, the development of standardised anti-listerial activity measurement procedures is useful because it helps to identify promising combinations of species or strains that limit the growth of *Listeria* and, subsequently, to devise new anti-listerial cultures for cheeses.

One of the main advantages of the methods described in the present study is that numerous samples can be tested simultaneously in small scale laboratory experiments. The experiments were performed with *L. innocua* test strains rather than with *L. monocytogenes*, because the former belong to risk group 1. *Listeria innocua* strains were used in several studies to replace *L. monocytogenes*, which belongs to risk group 2 (Belessi *et al*., 2008; Boucabeille *et al*., 1998; Carminati *et al*., 2000; Giraffa *et al*., 1994; Lauková & Czikková, 2001; Maisnier-Patin & Richard, 1995; Reviriego *et al*., 2007; Rodriguez *et al*., 1998; Ryser *et al*., 1994; Valdés-Stauber & Scherer, 1994). A cocktail of five indicator strains applied simultaneously may also be more effective for a simulation of a contamination since it exhibits a greater challenge to the microflora. Liu *et al*. (2009) showed that *L. innocua* is a suitable indicator for replacing *L. monocytogenes* during ripening of Camembert cheese, and Rudolf and Scherer (2001) observed a similar tendency in the occurrence of *L. monocytogenes* and *L. innocua* in smear-ripened cheeses. Similar occurrences and growth conditions are already convincing arguments for a classification of *L. innocua* as a marker organism for contaminated dairy plants.

Method A is easier to perform than Method B since it is faster and does not involve the production of model cheeses. The level of correlation between these methods was significant but moderate (correlation coefficient: 0.63). This may be explained by the wide dissimilarity of the conditions of the two methods. It is interesting to observe that in experiments where the microflora was propagated several times on the model cheeses before the competition with *Listeria* (Method C), a stable anti-listerial activity was reached

after two propagations and, in most cases, the resulting anti-listerial activity was higher than at the outset. Furthermore, in experiments where the microflora and *Listeria* were inoculated together in the cheese and propagation steps were then performed without new inoculation of *Listeria* (Method D), a stable anti-listerial activity was also observed, as shown by a linear decrease of $\log_{10}$ of the *Listeria* concentration after each propagation. The correlation between these two types of experiments was significant (correlation coefficient 0.84, Fig. 5). Performing such propagation steps may be advantageous. It is, in fact, likely that the propagation steps result in a better adaptation of the composition of the microflora to the model cheeses. In addition, the anti-listerial activity measurements are probably less influenced by the physiological state of the inoculated cells, in contrast to Experiments A and B, where the media are directly inoculated from cells stored at -80 °C.
The *Listeria* inoculation level in the anti-listerial activity measurements involving model cheeses was 10^4 cfu/g, which is probably much higher than the levels which contaminate cheeses in practice. The effect of lower inoculation levels was not investigated in the present study, but it is likely that the relative differences of efficiency between the various microfloras would be comparable when lower inoculation levels are used.
In Experiments C and D, FC12 had the highest anti-listerial activity. It was thus interesting to analyse its microbial composition. In comparison to the FC12 microflora recovered from the cheese and stored at -80 °C, the microflora obtained after four propagations on the model cheese contained a much higher proportion of cells belonging to the yeast species *Yarrowia lipolytica* and to the lactic acid bacteria *Vagococcus-Carnobacterium-Enterococcus*, which then constituted the dominant populations. 16SrRNA gene sequencing showed that *Vagococcus* isolates represented approximately 75 % of the isolates from the *Vagococcus-Carnobacterium-Enterococcus* group. *Yarrowia lipolytica* strains are frequently present in smear-ripened cheeses. They have competitive advantages for growth and dominance in dairy products, such as tolerance to high salt concentrations and low temperatures, lactate assimilation and production of extracellular proteolytic and lipolytic enzymes (Lanciotti *et al*., 2005). Inhibition of *Listeria* by yeasts has been reported by Goerges *et al.* (2006), who screened about 400 yeast strains for their anti-listerial potential. One of the ten *Y. lipolytica* strains examined displayed a slight inhibition of *Listeria*. A similar inhibition might be partly responsible for the *Listeria* inhibition in the propagated FC12 consortium. Contrary to *Yarrowia lipolytica,* the presence of *Vagococcus* strains in cheeses is not well documented. However, they have occasionally been isolated from different types of cheeses (Dolci *et al*., 2008; Mayr *et al*.,

2004). A bacteriocin-producing strain of *Vagococcus carniphilus* has been isolated from dry sausages from a small-scale facility and has been shown to inhibit the growth of *L. innocua*, *Staphylococcus aureus* and *Hafnia alvei* (Ammor *et al*., 2006). The authors suggested using this strain for its antagonistic performances rather than *Enterococcus* species, because the innocuity of *Vagococcus carniphilus* is more recognised. It is also important to note that the original composition of the cheese microflora could not be maintained for FC12 on model cheeses. This result once again highlights the great and poorly understood complexity of smear cheese microfloras. Changes in species composition justify further research since the anti-listerial potential could be improved.

In summary, the present study shows that anti-listerial activities considerably vary as a function of the microflora. Even if it is not possible to ensure that the anti-listerial activity measurements of microfloras recovered from cheeses reflect the anti-listerial potential during the manufacturing of the corresponding cheeses, it is of great interest to perform such experiments, which make it possible to identify combinations of strains that limit the growth of *Listeria*.

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3 High biodiversity and potent anti-listerial action of complex red smear cheese microbial ripening consortia

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Keywords: *Listeria monocytogenes*, red smear cheese, bactericidal activity, biodiversity, bacteriocin genes, lactic acid bacteria

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3.1 Abstract

When *Listeria monocytogenes* EGDe (serovar 1/2a) was cultivated in cell-free supernatants prepared from red smear cheese microbial ripening consortia grown for eight hours in liquid medium, eight out of 49 supernatants exhibited a bactericidal activity, sometimes even reducing the inoculum from 5×10^7 cfu/ml *L. monocytogenes* to zero after 24 hours of incubation. Another five consortia displayed a bacteriostatic capacity. No inhibition in supernatants was observed when the complex consortia were incubated for a ten-minute period only, indicating that the activity depends on active growing consortia. While the consortia displayed an extraordinarily high biodiversity (Simpson's diversity index up to 0.97), biodiversity did not correlate with anti-listerial activity. There was no obvious similarity between the anti-listerial consortia studied, and no general difference in comparison to non-inhibitory communities. The proportion of lactic acid bacteria (LAB) in the consortia ranged between 3 and 45 %. Therefore, 23 different LAB bacteriocin genes were assayed using specific PCR primers, identifying one to five bacteriocin genes in several consortia. In situ transcription of lactococcin G mRNA on the cheese surface was demonstrated by RT-PCR in five samples but these displayed no anti-listerial activity. Supernatants subjected to thermal and enzymatic treatment suggested the presence of heat-stable, non proteinaceous molecules as well as heat-labile compounds which are sensitive to proteolytic digestion. Probably, substances other than LAB bacteriocins are responsible for the anti-listerial action of some supernatants.

3.2 Introduction

Bacterial surface-ripened cheeses have a long tradition in Germany, Austria, France, Italy and Switzerland. Brands like Munster, Limburger, Romadur and Appenzeller are artisanal high quality foods. Microbial consortia dwelling on the surface of the cheese ("smear") consist of coryneform bacteria, micrococci, staphylococci and yeasts and contribute to cheese ripening by the production of proteolytic and lipolytic enzymes as well as aroma compounds (Bockelmann and Hoppe-Seyler, 2001; Brennan *et al.*, 2004). The smear is applied repeatedly by brushing, thereby beginning with the mature ("old") cheeses followed by the freshly produced ("young") cheeses. Old-young-smearing provides an inexpensive

technique to propagate these complex surface consortia and ensures the plant specific uniqueness of the product. However, the risk of spreading contaminating microorganisms by this procedure is a disadvantage of this approach (Little *et al.*, 2008; Loncarevic *et al.*, 1998).

Listeria monocytogenes, the causative agent of human listeriosis, is a Gram positive, non-sporeforming bacterium characterized by a remarkable resistance to environmental stresses (Gandhi and Chikindas, 2007). Its psychrophilic, acid- and halotolerant, facultatively anaerobic character allows the pathogen to overcome many common approaches of food preservation. Contrary to most other pathogens targeting the gastrointestinal tract, infections with *L. monocytogenes* may not be self-limiting. The clinical symptoms of human listeriosis mainly manifest in infections of the central nervous system (meningitis and encephalitis), bacteremia and endocarditis (Lecuit, 2005). The incidence of listeriosis in Europe is low but the mortality rate can reach 25-30 % among persons developing the disease (de Valk *et al.*, 2005).

The food-borne nature of *L. monocytogenes* was recognized not before the 1980s, when a number of listeriosis outbreaks were linked to the consumption of contaminated soft cheeses (Farber and Peterkin, 1991). The production and ripening parameters of salty red smear cheeses provide excellent growth conditions for undesirable microorganisms. As a consequence, smear cheeses are more frequently contaminated with *L. monocytogenes* than other soft cheeses, showing exceedingly high cell counts in some products (Jacquet *et al.*, 1993; Loncarevic *et al.*, 1995; Rudolf and Scherer, 2001). Therefore, there is considerable interest to develop microbial ripening consortia which display anti-listerial activity (Carnio *et al.*, 2000; Eppert *et al.*, 1997; Loessner *et al.*, 2003). Undefined cheese surface consortia with anti-listerial properties have been described in the past, sometimes containing bacteria producing bacteriocins (Saubusse *et al.*, 2007; Valdes-Stauber and Scherer, 1994). Bacteriocins are small, ribosomally synthesized peptides usually acting on the cytoplasmic membrane of target bacteria, thereby causing cell lysis (for review see Cotter *et al.*, 2005; Jack *et al.*, 1995). These findings raised hope that indigenous smear bacteria may serve as protective cultures to improve the microbial quality of these products (McAuliffe *et al.*, 1999; O'Sullivan *et al.*, 2006). Furthermore, unspecific and most likely synergistic inhibitory effects have been attributed to stable microbial consortia, preventing the establishment of undesired bacteria (Guillier *et al.*, 2008; Saubusse *et al.*, 2007).

The present study gives a survey over complex cheese microbial communities and their potential anti-listerial properties, reports on their biodiversity and explores the presence and transcription of 23 bacteriocin genes.

3.3 Materials and methods

3.3.1 Collection of microbial consortia

Smear cheeses produced in France, Italy and Germany were bought on the retail market or provided by Unité de Recherches Fromagères (INRA, Aurillac, France). The surface flora was recovered using a sterile knife and homogenized in saline solution containing 5 % NaCl using a Stomacher Lab-Blender. Suspensions were centrifuged at 6,600 × *g* and washed twice in saline solution to remove excessive cheese fat. After addition of glycerol to the cells in a final concentration of 15 %, aliquots were frozen at -80 °C. All samples underwent routine analysis according to DIN EN ISO 11290-1 (1997) to detect natural contamination with *Listeria* sp. (see table S1).

3.3.2 Supernatant preparation

500 µl of the thawed smear were used to inoculate 15 ml of Plate Count broth supplemented with 3 % sodium chloride (PC3+ broth, initial cell density was approx. 3×10^8 cfu/ml) to mimic the conditions of cheese brines (Valdes-Stauber *et al.*, 1991). The flasks were incubated for ten minutes or eight hours at 30 °C and 180 rpm. The cells were pelleted by centrifugation at 12,000 × *g*. The supernatants were filter-sterilized (Millex GP 0.2 µm filter units, Millipore, Schwalbach, Germany) and stored at -20 °C until use. The preparation of cell-free supernatants (CFS) was carried out at least three times independently. The pH values of the CFS were not adjusted to avoid possible inactivation of unknown compounds (Hütt *et al.*, 2006). The pH was measured after freezing using universal indicator strips (Macherey-Nagel, Düren, Germany), the content of lactic acid and hydrogen peroxide was determined using the analytical systems of Merck (Darmstadt, Germany). The enzymes lactate dehydrogenase and peroxidase, respectively, are immobilized on strips allowing quantification via a colorimetric reaction. Bacteriophage involvement was excluded by monitoring plaque formation in poured plates using soft agar seeded with 10^8 cfu/ ml *L. monocytogenes* mixed with CFS (data not shown).

3.3.3 Detection of anti-listerial activity in CFS

An overnight culture of *L. monocytogenes* EGDe WSLC 1993 from the Weihenstephan *Listeria* Collection WSLC (serovar 1/2 a) in Brain Heart Infusion broth (BHI, Merck) was diluted to an optical density of 0.1 at 600 nm (OD_{600}) with PC3+ broth and grown to a final OD_{600} of 0.3. Five ml of the CFS were inoculated with 0.5 ml of the cell suspension (the inoculation level was equivalent to 5×10^7 cfu/ml) and incubated for 24 h at 30 ℃ and 180 rpm. Samples of 100 µl were serially diluted and plated onto PC3+ agar in duplicate. Viable *Listeria* cells were counted after two days of incubation at 30 ℃ (summarized in table S1). In challenge experiments, CFS were subjected to dilution 1:10 in PC3+ broth and to 100 ℃ for 5 min. Sensitivity towards proteo lytic digest was checked by adding proteinase K in a final concentration of 0.5 mg/ml (30 U/mg, Merck) and incubation for 2 h at 50 ℃. The enzyme was heat inactivated for 30 mi n at 75 ℃ prior to an inoculation with *Listeria* (table 1).

3.3.4 Determination of biodiversity

The composition of six surface consortia was studied in detail using Fourier Transform-Infra Red (FT-IR) spectroscopy (Wenning *et al.*, 2008). Appropriate dilutions of the thawed smear were plated onto PC3+ agar plates. After 3-5 days of aerobic incubation at 30 ℃, 100 to 250 bacterial colonies were selected randomly for identification. The sample preparation to obtain reliable infrared spectra was carried out as described (Kümmerle *et al.*, 1998; Oberreuter *et al.*, 2002). Two independent measurements were conducted using a HTS-XT FT-IR spectrometer (Bruker, Karlsruhe, Germany). For data processing, OPUS software v. 6 (Bruker) was used. The spectral distances of the isolates, reflecting the similarity of two spectra by comparing the size of non-overlapping areas, were used to discriminate the isolates at the strain level. A subsequent cluster analysis was performed to visualize the abundance ratio of strains resulting in a dendrogram (average linkage algorithm). The diversity, taking into account both richness and evenness of the flora, was estimated according to the following equation

$$SID = 1 - \frac{\sum_{i=1}^{S} ni(ni-1)}{N(N-1)},$$

where N = total number of isolates analysed and n_i = number of isolates belonging to one strain, and S = total number of different strains in a consortium (Simpson, 1949). Simpson's Index of Diversity $SID = 1 - D$ was calculated.

3.3.5 Detection of bacteriocin genes

Genomic DNA of consortia 1-25 was extracted according to the Rotiphenol protocol (Roth, Karlsruhe, Germany), following the recommendations of the manufacturer. 500 µl of thawed smear were mixed with 1 ml Rotiphenol. After a proteinase K treatment for digestion of disturbing proteins, the enzyme was heat inactivated. PCR reactions were performed using specific primer pairs for 23 different bacteriocin genes, listed in table S2 (oligonucleotide primers purchased from Invitrogen, Karlsruhe, Germany). PCR amplification was carried out in a 50 µl reaction mixture containing 1 pmol of specific primers, 200 µM dNTP mixture, 1.5 mM $MgCl_2$, 1 U Thermoprime Plus Polymerase (Thermo Scientific) and 100 ng of template. PCR conditions were as described recently (see table S2). DNA extracts of bacteriocin producing strains, listed in table S3, served as positive controls. Amplified fragments were resolved by electrophoresis on 2 % agarose gels and visualized by staining with ethidium bromide. Resulting bands were excised and sequenced for verification. For a summary of all positive amplification reactions see table 2.

3.3.6 RNA extraction and reverse transcription

Total RNA of consortia tested positive in the above approach was extracted directly from the thawed smear using Trizol (Invitrogen, Karlsruhe, Germany), following the instructions of the manufacturer. Reverse transcription was performed on extracts standardized to 1 µg RNA by spectroscopic measurements at 260 nm (Nanodrop ND-1000, Peqlab, Erlangen, Germany). The reaction volume of 20 µl contained DNase digested RNA (TURBO DNase, Ambion, Darmstadt, Germany), dNTP mixture (500 µM), DDT (5 mM), RNase OUT (40 U, Invitrogen) and first strand buffer. 1 pmol of reverse primers was added for specific reverse transcription. Samples were completed by the addition of Superscript III (200 U,

Invitrogen); in blank samples no enzyme was added. The incubation conditions for the RT reaction were as follows: 25 ℃ for 5 min, 50 ℃ fo r 90 min and 70 ℃ for 15 min. The amplification reactions using cDNA as template were carried out as described for the gDNA samples. No PCR amplification could be achieved when RNA samples did not undergo reverse transcription (blank samples) or when water was added instead of template (blind samples).

3.4 Results and discussion

3.4.1 Anti-listerial action depends on actively growing consortia

When 5×10^7 cfu/ml *L. monocytogenes* EGDe (serovar 1/2a) were inoculated in the cell-free supernatants, eight consortia (16 %) displayed a bactericidal effect and another five (10 %) acted bacteriostatically (table S1). No inhibition was detected when CFS were prepared from microbial consortia cultured only for a ten-minute period (data not shown). To the contrary, growth of *Listeria* was often stimulated in this case. It is concluded that, in the frozen smear, no antagonistic substance is present which can be washed off the cells. Actively growing consortia are necessary to produce inhibitory substances. Inhibition of the CFS was generally abolished upon 1:10 dilution in PC3+ (data not shown). The pH values and the concentrations of lactic acid and H_2O_2 determined in anti-listerial CFS (table 1) were not considered to be listericidal (Gonzalez-Fandos and Dominguez, 2006; Romanova *et al.*, 2002, compare table 1). Therefore, substances other than lactic acid or hydrogen peroxide must be active. In challenge experiments, the supernatants were heated and subjected to proteolytic digest (table 1). The cell counts of viable *Listeria* in challenged CFS suggest the presence of either heat-stable, non proteinaceous molecules (CFS 5a, 6 and 11a) or heat-labile compounds sensitive to proteinase K.

Table 1: Characteristics and activity of CFS against *Listeria monocytogenes* EGDe. The concentration of hydrogen peroxide was below 15 µM in all supernatants tested.

Number of consortium	Variety of cheese	Cell-free supernatant (CFS)		number of viable *Listeria* after 24 h in CFS [cfu/ml]		
		pH	lactic acid [mM]	untreated [a]	5 min 100 °C [b]	proteinase K [c]
5a	Maroilles	6.0	5.5	0	0	0
6	Munster	6.0	4.6	4.2E+03	0	0
7	Munster	5.5	6.1	2.5E+03	3.3E+06	1.5E+07
9	Maroilles	6.0	3.4	1.9E+02	3.5E+06	8.7E+06
11a	Munster	5.5	8.4	0	0	0
15a	Maroilles	6.0	5.4	0	1.6E+07	0
20	Maroilles	6.0	3.9	0	9.6E+05	4.4E+06
21	Epoisses	5.5	3.2	3.0E+07	5.1E+07	3.0E+07
22	Munster	6.0	2.7	6.8E+06	5.1E+07	6.5E+06
23	Livarot	6.0	3.5	3.3E+06	2.5E+07	1.4E+07
24	Munster	6.0	4.4	0	9.8E+06	2.0E+06
11b	Munster	5.7	n.d.	2.3E+08	n.d.	n.d.
39b	St. Nectaire	6.0	n.d.	2.5E+09	n.d.	n.d.

[a] = average number, calculated from three independent trials (see also table S1)
[b] = absolute number, performed only once
[c] = average number, calculated from two independent trials
n.d. = not determined

3.4.2 Biodiversity of microbial consortia

Since only a fraction of the microbial consortia inhibited *Listeria*, the anti-listerial activity observed is not just a property of a complex microbial community due to general species competition, but depends critically on the species composition of a cheese microbial community. Therefore, in-depths taxonomical analyses were conducted using FT-IR spectroscopy. This technique has been applied successfully to analyse cheese surface floras even to the strain level (Larpin *et al.*, 2006; Wenning *et al.*, 2006). Cluster analysis allows grouping of identical isolates for comparison of complex bacterial communities. Four anti-listerial and two non-inhibitory consortia were investigated (table 1), revealing each flora having unique properties in terms of species composition and diversity (table 3). The diversity index of the consortia was calculated according to Simpson, yielding values between 0.88 and 0.97 (compare table 3).

Table 3: Species composition and biodiversity of six microbial ripening consortia. Species proportions are presented in percent of the total number of isolates analysed; numbers in brackets indicate number of strains of a given species / species group.

consortia		**6** [a]	**11a** [a]	**23** [a]	**24** [a]	**25** [b]	**39b** [b]
Number of isolates analysed		100	100	250	125	250	100
Coryneform bacteria	*Arthrobacter arilaitensis/ nicotianae/ protophormiae*	20 (9)	45 (9)	3 (4)	46 (19)	46 (9)	13 (10)
	Corynebacterium ammoniagenes/ casei/ variabilis	17 (7)	-	47.5 (7)	9 (4)	-	2 (2)
	Leucobacter komagatae	-	-	16 (2)	-	-	1 (1)
	Brachybacterium arcticum/ paraconglomeratum/ tyrofermentans	-	-	15 (7)	1 (1)	1 (1)	11 (3)
	Brevibacterium linens	1 (1)	1 (1)	2 (3)	-	5 (4)	20 (11)
	Microbacterium gubbeenense	2 (2)	-	5 (5)	-	-	1 (1)
	Staphylococcus equorum/ xylosus/ saprophyticus/ vitulinus	2 (1)	15 (1)	2 (2)	13 (6)	-	8 (6)
	Micrococcus luteus	1 (1)	-	-	-	-	-
	∑	**43**	**61**	**90.5**	**69**	**52**	**56**
Lactic acid bacteria	*Vagococcus carniphilus/ salmoninarum/ fluvialis*	22 (5)	6 (4)	1 (3)	6 (3)	-	3 (1)
	Marinilactibacillus psychrotolerans	16 (9)	-	2 (1)	16 (6)	16 (2)	4 (1)
	Enterococcus spec.	4 (3)	15 (7)	-	-	-	8 (4)
	Lactococcus lactis	3 (2)	7 (1)	-	1 (1)	-	2 (1)
	Lactobacillus spec.	-	1 (1)	-	-	-	1 (1)
	∑	**45**	**29**	**3**	**23**	**16**	**18**
Gram negative bacteria	pseudomonads	2 (1)	8 (2)	4	7 (5)	31 (5)	6 (4)
	enterobacteria	10 (7)	2 (1)	2	1 (1)	1 (3)	17 (5)
	others (ß-proteobacteria, γ-proteobacteria)	-	-	0.5 (1)	-	-	3 (2)
	∑	**12**	**10**	**6.5**	**8**	**32**	**26**
Total number of strains	∑	**48**	**27**	**41**	**46**	**24**	**53**
Simpson's index of diversity		**0.97**	**0.92**	**0.90**	**0.96**	**0.88**	**0.97**

[a] = anti-listerial, [b] =non-inhibitory

As an example, the species composition of consortium 11a, having the lowest biodiversity and being anti-listerial, and consortium 39b, displaying the highest biodiversity but no anti-listerial activity, is shown in figure 1 for comparison. The non-inhibitory consortium 25 showed a low diversity (the flora consisted of only 24 strains) with a high amount of Gram negative bacteria (32 % of all isolates). Consortium 39b, on the other hand, was not anti-listerial although the number of strains reported (53) was the highest for all consortia. Gram negative bacteria accounted for 26 % of all isolates in this flora. In the anti-listerial consortia 6, 11a, 23 and 24, Gram negative bacteria were less frequently detected (between 6.5 and 12 % of all isolates). There was no obvious correlation between biodiversity or species composition on the one hand and anti-listerial potential at the other hand.

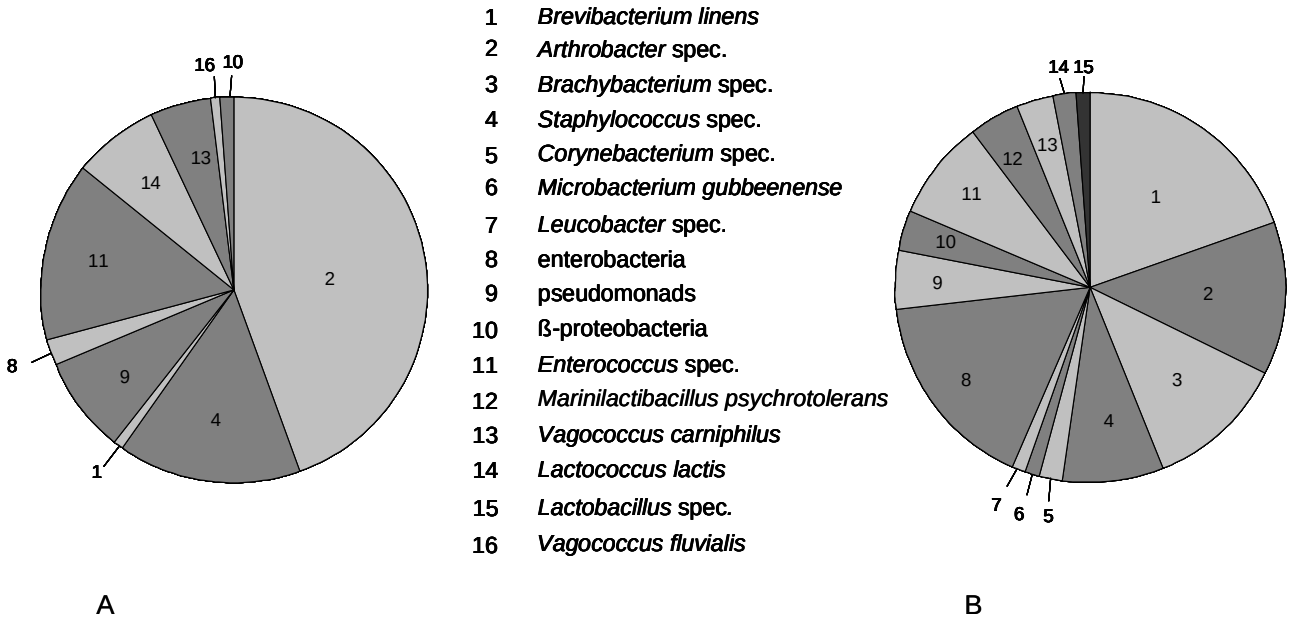

Figure 1. Biodiversity at the species level of the anti-listerial consortium 11a (A) and the non-inhibitory consortium 39b (B), when 100 isolates were randomly analysed.

Several ripening consortia, differing in their species mixture and also in terms of diversity, have been analysed in the past. A low diversity and high temporal stability was reported by Goerges *et al.* (2008) for a Limburger consortium; a high species diversity was reported by Feurer *et al.* (2004a) and Rea *et al.* (2007) for a French and an Irish smear cheese variety, respectively. There is no evidence that uncultivable organisms contribute significantly to such ripening consortia (Carnio *et al.*, 1999), but the number of subdominant species

detected and hence the diversity index may be larger when molecular techniques for species detection in situ are used (Feurer *et al.*, 2004a). In recent studies, flora analysis using strain typing methods revealed species were often present as single clones (Brennan *et al.*, 2002; Mounier *et al.*, 2005). This was not the case for the bacterial communities examined in this work. An unexpectedly high fraction of lactic acid bacteria (LAB) was detected in consortium 6 (45 %, nineteen different strains), followed by consortium 11 (29 %, 13 strains) and consortium 24 (23 %, 10 strains). While this may partly be due to some carry over of LAB from the cheese curd during sampling of the microbial consortia, it should be noted that the majority of the lactic acid bacteria detected are not members of starter cultures typically used for cheese production.

3.4.3 Presence of bacteriocin genes in RNA and DNA extracts

Three of the four anti-listerial consortia comprised a high proportion of LAB (table 3). The ability of these organisms to produce bacteriocins in situ has been exploited to confer microbial safety to red smear cheeses (Izquierdo *et al.*, 2009; Laukova *et al.*, 2001). As shown by Loessner *et al.* (2003), the addition of *Lactobacillus plantarum* ALC01 culture supernatants containing pediocin resulted in a complete eradication of *L. monocytogenes* from the surface of soft cheeses. These findings encouraged us to investigate the presence of bacteriocin genes in the cheese surface communities. The presence of 23 bacteriocin genes typically produced by *Lactobacillus*, *Lactococcus* and *Enterococcus* species was explored in a PCR based screening of 25 consortia. First, genomic DNA, extracted directly from the thawed smear, was used as a template. Nine consortia yielded positive results for one bacteriocin, five consortia for two bacteriocins and another five for three or more bacteriocin genes. The eight bacteriocins identified comprised classes I, IIa, IIb, IIc and III according to the classification of Drider *et al.*, 2006 (table 2). Only six consortia were tested negative for all 23 primer pairs applied. As recently shown by Trmčić *et al.* (2008), the microbial consortia of two Slovenian raw milk cheeses harboured three to nine bacteriocin genes. Similar to this study, enterocins A and B as well as cytolysin were commonly detected. In contrast, the predominant genetic determinant plantaricin A could not be amplified in the current study.

Table 2: Presence of bacteriocin genes as determined by PCR, using genomic DNA extracted from consortia 1-25 as a template. Amplification products were sequenced for verification.

consortium	enterocin A	enterocin B	cytolysin	lactococcin G	helveticin J	curvacin A	acidocin B	nisin
1	+	-	-	+/-	-	-	-	-
2	-	-	-	+	+	-	-	-
3	-	-	-	+/-	+	-	-	-
4	+	+	+	+	-	-	-	-
5a	+	-	-	+/-	-	-	-	-
6	-	-	-	+	+	+	-	-
7	-	-	+	+/-	-	+	-	-
8	+	-	-	+/-	-	-	-	-
11a	-	-	+	+/-	+	+	-	-
12	-	-	+	+/-	-	-	+	-
13	-	-	-	+/-	-	-	+	-
14	-	-	+	-	-	-	-	-
15a	-	-	-	+/-	+	-	-	-
17	-	-	-	+/-	+	-	+	-
18	-	-	-	+/-	-	+	-	+
19	-	-	-	-	+	-	-	-
22	-	-	-	+/-	+	-	-	-
23	+	+	+	+	-	-	+	-
24	-	-	+	+/-	+	-	+	-

+ amplification positive, +/- weak bands, - no amplification product

Second, for samples tested positive in genomic DNA, the presence of bacteriocin mRNA in the smear was assayed. Amplification using cDNA was successful for only one of the eight genes investigated, i.e., lactococcin G. Products were obtained for five samples (consortia 2, 4, 7, 11a and 12) and could be verified by sequencing. To our knowledge, this is the first time that in situ transcription of bacteriocins on a cheese surface is demonstrated. In other studies, bacteriocin activity in the food matrix was tested indirectly (Ryan *et al.*, 1996 for lacticin 3147; Benech *et al.*, 2002 for nisin and Foulquié-Moreno *et al.*, 2003 for enterocin). Lactococcin G is produced by *Lactococcus lactis* LMG 2081 and displays a narrow inhibitory spectrum, acting only against other lactococci and *Clostridium* (Nissen-Meyer *et al.*, 1992), but not against *Listeria*. Other bacteriocins are either not expressed on cheese surfaces or DNA from dead cells was amplified in the first approach. Eventually, mRNA

species of bacteriocin genes detected in the DNA were degraded during the storage of the consortia at -80 ℃.

No bacteriocin gene amplification was achieved using reversed transcribed RNA, which had been extracted after eight hours of incubation in liquid medium, was used as a template (data not shown). The regulation of bacteriocin expression is often mediated via a quorum-sensing mechanism involving peptide pheromones (Kleerebezem, 2004). Upon cultivation in broth, cells were diluted 1:30 and the external stimulus for bacteriocin production may thus have been eliminated and bacteriocin gene expression was down regulated.

Thus, we have no indication yet that the anti-listerial activity reported in this study is due to one or more of the 23 LAB bacteriocin genes investigated. It may either be due to unknown or non-investigated LAB bacteriocins or to inhibitory substances produced by coryneform bacteria (Maisnier-Patin and Richard, 1995; Valdes-Stauber and Scherer, 1994) or Gram negative bacteria.

3.5 Concluding remarks

In eight out of 49 complex microbial consortia from the surface of red smear cheeses the potential to produce highly potent anti-listerial substances was demonstrated. Challenge experiments revealed the participation of proteinaceous molecules but also heat-stable, most likely non-peptide substances, but their chemical nature remains to be determined. Intrinsic microbial food protection, often mediated by natural bacteriocinogenic starter cultures, becomes more and more important due to an increasing consumer's awareness of genetically modified organisms and chemical food preservatives. However, the development of resistances to single bacteriocins is a major concern in bacteriocin-based strategies for food preservation. Therefore, complex indigenous microbial ripening consortia, harbouring several producers of inhibitory substances or exhibiting unspecific inhibitory activity, are a promising approach to develop an innate anti-listerial capacity while preventing the evolution of resistant pathogenic or spoilage bacteria. However, due to the enormous biodiversity of such consortia, there is still a long way to go in order to understand the chemical basis of anti-listerial activity as well as the temporal stability of the species composition and anti-listerial action.

3.6 Acknowledgements

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3.7 Supplementary material

Table S1: Source of 49 cheese surface consortia and activity of CFS against *Listeria* in three independent measurements. Samples indicated with an asterisk revealed natural contamination with *Listeria* sp. in routine analysis.

					surviving cells of *L. monocytogenes* EGDe after 24 h		
no.	**name of cheese**	**source**	**days to expiry date**	**number of cheeses pooled**	**first trial**	**second trial**	**third trial**
1	Langres Germain Boites FR 52.092.01 CE	French retail market	5	4 x 180 g	1.45E+09	2.01E+09	3.40E+09
2	Epoisses Berthaut F 21.247.01 CEE	French retail market	10	3 x 250 g	5.00E+08	8.75E+08	7.41E+08
3	Livarot FR 14.371.01 CE	French retail market	-21	2 x 500 g	9.27E+07	7.82E+07	5.89E+07
4	Palet de Bourgogne F 21.110.01 CEE	French retail market	0	2 x 125 g	1.63E+09	2.13E+09	2.35E+09
5	Maroilles Le Royal F 02.269.01 CEE	French retail market	30	760 g	0.00E+00	0.00E+00	0.00E+00
5b	Maroilles Le Royal, second batch F 02.269.01 CEE	French retail market	35	2 x 760 g	2.28E+09	2.60E+09	2.36E+09
6	Munster Géromé Frech F 67.216.01 CEE	French retail market	3	750 g	1.27E+04	0.00E+00	0.00E+00
7	Munster Géromé Fermier F 02.269.011 CEE	French retail market	15	750 g	0.00E+00	0.00E+00	7.36E+03
8	Vieux Gris F 02.269.01 CEE	French retail market	30	750 g	1.65E+09	1.46E+09	1.82E+09
9	Maroilles Lesire F 02.495.01 CEE	French retail market	42	2 x 375 g	0.00E+00	0.00E+00	5.55E+02
10	Vieux Lille Fauquet F 02.558.02 CE	French retail market	17	3 x 200 g	1.94E+09	2.08E+09	2.62E+09
11a	Petit Munster Ermitage, F 88.079.01 CE	French retail market	6	3 x 200 g	0.00E+00	0.00E+00	0.00E+00

11b	Petit Munster Ermitage, second batch F 88.079.01 CE	French retail market	20	6 x 200 g	3.40E+07	4.19E+08	2.23E+08
11c	Petit Munster Ermitage, third batch F 88.079.01 CE	French retail market	6	5 x 200 g	0.00E+00	0.00E+00	0.00E+00
12	Epoisses Lincet Cave F 52.092.01 CEE	French retail market	14	3 x 250 g	7.56E+08	1.51E+09	1.26E+09
13	Petit Munster Lisbeth FR 57.060.01 CE	French retail market	15	3 x 200 g	2.69E+09	3.39E+09	3.13E+09
14	Le Paillou F 52.092.01 CEE	French retail market	7	4 x 150 g	1.74E+09	2.22E+09	2.30E+09
15a	Maroilles Fauquet F 02.558.02 CE	French retail market	60	750 g	0.00E+00	0.00E+00	0.00E+00
15b	Maroilles Fauquet, second batch F 02.558.02 CE	French retail market	33	2 x 750 g	0.00E+00	0.00E+00	0.00E+00
15c	Maroilles Fauquet, third batch F 02.558.02 CE	French retail market	55	27 x 750 g	1.05E+03	3.51E+04	1.87E+04
16	Langres FR 52.092.01 CE	French retail market	12	4 x 150 g	1.39E+08	1.55E+09	2.44E+09
17	Epoisses FR 52.092.01 CE	French retail market	14	1066 g	4.68E+08	6.55E+08	5.61E+08
17b	Epoisses FR 52.092.01 CE	German retail market	21	1056 g	1.02E+09	2.95E+09	2.29E+09
18	Munster Remy-Rudler FR 88.391.01	French retail market	9	900 g	3.67E+08	1.51E+08	1.36E+08
19	Les Sauveurs Petit Livarot FR 14.371.01 CE	French retail market	17	3 x 270 g	1.80E+09	3.02E+09	3.32E+09
20	Maroilles Quart FR 02.495.01 CE	French retail market	12	4 x 200 g	0.00E+00	0.00E+00	0.00E+00
21	Epoisses Germain FR 52.092.01 CE	French retail market	10	3 x 250 g	2.18E+07	3.47E+07	3.50E+07
22	Munster Géromé de Grand-Père Fischer F 68.023.01 CEE	French retail market	18	750 g	0.00E+00	7.70E+04	2.03E+07

23	Reflets de France Petit Livarot * FR 14.081.01 CE	French retail market	11	3 x 250 g	0.00E+00	7.24E+06	2.55E+06
24	Reflets de France Petit Munster Géromé * FR 68.175.01 CE	French retail market	12	5 x 200 g	0.00E+00	0.00E+00	0.00E+00
25	Munster Les Petits Amis F 68.252.02 CEE	French retail market	18	3 x 200 g	3.68E+09	3.32E+09	3.50E+09
26	Chaumes FR 24.370.02 CE	German retail market	9	4 x 150 g	4.96E+05	5.15E+05	2.10E+05
27	Allgäuer Limburger DE BY 709 EG	German retail market	27	6 x 100 g	2.60E+09	3.21E+09	4.97E+09
28	St. Mang Klosterkäse DE BY 709 EG	German retail market	27	6 x 100 g	2.11E+09	2.07E+09	1.79E+09
29	Reblochon Savoie F 74.280.050 CEE	German retail market	22	550 + 590 g	2.51E+09	3.18E+09	3.89E+09
30	Weihenstephaner Rahmromadur DE BY 709 EG	German retail market	7	8 x 100 g	1.55E+09	1.80E+09	1.30E+09
31	St. Mang Romadur DE BY 709 EG	German retail market	-1	8 x 100 g	2.65E+09	1.97E+09	1.98E+09
32	Trésor de Normandie FR 61.402.01	German retail market	37	1000 g	5.02E+09	5.16E+09	5.20E+09
33	Mini Mont d´Or FR 25.041.01 CE	German retail market	15	2 x 500 g	3.93E+09	3.46E+09	3.14E+09
34	Cremoulin Les Caves de Breuilh Fromageries de Chaumes	German retail market	16	468 g	2.17E+09	3.87E+09	2.42E+09
35	Vera Paglietta * I CEE 01/254	German retail market	15	2 x 250 g	9.18E+07	3.50E+09	1.88E+09
36	Robiola Bosina IT 01/204	German retail market	28	4 x 280 g	3.35E+09	3.91E+09	1.42E+09
37	St. Marcellin FR 38.559.01 CE	German retail market	?	5 x 80 g	1.98E+09	4.47E+09	9.64E+08
38	Andechser Natur Romadur DE BY 117 EG	German retail market	19	8 x 100 g	0.00E+00	0.00E+00	1.89E+06

39a	St. Nectaire * sample TR15 a	INRA	young	one quarter of surface	2.05E+09	1.22E+09	1.63E+09
39b	St. Nectaire * sample TR15 b	INRA	fully ripenend	one quarter of surface	2.88E+09	2.44E+09	2.25E+09
39c	St. Nectaire sample TR15 c	INRA	fully ripenend	one quarter of surface	3.14E+09	2.67E+09	2.79E+09
39d	St. Nectaire sample TR26 a	INRA	young	one quarter of surface	2.57E+09	2.83E+09	2.79E+09
39e	St. Nectaire sample TR26 b	INRA	medium	one quarter of surface	2.45E+09	2.59E+09	2.10E+09

Table S2: Bacteriocins and their classification selected for a PCR based screening using genomic and copy DNA templates. Sequences of the oligonucleotide primers are either retrieved from the given references or designed on the basis of gene sequences submitted to the GenBank database using the software Primer3.

For table S2 see table S2 in Chapter 4

Table S3: Control strains used in PCR-based screening for bacteriocin genes.

organism	strain	producer of	source
Lactobacillus bavaricus	TMW 1.5	curvacin A	R. Vogel, Chair for Technical Microbiology, Technical University of Munich
Lactococcus lactis	TMW 2.25	nisin	
Lactobacillus plantarum	TMW 1.25	plantaricin 1.25	
Lactobacillus sakei	TMW 1.454	sakacin P	
Lactobacillus helveticus 481	NCK 228	helveticin J	I. Rogelj, Chair of Dairy Science,University of Ljubljana
Enterococcus faecium	LMG 11423	enterocins A, B, P	
Lactococcus lactis	LMG 2081	lactococcin G	

4 Potent anti-listerial cell-free supernatants produced by complex red smear cheese microbial consortia

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Keywords: *Listeria monocytogenes*, red smear cheese, inhibition, cell-free supernatant

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4.1 Abstract

The microbial surface ripening consortia of 49 soft cheeses were investigated with respect to their inhibition of *Listeria monocytogenes*. When *L. monocytogenes* EGDe (serovar 1/2a) was cultivated in cell-free supernatants (CFS) obtained from consortia grown for eight hours in liquid medium, a strong bactericidal activity was observed in several cases. The CFS of two of these consortia reduced an initial *L. monocytogenes* inoculum of 5×10^7 cfu/ml to zero after 24 hours of incubation. No inhibitory substances could be washed off the complex consortia when incubated for a ten-minute period. A taxonomical analysis of the anti-listerial consortia I and II using FT-IR spectroscopy yielded a considerable species diversity with lactic acid bacteria (LAB) increasing strongly during the 8-hour cultivation. Therefore, 23 LAB bacteriocin genes were assayed using specific PCR primers, identifying three bacteriocin genes in both microbial communities. However, no transcription of these genes was found on cheese surfaces or in consortia propagated in liquid culture. Individual LAB isolates of both consortia displayed no or only weak inhibition of *L. monocytogenes* on solid medium. The complex CFS, in contrast, exhibited an unusually broad inhibitory spectrum, killing *L. monocytogenes* ssp., *Bacillus* sp., *Staphylococcus aureus*, as well as Gram negative bacteria such as *Escherichia coli* DH5α and *Salmonella enterica* serovar Typhimurium. Inhibition could not be abolished by heating to 100 ℃ or by proteinase K treatment. Initial pur ification of an inhibitory substance from consortium I by solid phase extraction and HPLC indicate the presence of rather small, extremely stable compounds, which, most probably, are not bacteriocins.

4.2 Introduction

Listeria monocytogenes, the causative agent of human listeriosis, is a Gram positive, non-sporeforming bacterium characterized by a remarkable resistance to environmental stresses (Low & Donachie, 1997). Its psychrophilic, acid- and halotolerant, facultatively anaerobic character allows the pathogen to overcome many common approaches of food preservation. The pathogenicity of *L. monocytogenes* is characterized by a switch from saprophytic to intracellular lifestyle. Its ability of spreading from cell to cell and thus avoiding the host's humoral immunity response may result in systemic infections (Freitag

et al., 2009). The incidence of listeriosis in Europe is low but the mortality rate can reach 25-30 % among persons developing disease (de Valk *et al*., 2005).

The food-borne nature of *L. monocytogenes* was not recognized before the 1980s, when a number of listeriosis outbreaks were linked to the consumption of contaminated soft cheeses (Farber &Peterkin, 1991). The ripening parameters of salty red smear cheeses at temperatures ranging from 14 to 19 °C and a relative humidity of at least 95 % provide excellent growth conditions for undesirable microorganisms. The practice of “old-young-smearing”, i. e., the transfer of complex microbial consortia from ripe to fresh cheeses, makes controlling *Listeria* further complicated. As a consequence, smear cheeses are more frequently contaminated with *L. monocytogenes* than other soft cheeses, showing exceedingly high cell counts in some products (Jacquet *et al*., 1993; Loncarevic *et al*.; 1995, Rudolf & Scherer, 2001). Undefined cheese surface consortia with anti-listerial properties have been described in the past, sometimes containing bacteria producing bacteriocins (Saubusse *et al*., 2007; Valdes-Stauber & Scherer, 1994). Bacteriocins are small, ribosomally synthesized peptides often acting on the cytoplasmic membrane of target bacteria, thereby causing dissipation of the proton motive force, leakage of small molecules and subsequent cell lysis (for review see Cotter *et al*., 2005; Jack *et al*., 1995). These findings raised hope that indigenous smear bacteria may serve as protective cultures to improve the hygienic quality of these products (McAuliffe *et al*., 1999; O'Sullivan *et al*., 2006). Some approaches pursued a strategy of external microbial protection using bacteriocinogenic starter cultures when the intrinsic protection of a product was inadequate (Eppert *et al*., 1997; Loessner *et al*., 2003).

However, the application of single inhibitory strains is limited since they have to compete with the resident, often very stable “house flora” of a certain ripening cellar (Feurer *et al*., 2004b; Goerges *et al*., 2008). It has been shown that the addition of starter cultures to an established microbial consortium may not result in the expression of the desired functions which the pure cultures displayed. The present study therefore focuses on the antagonistic properties of the complex ripening flora. Our aims were i) to develop a testing system to identify anti-listerial cheese surface consortia, ii) to analyse their microbial composition in terms of species composition and diversity and iii) to characterize the substances produced by the complex microbial populations.

4.3 Materials and methods

4.3.1 Collection of microbial consortia

Smear cheeses produced in France, Italy and Germany were bought on the retail market or provided by Unité de Recherches Fromagères (INRA, Aurillac, France). The surface flora was recovered using a sterile knife and homogenized in saline solution containing 5 % NaCl using a Stomacher Lab-Blender. Suspensions were centrifuged at 6,600 × g and washed twice in saline solution to remove excessive cheese fat. After addition of glycerol to a final concentration of 15 %, aliquots were frozen at -80 °C (glycerol culture). All samples underwent routine analysis according to DIN EN ISO 11290-1 (1997) to detect natural contamination with *Listeria* sp.

4.3.2 Supernatant preparation

500 µl of the glycerol culture were used to inoculate 15 ml of Plate Count broth supplemented with 3 % sodium chloride (PC3+ broth) to mimic the conditions of cheese brines (Valdes-Stauber *et al*., 1991). The flasks were incubated for ten minutes or eight hours at 30 °C with shaking (180 rpm). The cells we re pelleted by centrifugation at 12,000 × g. The supernatants were filter-sterilized (Millex GP 0.2 µm filter units, Millipore, Schwalbach, Germany) and stored at -20 °C until use . The preparation of cell-free supernatants (CFS) was carried out at least three times independently. The pH was measured after freezing using universal indicator strips (Macherey-Nagel, Düren, Germany), the content of lactic acid and hydrogen peroxide was determined using the analytical systems of Merck (Darmstadt, Germany) The enzymes lactate dehydrogenase and peroxidase, respectively, are immobilized on strips allowing quantification via a colorimetric reaction. Bacteriophage involvement was excluded by monitoring plaque formation in poured plates using TSS (see below).

4.3.3 Detection of anti-listerial activity

In the cultivation assay, indicator strains (listed in table S1) were grown overnight in Brain Heart Infusion broth (BHI, Merck) and inoculated 1:20 in PC3+ broth. After 2 h of incubation cells were diluted to obtain a cell density of 10^4 cfu/ml. Five ml of the CFS were inoculated with 0.5 ml of the indicator cell suspensions and incubated for 24 h at 30 °C and 180 rpm. Samples of 100 µl were serially diluted and plated onto PC3+ agar in duplicate.

Viable cells of the indicator strains were counted after two days of aerobic incubation at 30 °C. In the overlay assay, an overnight culture of *L. monocytogenes* EGDe was used to inoculate Tryptic Soy Softagar (TSS, 17.0 $g \cdot L^{-1}$ tryptone, 6.0 $g \cdot L^{-1}$ yeast extract, 3.0 $g \cdot L^{-1}$ soy peptone, 5.0 $g \cdot L^{-1}$ NaCl, 2.5 $g \cdot L^{-1}$ D-glucose, 2.5 $g \cdot L^{-1}$ K_2HPO_4 and 7.5 $g \cdot L^{-1}$ agar-agar) at 1:100. Ten ml of the seeded softagar were poured over agar plates containing picks or streaks of testing bacteria (incubation see below). After an overnight incubation at 30 °C under aerobic conditions, inhibitory strains were identified on the basis of clearing zones they caused in the confluent lawn of *L. monocytogenes* EGDe.

4.3.4 Isolation of inhibitory lactic acid bacteria

Eight-hour liquid cultures of consortia I and II were plated onto All Purpose Tween 80 agar plates (APTA, Merck) and incubated for 2 days at 34 °C in an anaerobic jar containing the catalyst Anaerocult IS (Merck). Several hundred isolates were randomly picked and transferred to new APTA plates. A two day-period for the production of inhibitory substances was given; afterwards the plates were subjected to the overlay assay. Anti-listerial colonies were recovered from the plates, streaked to convince purity and applied to the assay a second time.

4.3.5 Fourier Transform Infrared spectroscopy

The composition of both surface floras was studied in detail using Fourier Transform-InfraRed (FT-IR) spectroscopy. 100 bacterial colonies were picked randomly from PC3+ agar plates after 3-5 days of aerobic incubation at 30 °C. Lactic acid bacteria were identified after anaerobic cultivation on APTA at 34 °C. The sample preparation to obtain reliable infrared spectra was carried out as described (Kümmerle *et al*., 1998; Oberreuter *et al*., 2002). Two independent measurements were conducted using a HTS-XT FT-IR spectrometer (Bruker, Karlsruhe, Germany). For data processing, OPUS software v. 6 (Bruker) was used. The spectral distances of the isolates, reflecting the similarity of two spectra by comparing the size of non-overlapping areas, were used to discriminate the isolates at strain level. A subsequent cluster analysis was performed to visualize the abundance ratio of strains resulting in a dendrogram calculated with the average linkage algorithm.

4.3.6 PCR detection of bacteriocin encoding genes

Genomic DNA of consortia I and II was extracted according to the Rotiphenol protocol (Roth, Karlsruhe, Germany), following the recommendations of the manufacturer. 500 µl of glycerol culture were mixed with 1 ml Rotiphenol. After proteinase K treatment for digestion of disturbing proteins, the enzyme was heat inactivated. PCR reactions were performed using specific primer pairs for 23 different bacteriocin genes, listed in table S2 (oligonucleotide primers purchased from Invitrogen, Karlsruhe, Germany). PCR amplification was carried out in a 50 µl reaction mixture containing 1 pmol of specific primers, 200 µM dNTP mixture, 1.5 mM $MgCl_2$, 1 U Thermoprime Plus Polymerase (Thermo Scientific) and 100 ng of template. PCR conditions were as described recently (see table S2). DNA extracts of bacteriocin producing strains, listed in table S1, served as positive controls. Amplified fragments were resolved by electrophoresis on 2 % agarose gels and visualized by staining with ethidium bromide. Resulting bands were excised and sequenced for verification.

4.3.7 Initial characterization of anti-listerial substances

The complex CFS were exposed to challenge conditions prior to their inoculation with 1×10^3 cfu/ml *L. monocytogenes* EGDe. All experiments were conducted at least twice. CFS were subjected to serial twofold dilutions up to 1:16 in PC3+ broth. For thermal treatment, the CFS were exposed to 100 °C for 1 min and 121 °C for 15 min. Proteinase K was added in a final concentration of 0.5 mg/ml (30 U/mg, Merck) and incubated for 2 h at 50 °C. The enzyme was heat inactivated for 30 min at 75 °C. Catalase treatment was performed for 2.5 h at 37 °C using a final concentration of 1 mg/ml (3800 U/mg, Sigma-Aldrich, Hamburg, Germany). In a combined approach, proteinase K was added after the reaction of catalase was completed. The inhibitory spectrum was determined using the alternative indicator strains listed in table S1 and conducted as described for *L. monocytogenes* EGDe.

4.3.8 Fractionation of complex supernatant I by Solid-Phase Extraction (SPE)

Aliquots (2.5 ml each) of CFS I were applied onto the top of a C18-SPE cartridge (6 ml, 1g, Strata C18-E, Phenomenex, Aschaffenburg, Germany) preconditioned with methanol, followed by water. Water for chromatographic separations was purified with a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany), and solvents used were of HPLC-grade (Merck, Darmstadt, Germany). Fractionation was performed by flushing the column with water (6 ml, fraction S1), followed by increasing methanol/water mixtures starting with a mixture of 20 % (20/80, v/v; 6 ml; fraction S2) in 20 % steps (S3-5, 6 ml each), and methanol (10 ml; fraction S6). The fractions S1-S6 collected were concentrated in a vacuum (40 °C) and, after freeze-drying twice, resu spended in 1ml water. Bioactivity of individual fractions was determined in the cultivation assay. Fractions active against *L. monocytogenes* EGDe were suspended in water (1 ml) and aliquots (250 µl) were separated by means of semi-preparative HPLC.

4.3.9 High Performance Liquid Chromatography (HPLC) of CFS I. Separation of SPE fraction S2 and S3

The HPLC system consisted of a HPLC-pump system PU 2087, a high-pressure gradient unit and a PU-2075 UV-detector using a semipreparative RP column, Luna Phenyl hexyl, 10.0×250 mm, 5 µm (Phenomenex, Aschaffenburg, Germany) as the stationary phase. Monitoring the effluent at 210 nm, chromatography was performed starting with aqueous formic acid (0.1 %) for 3 min, increasing the methanol content to 100 % over 10 min, and, thereafter, eluting with methanol for 5 min at a flow rate of 4.2 ml/min. After removing the solvent in vacuum, 40 °C, fractions S2/H1-10 and S3 /H1-10 were suspended in water (10 ml), and freeze-dried twice to determine the bioactivity of the individual fractions (see above).

4.4 Results and discussion

4.4.1 Anti-listerial substances are produced in short-time liquid cultures

Microbial cheese ripening consortia were suspended in PC3+ broth and incubated for a 10 min period to prepare complex supernatants. No anti-listerial effect was detected in these supernatants. In contrast, growth of *Listeria* was often stimulated and reached levels higher than in nutrient broth (data not shown). It is concluded that, in frozen microbial consortia, no antagonistic substance is present which can be washed off the cells. Therefore, a longer incubation period of 8 h at a high initial cell density (approx. 3×10^8 cfu/ml) was applied. When 5×10^7 cfu/ml *L. monocytogenes* EGDe (serovar 1/2a) were inoculated in 8-h CFS, eight of 49 supernatants displayed a bactericidal effect, sometimes reducing the inoculum to zero after 24 h, and five CFS acted bacteriostatically. The supernatants from consortia I and II (Munster Géromé Frech and Reflets de France and Petit Munster Géromé, respectively, from the French retail market) were chosen for study. Both CFS had a content of lactic acid of 4.4 mM and a content of H_2O_2 of less than 15 µM; pH values were 6.0. The minimal inhibitory concentration (MIC) of H_2O_2 in *L. monocytogenes* lies between 0.26-2.2 mM (Romanova *et al*., 2002), the MIC of lactic acid has been determined around 220 mM but only at refrigeration temperatures (Gonzalez-Fandos & Dominguez, 2006). However, lactic acid acts only bacteriostatically, not bactericidal. Obviously, the actively growing consortia produce soluble anti-listerial substances other than lactic acid or hydrogen peroxide.

The serially two-fold diluted CFS of consortia I and II, inoculated with 10^3 cfu/ml Listeria, exhibited different bactericidal potentials with CFS I clearly being more active (figure 1, white symbols). CFS I was also more active when a kinetics of inhibition of 10^7 cfu/ml *Listeria* was measured (figure 1, black symbols).

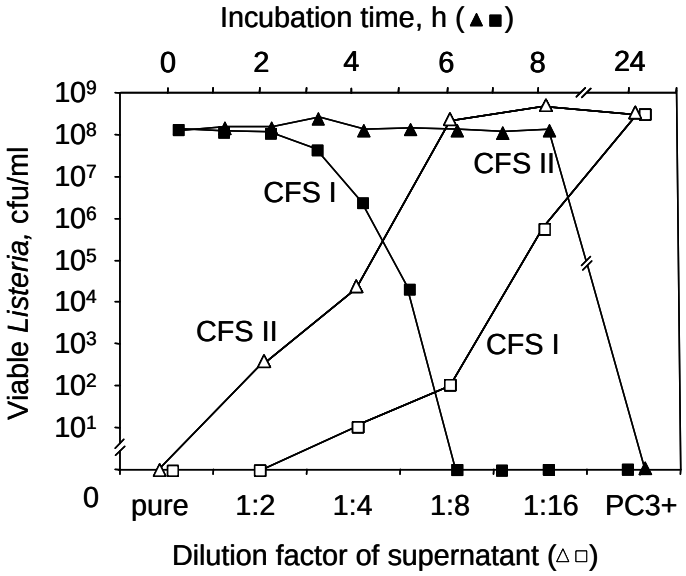

Figure 1. *L. monocytogenes* EGDe counts inoculated either with 10^7 cfu/ml in cell-free supernatants (CFS) and sampled every hour (black symbols), or inoculated with 10^3 cfu/ml in serially twofold dilutions of CFS and sampled only after 24 h (white symbols).

In addition, supernatants I and II differed in other respects. Whereas boiling did not affect the anti-listerial potential of CFS I, the bactericidal, but not the bacteriostatic effect of CFS II was abolished, suggesting a mixture of active components. Upon autoclaving, CFS I maintained a bacteriostatic action whereas in CFS II inhibition of listerial growth was nearly completely abolished. A treatment with proteinase K left CFS I unaffected, indicating the involvement of non-peptide molecules, while CFS II was partially susceptible to proteinase K, indicating the participation of a proteinaceous compound (results summarized in table 1).

Table 1: Characteristics of supernatants I and II and viable cell counts after 24 h when 10^3 cfu/ml *L. monocytogenes* EGDe were cultured in untreated, heat-treated and enzyme-treated CFS. Growth of *L. monocytogenes* EGDe in PC3+ broth with addition of enzymes resulted in 10^9 cfu/ml (data not shown). Results of a representative experiment are given.

characteristics/ treatment of CFS	viable *L. monocytogenes* EGDe after 24 h of incubation in	
	CFS I	CFS II
untreated	0	0
1 min 100 ℃	0	1.1E+04
15 min 121 ℃	1.8E+03	2.3E+06
proteinase K	0	9.0E+02

The inhibitory spectrum of both consortia was unexpectedly broad (table 2), comprising *L. monocytogenes* isolates of four different serovars (1/2a, 1/2b, 3a and 4b), *Bacillus* sp., *S. aureus* as well as the Gram negative bacteria *Salmonella enterica* Typhimurium and *E. coli* DH5α. While bacteria exposed to CFS I were often killed within 6 h, CFS II acted bacteriostatically and displayed a bactericidal action only after 24 h (data not shown). Taken together, these features indicate that heat-stable molecules with a broadband antagonistic activity were produced during propagation of the complex consortia.

Table 2: Viable cell counts after 24 h of Gram positive and Gram negative indicator strains cultured in cell-free supernatants I and II. Results of a representative experiment are given.

indicator strain	serovar	CFS I	CFS II
L. monocytogenes EGDe	1/2a	0	0
L. monocytogenes WSLC 1416	1/2a	0	7.1E+02
L. monocytogenes WSLC 1499	1/2b	0	1.2E+03
L. monocytogenes WSLC 11149	3a	0	7.6E+02
L. monocytogenes WSLC 1364	4b	0	1.8E+03
S. aureus ATCC12600		0	0
B. megaterium		0	0
B. cereus F4810/72	emetic	0	2.3E+01
B. cereus ATCC 10987	non-emetic	0	0
S. enterica ATCC 14028	Typhimurium	0	0
E. coli DH5α ATCC PTA-1798		0	0

4.4.2 Distinct shifts of biodiversity upon cultivation in liquid medium

A taxonomic analysis of both consortia was conducted using FT-IR spectroscopy (Wenning *et al*., 2006). Both consortia grown on the cheese surface showed a high biodiversity (figure 2, grey bars). Forty-eight strains of different physiological groups could be distinguished in consortium I with 45 % belonging to the lactic acid bacteria, 43 % to coryneform species, and 12 % to Gram negative enterobacteria and pseudomonads. Consortium II was composed of 46 different strains with 69 % belonging to the coryneform bacteria, 24 % to the lactic acid bacteria and 8 % to the Gram negative bacteria. *Arthrobacter*, *Corynebacterium* and *Staphylococcus* species were represented in high numbers. Gram negative bacteria have shown to constitute an important group in ripening consortia of Irish and French smear cheeses (Feurer *et al*., 2004b; Mounier *et al*., 2005). Compared to recent biodiversity studies (Maoz *et al*., 2003; Rea *et al*., 2007), the high number of lactic acid bacteria is unusual. This may be due to some carry over of LAB during sampling of microbial consortia.

The initial composition of the consortia changed considerably upon the 8-h cultivation in broth (figure 2, black bars). The coryneform bacteria which constituted the predominant group in the biofilms on cheese did not multiply or even disappeared, while the growth of Gram negative and lactic acid bacteria was favoured, resulting in a doubling of their numbers. Taking into account that all isolates less than 1 % of the total cell count are below the detection limit of the method, the biodiversity in broth is very different compared to the one in the smear biofilm. Similar data were reported by Monnet *et al*. (2010), who performed successive propagations of 14 cheese surface consortia on model cheese. An increase of the anti-listerial potential was reported after repeated inoculation of the consortia on the cheese, concomitant with an increase of lactic acid bacteria and Gram negative cell counts. We suggest that coryneform bacteria did not produce the inhibitory substances. Rather, the inhibition observed may be due to bacteriocin production of the lactic acid bacteria.

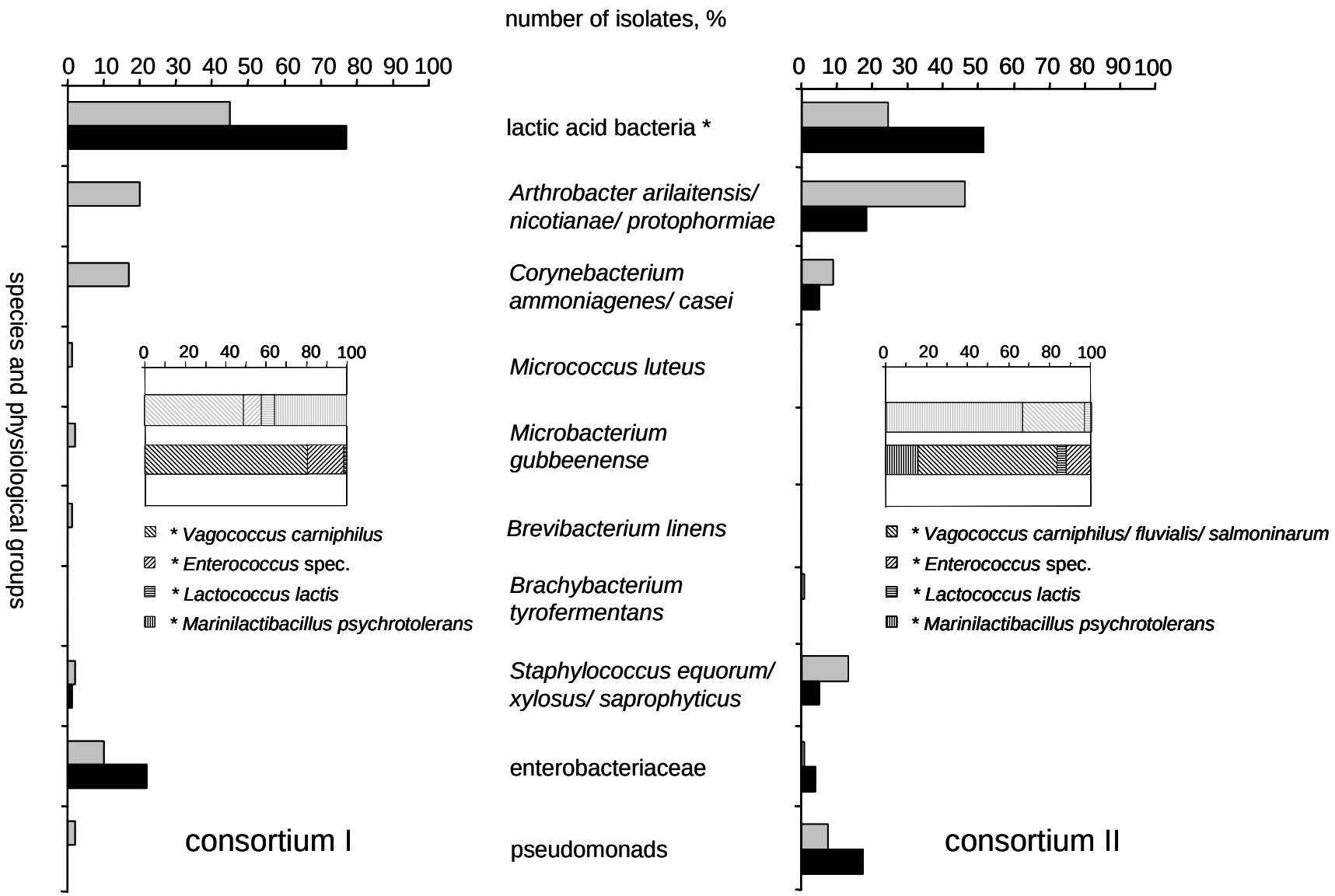

Figure 2. Biodiversity of consortium I and II. Grey bars indicate for the original species composition, black bars reflect the composition after eight hours of incubation in broth. The identity of 100 isolates per consortium and per time point were analysed.

4.4.3 Detection of bacteriocin genes by PCR

The presence of genes encoding bacteriocins was explored by a PCR based screening using genomic DNA, extracted directly from microbial consortia frozen in glycerol, as a template. PCR reactions for consortium I yielded positive results for lactococcin G, helveticin J and curvacin A. For consortium II, amplification of helveticin J, acidocin B and cytolysin was successful. The *in situ* expression of the bacteriocin genes detected in consortia I and II could not be proven using total RNA extracts of 8-h liquid culture microbiota in PCR (data not shown).

Therefore, a large-scale screening for anti-listerial LAB was performed using anaerobic incubation on APT agar. Among 776 LAB colonies of the 8-h propagated consortium I, only 25 anti-listerial colonies were found, comprising four different strains identified as *Lactococcus lactis* or *Lactococcus lactis lactis*. No *Lactobacillus curvatus* (producer of curvacin A) or *Lactobacillus helveticus* (producer of helveticin J) was identified and also amplification of lactococcin G was not successful in *L. lactis* strains. Among 889 isolates of consortium II, 92 colonies showed clearing zones in the first test. Seven different strains belonging to *Lactococcus lactis* or *Lactococcus lactis lactis* (3), *Enterococcus faecalis* (3) and *Enterococcus durans* (1) could be distinguished, one *L. lactis* being identical to a strain found in consortium I. Neither were *L. helveticus*, *Lactobacillus acidophilus* (producer of acidocin B) found nor was PCR detection of cytolysin possible in the *E. faecalis* strains.

The inhibitory phenotype of the LAB on solid medium was unstable. Clearing zones became smaller or even disappeared when producer strains were propagated. Bacteriocin-sensitive bacteria acting as environmental stimuli inducing bacteriocin production were previously reported (Maldonado *et al*., 2004b; Rojo-Bezares *et al*., 2007). Most bacteriocin producers have been isolated from sources that contain a heterogeneous microflora and bacteriocin expression is often regulated via a quorum-sensing mechanism mediated by peptide pheromones (Kleerebezem, 2004; Navarro *et al*., 2008). Possibly, the high cell density and the mixed culture during CFS production triggered bacteriocin production in some strains but upon withdrawal of concomitant cells, the external stimulus of the signal cascade was eliminated and bacteriocin gene expression ceased.

Bacteriocins belonging to class I (e. g. cytolysin) and class II (as curvacin A, acidocin B and lactococcin G) generally are resistant towards heat (Nes *et al*., 2007), but lose their

activity upon treatment with proteinase K (Contreras *et al*., 1997; Noonpakdee *et al*., 2003; Yamamoto *et al*., 2003). Helveticin J as a member of class III bacteriocins is sensitive towards both heat and proteolytic enzymes (Joerger & Klaenhammer, 1986). Proteinase K as well as heating to 100 °C decreased the inhibito ry activity of CFS II at least partly, which may indicate that bacteriocins could be responsible for part of the inhibition observed. However, CFS I remained active after proteinase K addition, suggesting that substances different to bacteriocins are involved in the strong listericidal effect. Therefore, supernatant I was further investigated.

4.4.4 Partial purification of inhibitory fractions from CFS I

The complex supernatant of consortium I was separated into six fractions (S1-S6) using solid phase extraction on RP-18 material. The inhibitory activity was eluted with 20 % and 40 % methanol (S2 and S3, respectively), indicating the presence of polar substances. Subsequently, S2 was separated by RP-HPLC (figure 3). Activity was recovered from fractions H3 and H8 with H8 having the best inhibitory potential against *L. monocytogenes* EGDe in the cultivation assay (figure 3, inset). Our data indicate a mixture of substances being present in H3 and H8. At this state fractions are far too complex for direct peptide sequencing. The inhibitory potential of native CFS I was neither destroyed by addition of 30 % methanol nor by freeze-drying and it was stable over a period of 3 years at -20 °C (data not shown). This indicates that loss of activity after SPE and HPLC fractionation was not due to the denaturation of inhibitory proteins. The use of SPE cartridges and HPLC columns with pore sizes of 100 Å further cast doubt on the involvement of proteins in the active fractions. Short-chain fatty acids (SFCA, Caplice & Fitzgerald, 1999) or anti-bacterial peptides (Lopez-Exposito *et al*., 2006) may be involved in this inhibition.

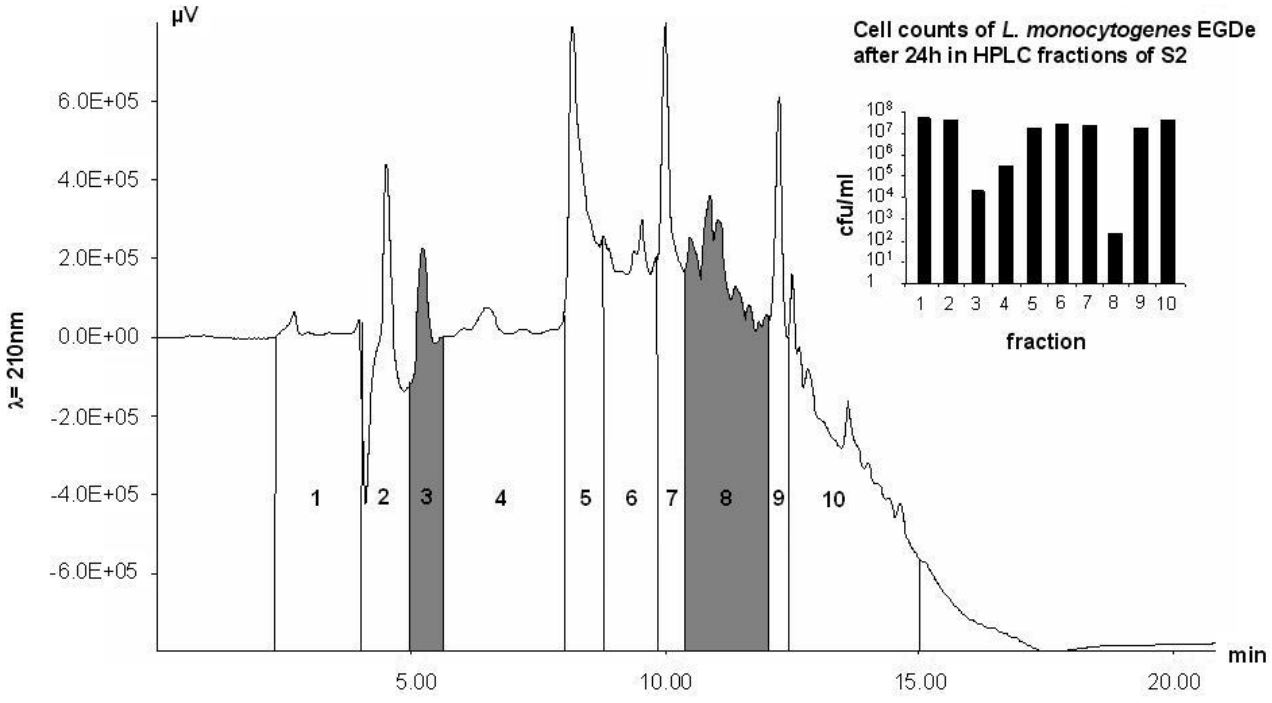

Figure 3. HPLC run of SPE fraction eluted with 20 % methanol (= S2). Fraction 8 (H8) showed the highest bioactivity in the cultivation assay, reducing 1 × 10^3 cfu/ml *L. monocytogenes* EGDe to 2 × 10^2 cfu/ml after 24 h of incubation.

4.5 Conclusion

Intrinsic microbial food protection, often mediated by bacteriocinogenic starter cultures, becomes more important upon the increasing consumer's awareness of genetically modified organisms and questionable additives. In the present study, the production of potent inhibitory substances by two cheese ripening consortia in short-time liquid culture was observed. Their antagonistic potential against Gram negative bacteria and their resistance to proteinase K distinguishes them from classical bacteriocins. A preliminary characterization of the inhibitory substances was achieved through fractionation of the complex CFS I. Further research is needed in order to identify these substances and their producers and figure out a potential antagonistic function *in situ* in cheese microbial consortia.

4.6 Acknowledgments

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4.7 Supplementary material

Table S1: Strains used in this study.

organism	strain	other characteristics	source
1. indicator strain in cultivation assay			
Listeria monocytogenes	EGDe	1/2 a	T. Chakraborty, University of Gießen
Listeria monocytogenes	WSLC 1416	1/2 a	German cheese (Rehkopf)
Listeria monocytogenes	WSLC 1499	1/2 b	Austrian cheese
Listeria monocytogenes	WSLC 11149	3a	smoked salmon
Listeria monocytogenes	WSLC 1364	4b	Vacherin Mont d`Or outbreak 1987
Staphylococcus aureus aureus	WS 2438		ATCC 12600
B. megaterium	WS 1537		ATCC 14581
Bacillus cereus	F4810/72	emetic	
Bacillus cereus		emetic-like	ATCC 10987
Salmonella enterica	WS 4170	Typhimurium	ATCC 14028
Escherichia coli	DH5α		ATCC PTA-1798
2. PCR positive controls			
Lactobacillus bavaricus	TMW 1.5	curvacin A	R. Vogel, Chair for Technical Microbiology, Technical University of Munich
Lactococcus lactis	TMW 2.25	nisin	
Lactobacillus plantarum	TMW 1.25	plantaricin 1.25	
Lactobacillus sakei	TMW 1.454	sakacin P	
Lactobacillus helveticus 481	NCK 228	helveticin J	I. Rogelj, Chair of Dairy Science, University of Ljubljana
Enterococcus faecium	LMG 11423	enterocins A, B, P	
Lactococcus lactis	LMG 2081	lactococcin G	

Table S2: Bacteriocins and their classification selected for a PCR based screening using genomic and copy DNA templates. Sequences of the oligonucleotide primers are either retrieved from the given reference publications or designed on the basis of gene sequences submitted to the GenBank database using the software Primer3.

bacteriocin	class	name and sequence of forward primer (5´→ 3´)	name and sequence of reverse primer (5´→ 3´)	length of product (bp)	primer design and conditions for amplification
lactococcin A	IId	lcnA-F CAA TCA GTA GAG TTA TTA ACA TTT G	lcnA-R GAT TTA AAA AGA CAT TCG ATA ATT AT	771	Rodríguez *et al*., 2000
lactococcin B	IIc	lcnB-F GCT TGC AGT ATG TTA TGA GTG	lcnB-R CCT ACC ATC CAG GAT TTT CTT	431	van Belkum *et al*., 1992
lactococcin G	IIb	lcnG-F AGA ATT ACG AGA ATG CGT TGG	lcnG-R GAA GCT TGA TTA ACA TCG CTC A	117	this study (Nes, personal correspondance)
lactococcin 513		lcn513-F GCT CCA AAA AGC GCT AGA TC	lcn513-R GCT GGC TAC GAT ATT GCT AG	466	this study (sequence from GenBank AF056207)
lactococcin 972	IIb	lcn972-F GCG CTC TCT TGC ATA GTG AG	lcn972-R ACT CCT CCA TTA GTA CCA GC	531	Martinez *et al*., 1999
lacticin RM		lacRM-F ATC CTA TCC GAT ACC GTC AG	lacRM-R GTT TTC CCT GAA CCA TTG GG	644	GenBank AF080265
lacticin 481	I	lac481-F TCT GCA CTC ACT TCA TTA GTT A	lac481-R AAG GTA ATT ACA CCT CTT TTA T	366	Rodríguez *et al*., 2000
enterocin A	IIa	entA-F GGT ACC ACT CAT AGT GGA AA	entA-R CCC TGG AAT TGC TCC ACC TAA	150	De Vuyst *et al*. 2003
enterocin B		entB-F CAA AAT GTA AAA GAA TTA AGT ACG	entB-R AGA GTA TAC ATT TGC TAA CCC	200-250	De Vuyst *et al*. 2003
enterocins 1071A, B	IIb	ent1071AB-F CCT ATT GGG GGA GAG TCG GT	ent1071AB-R ATA CAT TCT TCC ACT TAT TTT T	117, 129	Balla *et al*., 2000
enterocin P	IIa	entP-F GCT ACG CGT TCA TAT GGT AAT	entP-R TCC TGC AAT ATT CTC TTT AGC	100	De Vuyst *et al*. 2003
enterocin 31	IIa	ent31-F CCT ACG TAT TAC GGA AAT GGT	ent31-R GCC ATG TTG TAC CCA ACC ATT	201	De Vuyst *et al*. 2003
enterocin AS 48	IIc	entAS48-F GAG GAG TAT CAT GGT TAA AGA	entAS48-R ATA TTG TTA AAT TAC CAA	210	De Vuyst *et al*. 2003

enterocin L50A	IIb	entL50A-F ATG GGA GCA ATC GCA AAA TTA	entL50A-R TTT GTT AAT TGC CCA TCC TTC	132	De Vuyst *et al*. 2003
enterocin L50B	IIb	entL50B-F ATG GGA GCA ATC GCA AAA TTA	entL50B-R TAG CCA TTT TTC AAT TTG ATC	129	De Vuyst *et al*. 2003
plantaricin A	IIa	plnA-F A TGA AAA TTC AAA TTA AAG GTA TGA AGC	plnA-R TTA CCA TCC CCA TTT TTT AAA CAG TTT C	145	Maldonado *et al*., 2004
plantaricin S	IIb	plnS-F AAY AAR YTI GCI TAY AAY ATG	plnS-R GCY TTY AAR RAI CCY TCI CC	380	Remiger *et al*., 1996
cytolysin	I	cyt-F GGC GGT ATT TTT ACT GGA GTN	cyt-R CCT ACT CCT AAG CCT ATG GTA	250	De Vuyst *et al*. 2003
nisin	I	nis-F AAG AAT CTC TCA TGA GT	nis-R CCA TGT CTG AAC TAA CA	898	Rodríguez *et al*., 2000
acidocin A		aciA-F TGG TGT GCA TTG TAC T	aciA-R TTG ATC GGC AAC GAT T	96	Majhenič *et al*., 2003
acidocin B	IIc	aciB-F AGA TGC AGT GGC TTC T	aciB-R CCA TGC AGG TAA TGT C	73	Majhenič *et al*., 2003
sakacin P	IIa	sakP-F ATG GAA AAG TTT ATT GAA TTA	sakP-R TTA TTT ATT CCA GCC AGC GTT	186	Remiger *et al*., 1996
curvacin A	IIa	curA-F GTA AAA GAA TTA AGT ATG ACA	curA-R TTA CAT TCC AGC TAA ACC ACT	171	Remiger *et al*., 1996
helveticin J	III	helJ-F AGA CAT GGG AAT TTG CTG GT	helJ-R GGC GCG ATT CAA GTA GGA TA	203	Trmčić *et al*., 2008

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5 *Vibrio casei* sp. nov., isolated from the surface of two French red smear soft cheeses

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5.1 Abstract

Three Gram-negative, rod shaped, catalase and oxidase positive, facultatively anaerobic and motile bacteria, WS 4538, WS 4539^T and WS 4540 were isolated from the surface of two fully ripened French red smear soft cheeses. Based on 16S rRNA gene sequence similarity, all three strains were shown to belong to the genus *Vibrio*. They are most closely related to *Vibrio rumoiensis* S-1^T (96·3 %) and *Vibrio litoralis* MANO22D^T (95·9 %). DNA-DNA hybridisation confirmed that all three isolates belong to the same species and clearly separated WS 4539^T from *V. rumoiensis* S-1^T (38-42 % similarity) and *V. litoralis* MANO22D^T (28-37 % similarity). In contrast to their next relatives, the strains exhibited ß-galactosidase and esculin hydrolase activity. A 14-bp insertion in the 16S rRNA gene sequence forms an elongated structure at helix 10 in the rRNA molecule and provides a tool for PCR based identification of the novel species. Partial sequences of the housekeeping genes *atpA*, *recA*, *rpoA* and *pyrH* supported the three isolates to constitute a separate species within the genus *Vibrio*. The name *Vibrio casei* is proposed for the novel taxon. WS 4539^T (= DSM 22364^T = LMG 25240^T, DNA G+C content of 41·8 %) is the type strain and WS 4540 (= DSM 22378 = LMG 25241) is the reference strain.

The family of the *Vibrionaceae* (Baumann & Schubert, 1984) belongs to the Gammaproteobacteria and includes, amongst others, the genera *Photobacterium* (Baumann & Baumann, 1984), *Salinivibrio* (Mellado *et al*., 1996) and *Vibrio*. The genus *Vibrio* currently consists of 72 species which form nine phylogenetically different clades (AViB, Association of *Vibrio* Biologists, http://www.vibriobiology.net/). The clearly separated *rumoiensis* clade consists of *V. rumoiensis* (Yumoto *et al*., 1999) and *V. litoralis* (Nam *et al*., 2007) and forms a distant lineage within the genus *Vibrio*. Microorganisms belonging to the genus *Vibrio* are known to live either freely or associated as symbionts with aquatic animals in marine or estuarine waters (Browne-Silva & Nishiguchi, 2008), or as parasites of fish, crustaceans and molluscs (Thompson *et al*., 2004). *Vibrio* species as members of microbial consortia on cheese surfaces are less frequently reported (Feurer *et al*., 2004a), but some studies suggest them to play a role in the ripening process (El-Baradei *et al*., 2007).

The composition of the surface microbial consortia of two French smear-ripened cheeses, Petit Munster Ermitage and Reflets de France Petit Livarot, was analysed using Fourier-

transform infrared (FT-IR) spectroscopy (Kümmerle *et al*., 1998; Naumann *et al*., 1991). After 5d of aerobic incubation at 10-30 ℃, isolate s were picked randomly from fully-grown plate count agar plates, supplemented with 3 % sodium chloride (PC3+). The cell density of the surface consortia was 6×10^9 cfu/ml (Munster) and 4×10^{10} cfu/ml (Livarot), respectively. The novel species was estimated to contribute with approximately 10^8 cfu/ml to the surface consortia of both cheeses.

Unless stated otherwise, cells were cultured in marine broth (MB, Difco) at 30 ℃ with shaking at 180 rpm. All experiments were conducted at least twice. Tolerance towards temperature (2, 4, 10, 14, 30, 37 and 43 ℃) was re corded for five days. Growth occurred between 2 and 30 ℃. Tolerance towards pH as well a s sodium requirement and sodium tolerance were tested in 2 % (w/v) peptone broth (15g·L^{-1} peptone from casein, Oxoid, Wesel, Germany and 5 g·L^{-1}soy peptone, BD, Heidelberg, Germany), adjusted to pH 3-11 or supplemented with NaCl (2, 4, 6, 8, 10, 12, 14 % w/v). To compensate effects due to osmolarity in case of 0 % NaCl, the basal medium according to Baumann & Baumann (1981) was used for inoculation, were Na^+ is replaced by K^+ ions. Growth was observed between pH 6-8·5 and 2-10 % NaCl.

The Gram reaction was determined by addition of 3 % KOH to a colony grown one day on marine agar (MA, Difco), and by Gram staining. Catalase and oxidase activities were documented by gas production in a 3 % hydrogen peroxide solution and by using test stripes of Bactident Oxidase (Merck), respectively. Sensitivity towards the vibriostatic agent O/129 was determined using Oxoid discs (150 mg/ disc). Motility was tested on freshly poured MA plates dried for one hour where swarming of the cells was checked after incubation at 30 ℃ overnight. The ability to grow under anaerobic conditions was determined over a 3 day period in an anaerobic jar containing the catalyst Anaerocult IS (Merck), prepared according to the manufacturer's instructions.

API 20NE and API ZYM test stripes (bioMérieux) were used to analyse substrate utilisation from sole carbon sources, acid production from carbohydrates and enzymatic activities. Since a misidentification using API systems was reported when suspending marine organisms in the solutions provided by the manufacturer (Martinez-Urtaza *et al*., 2006), the following modifications were introduced to ensure optimal growth conditions: sodium chloride was added in a final concentration of 1·5 % to the API 20NE suspension medium and the AUX solution. Cell suspensions with a standard opacity equivalent to McFarland 3 were used to inoculate the stripes which were incubated for 24 h at 30 ℃ prior to

evaluation. For API ZYM the incubation conditions were modified (6 h at 30 ℃ instead of 4 h at 37 ℃).

Bacteria were grown for 24 h at 28 ℃ on MA to analyse cellular fatty acid composition. Fatty acids were extracted and analysed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) as described recently (Verbarg *et al*., 2008). Analysis of the fatty acid content showed the predominance of three major fatty acids (Table 1), which is similar but not identical to *V. rumoiensis* S-1^T and *V. litoralis* MANO22D^T. No significant differences were found between the three new isolates.

Table 1: Major fatty acid composition in percent of the three *V. casei* isolates and their closest phylogenetic relatives *V. rumoiensis* S-1^T and *V. litoralis* MANO22D^T (data obtained in this study).

fatty acid content [%]	*V. casei* WS 4539^T	*V. casei* WS 4538	*V. casei* WS 4540	*V. litoralis* MANO22D^T	*V. rumoiensis* S-1^T
16:1 *ω7c* and/ or 15:0 iso 2-OH	41.0	41.3	40.8	43.4	40.3
16:0	22.0	21.2	20.4	23.9	23.2
18:1 *ω7c*	15.6	15.9	15.4	12.0	14.0
14:0 3-OH and/ or 16:1 iso I	3.2	3.3	3.2	7.7	7.0
14:0	1.5	1.8	1.7	1.8	4.0
12:0	3.8	3.8	3.6	4.0	3.8

The G+C content (mol%) of genomic DNA was examined by the Identification Service of the DSMZ using HPLC as described by Mesbah & Whitman (1989). The values obtained were 41·8 mol% for WS 4539^T and 40·7 mol% for WS 4538 and WS 4540, respectively. Table 2 summarises the phenotypic differences between *V. casei* WS 4539^T and its two closest phylogenetic relatives.

Table 2: Differential phenotypic characteristics of the three novel isolates and their phylogenetically closest relatives (data obtained in this study).

Characteristic	*V. casei* WS 4539^T	*V. litoralis* MANO22D^T	*V. rumoiensis* S-1^T
Expression of:			
ß-galactosidase	+	-	-
esculin hydrolase	+	-	+
Growth at 12 % NaCl	-	+	-
Growth at 37 ℃	-	+	+
Growth at pH 5	-	+	+
fatty acid content [%]			
14:0	1.5	1.8	4.0
14:0 3-OH and/ or 16:1 iso I	3.2	7.7	7.0

DNA-DNA hybridisation was performed between the strains isolated from smear cheeses and *V. litoralis* MANO22D^T as well as *V. rumoiensis* S-1^T. The studies were carried out by the Identification Service of the DSMZ based on renaturation curves. DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). Hybridisation studies were performed as described by De Ley *et al*. (1970*)* with the modifications introduced by Huß *et al.* (1983), using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier temperature controlled 6x6 multicell changer with an *in-situ* temperature probe (Varian) in 2 x SSC at 67 ℃. Following the recomme ndations of Wayne *et al.* (1987), the threshold value of DNA-DNA-relatedness is 70 % for the definition of a species. DNA-DNA hybridisation showed that WS 4539^T, WS 4538 and WS 4540 belong to the same species. This is also corroborated by the fact that we were unable to find physiological and biochemical differences between the three isolates. DNA pairing of isolates WS 4539^T versus *V. litoralis* MANO22D^T and *V. rumoiensis* S-1^T revealed values for DNA-DNA reassociation between 28 and 42 % (see also Table S1 in the online version of this article). Therefore, the three strains isolated from the surface of two smear-ripened cheeses constitute a new species within the genus *Vibrio.*

Table S1: DNA-DNA hybridisation based similarity as determined by Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig. Numbers in brackets indicate the values obtained in a second, independent analysis. Data obtained in this study.

	V. rumoiensis S-1^T	*V. litoralis* MANO22D^T	*V. casei* WS 4538	*V. casei* WS 4540
V. casei WS 4539^T	38 (42)	28 (37)	92 (89)	87 (83)

The phylogenetic position of the three isolates within the *Vibrionaceae* was determined by 16S rRNA, *atpA*, *recA*, *rpoA* and *pyrH* gene sequence analyses. Genomic DNA was extracted as described by Wenning *et al.* (2006). Cycle sequencing PCR of the 16S rRNA gene was performed by Sequiserve (Vaterstetten, Germany) using the 5´-primer 609V (5´-GGA TTA GAT ACC CBD GTA-3´, corresponding to positions 785-802 in *E. coli* 16S rRNA gene) and the 3´-primer 907R (5´-CCG TCA ATT CMT TTG AGT TT-3´, corresponding to positions 907- 926 in the *E. coli* 16S rRNA gene). A 1519 bp amplicon revealed the presence of 11 heterogenic positions scattered over the rRNA gene, indicating the existence of at least two *rrn* copies. A sequence similarity of 96·3 % to the closest relative *V. rumoiensis* S-1^T was found. The sequences were aligned using CLUSTAL_X v. 2.09 (Larkin *et al*., 2007). A rooted phylogenetic tree was calculated according to Kimura´s 2-parameter model using Treecon v. 1.3b (Van de Peer & De Wachter, 1997) based on the 16S rRNA gene sequences using the neighbour-joining algorithm (Fig. 1). A bootstrap analysis based on 1000 replicates was performed to test the stability of individual branches (Felsenstein, 1985). As suggested by Thompson *et al*. (2005), *Campylobacter jejuni* ssp. *jejuni* was used as an outgroop. The isolates cluster with the clearly separated *V. rumoiensis* group. UPGMA and maximum likelihood calculations produced the same grouping of the novel isolates (data not shown).

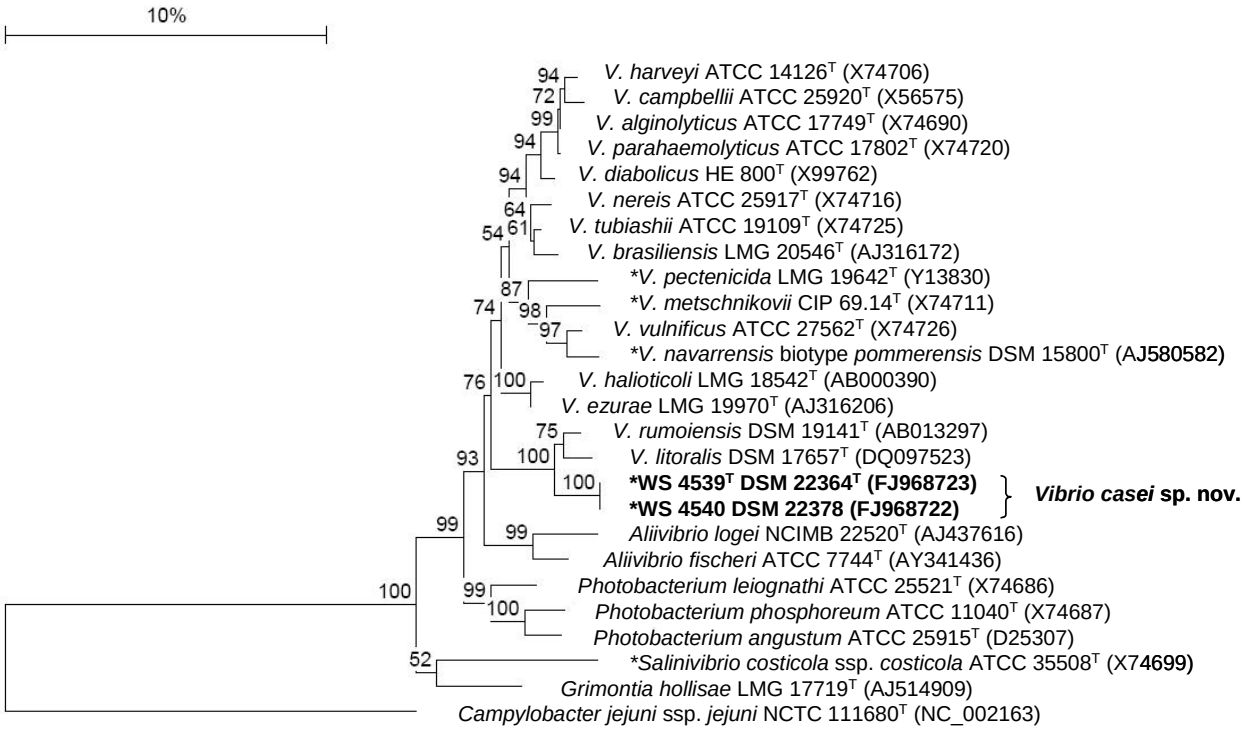

Figure 1. 16S rRNA gene based consensus neighbour-joining phylogenetic tree illustrating the position of WS 4539^T and WS 4540 within the *Vibrionaceae*. *Campylobacter jejuni* ssp. *jejuni* NCTC 11168^T was chosen as an outgroup. Bootstrap values, expressed as percentage of 1000 replications, are given at branching points. The bar indicates a sequence divergence of 10 %. Species containing a 14 or 15-bp insert in their 16S rRNA gene sequence are marked by an asterisk (for details see text).

The primer sequences and the thermal cycling conditions for *atpA*, *recA*, *rpoA* and *pyrH* were chosen according to Thompson *et al*. (2005) and Thompson *et al.* (2007). Phylogenetic trees based on these housekeeping genes supported the status of a novel species and are available as supplementary material in the online version of this article (Fig. S1 A-D).

All isolates shared a 14-bp insertion in their 16S rRNA genes, located between positions 188 and 189 in the 16S rRNA gene sequence *of E. coli* K12 MG1655 (Cannone *et al*., 2002, accession number J01695). The presence of this fragment in the mature rRNA could be demonstrated by PCR amplification of cDNA samples using the universal primer 27f and the insertion-specific primer 188r (for a detailed description see supplementary material). This insertion results in an elongated structure at helix 10 (calculations carried out using the ARB software; Ludwig *et al.,* 2004; Fig. S2 in the online version of this article). The insertion further distinguishes the isolates from their closest phylogenetic neighbours and provides a useful tool for quick identification. Such an insertional fragment

may move between species through horizontal gene transfer followed by recombination. Among the family of the *Vibrionaceae*, comprising 105 species, a 14 or 15-bp fragment was also detected in five other species which are marked by an asterisk in Fig. 1 (see also Table S2, supplementary material). Since these species inhabit different environments, the opportunity for horizontal gene transfer must remain speculative.

Based on these results we propose the name *Vibrio casei* for the novel species. *Vibrio* spec. R-27449 (accession number AJ967016) shares a 100 % identity of its 16S rRNA gene sequence to *V. casei* sp. nov.. It was found by Mounier *et al.* (2005) on the surface of the Irish smear-ripened cheese Milleens. While this report adds emphasis to the incidence of *V. casei* sp. nov. on cheese surfaces, this isolate was not described as a novel species.

5.2 Description of *Vibrio casei* sp. nov.

Vibrio casei (ca´se.i. L. gen. masc. n. casei of/ from cheese, because the organism was isolated from the surface of smear-ripened cheeses produced at geographically different places). The isolation place of the type strain was the Fromagerie de l´Ermitage in F-88140 Bulgnéville (N 48° 12´16” and W 5° 49´17”). The ref erence strain has been isolated at Les Fromage de Tradition in F-14127 Mondeville Cedex (N 49° 10´31” and W 0° 19´22”). Cells are Gram-negative, straight rods that are 0·6-1·2 µm wide and 1·5-1·8 µm long. Colonies grown aerobically on MA are cream, smooth, round or slightly irregular in shape and measure 1-2 mm in diameter after 3 days of culture on MA at 30 °C. Growth occurs between 2-30 °C and pH 6-8·5. Isolates were shown to require sodium ions and grew between 2 and 10 % NaCl. Motile but not swarming. Bioluminescence was not observed. Susceptible to the vibriostatic agent O/129. Catalase and oxidase positive. Facultatively anaerobic, nitrate is reduced to nitrite. The following substrates can be utilised as sole energy and carbon sources: glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose, gluconate, malate, citrate. Presence of ß-galactosidase, esculin hydrolase, alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosamidase. The predominant fatty acids are 16:1 *ω7c* and/ or 15:0 iso 2-OH, 18:1 *ω7c* and 16:0. The DNA G+C-content of the type strain is 41·8 mol%.

The GenBank accession numbers for the 16S rRNA, *atpA, recA, rpoA* and *pyrH* gene sequences of *V. casei* WS 4539^T, *V. casei* WS 4540 and *V. litoralis* MANO22D^T are FJ968710-FJ968723.

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5.4 Supplementary material

5.4.1 Phylogenetic analysis

Phylogenetic trees based on all five loci were calculated using representative type strains of *Vibrio* species. Sequences were either retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide) or from the Taxonomy of the *Vibrios* website (http://www.taxvibrio.lncc.br). The sequences were aligned using CLUSTAL_X v. 2.09 (Larkin *et al*., 2007). The distance matrices of the resulting multiple sequence alignments were calculated according to Kimura´s 2-parameter model using Treecon v. 1.3b (Van de Peer & De Wachter, 1997)

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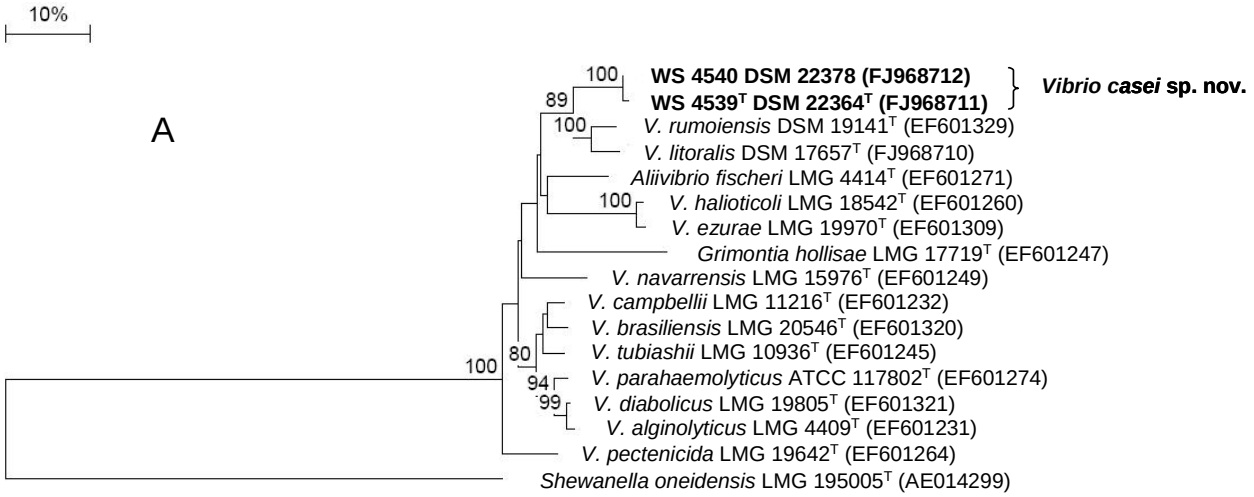
Table S1: Members and habitats of the *Vibrionaceae* carrying an insertional fragment in their 16S rRNA gene sequences.

organism	*Photobacterium profundum*	*Salinivibrio costicola* ssp. *costicola*	*Salinivibrio costicola* ssp. *vallismortis*	*Vibrio metschnikovii*	*Vibrio navarrensis* biotype *pommerensis*	*Vibrio pectenicida*	*Vibrio casei* sp. nov.
isolation source	sediment in a depth of over 5000 m	saline or hypersaline seas and soils, salted meats or brines	hypersaline pond located in Death Valley, California	fowl died of a choleraic disease	Baltic Sea, North Germany	pathogen of scallop larvae	surface of two French red smear cheeses (this study) and an Irish soft cheese (Mounier *et al*., 2005)
insert size	14 bp	15 bp	15 bp	15 bp	15 bp	15 bp	14 bp


10%
A
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100
80
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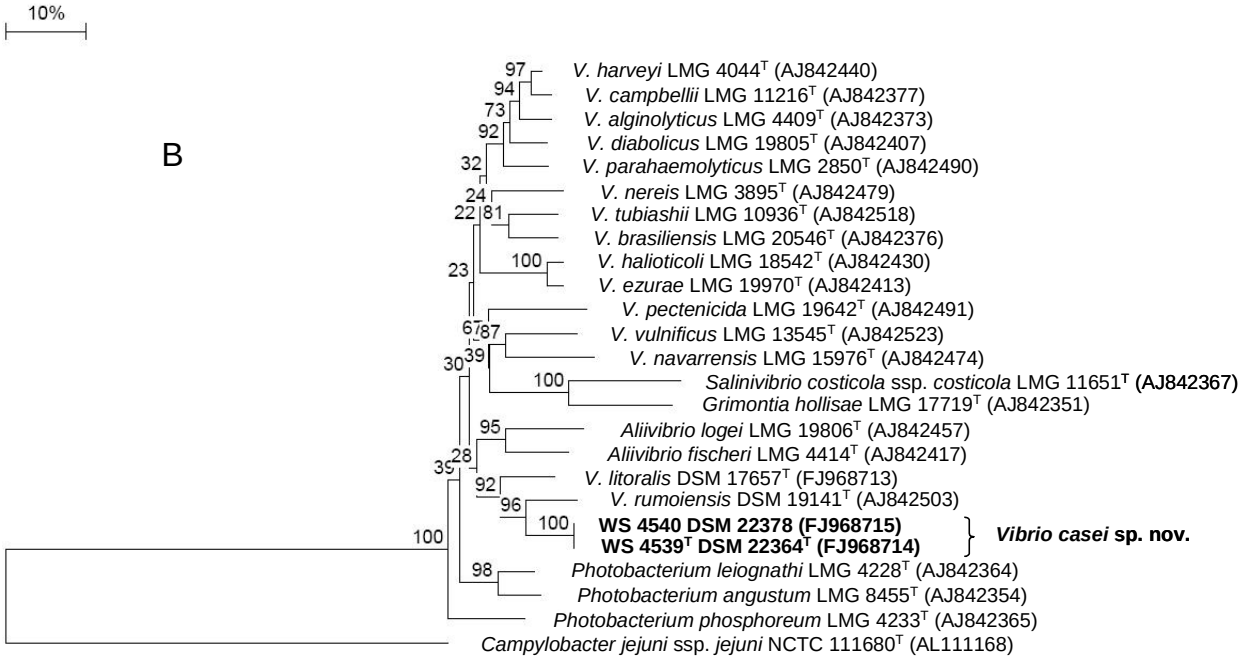
94
99
WS 4540 DSM 22378 (FJ968712)
WS 4539^T DSM 22364^T (FJ968711)
***Vibrio casei* sp. nov.**
V. rumoiensis DSM 19141^T (EF601329)
V. litoralis DSM 17657^T (FJ968710)
Aliivibrio fischeri LMG 4414^T (EF601271)
V. halioticoli LMG 18542^T (EF601260)
V. ezurae LMG 19970^T (EF601309)
Grimontia hollisae LMG 17719^T (EF601247)
V. navarrensis LMG 15976^T (EF601249)
V. campbellii LMG 11216^T (EF601232)
V. brasiliensis LMG 20546^T (EF601320)
V. tubiashii LMG 10936^T (EF601245)
V. parahaemolyticus ATCC 117802^T (EF601274)
V. diabolicus LMG 19805^T (EF601321)
V. alginolyticus LMG 4409^T (EF601231)
V. pectenicida LMG 19642^T (EF601264)
Shewanella oneidensis LMG 195005^T (AE014299)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V. harveyi LMG 4044^T (AJ842440)
V. campbellii LMG 11216^T (AJ842377)
V. alginolyticus LMG 4409^T (AJ842373)
V. diabolicus LMG 19805^T (AJ842407)
V. parahaemolyticus LMG 2850^T (AJ842490)
V. nereis LMG 3895^T (AJ842479)
V. tubiashii LMG 10936^T (AJ842518)
V. brasiliensis LMG 20546^T (AJ842376)
V. halioticoli LMG 18542^T (AJ842430)
V. ezurae LMG 19970^T (AJ842413)
V. pectenicida LMG 19642^T (AJ842491)
V. vulnificus LMG 13545^T (AJ842523)
V. navarrensis LMG 15976^T (AJ842474)
Salinivibrio costicola ssp. *costicola* LMG 11651^T **(AJ842367)**
Grimontia hollisae LMG 17719^T (AJ842351)
Aliivibrio logei LMG 19806^T (AJ842457)
Aliivibrio fischeri LMG 4414^T (AJ842417)
V. litoralis DSM 17657^T (FJ968713)
V. rumoiensis DSM 19141^T (AJ842503)
WS 4540 DSM 22378 (FJ968715)
WS 4539^T DSM 22364^T (FJ968714)
***Vibrio casei* sp. nov.**
Photobacterium leiognathi LMG 4228^T (AJ842364)
Photobacterium angustum LMG 8455^T (AJ842354)
Photobacterium phosphoreum LMG 4233^T (AJ842365)
Campylobacter jejuni ssp. *jejuni* NCTC 111680^T (AL111168)

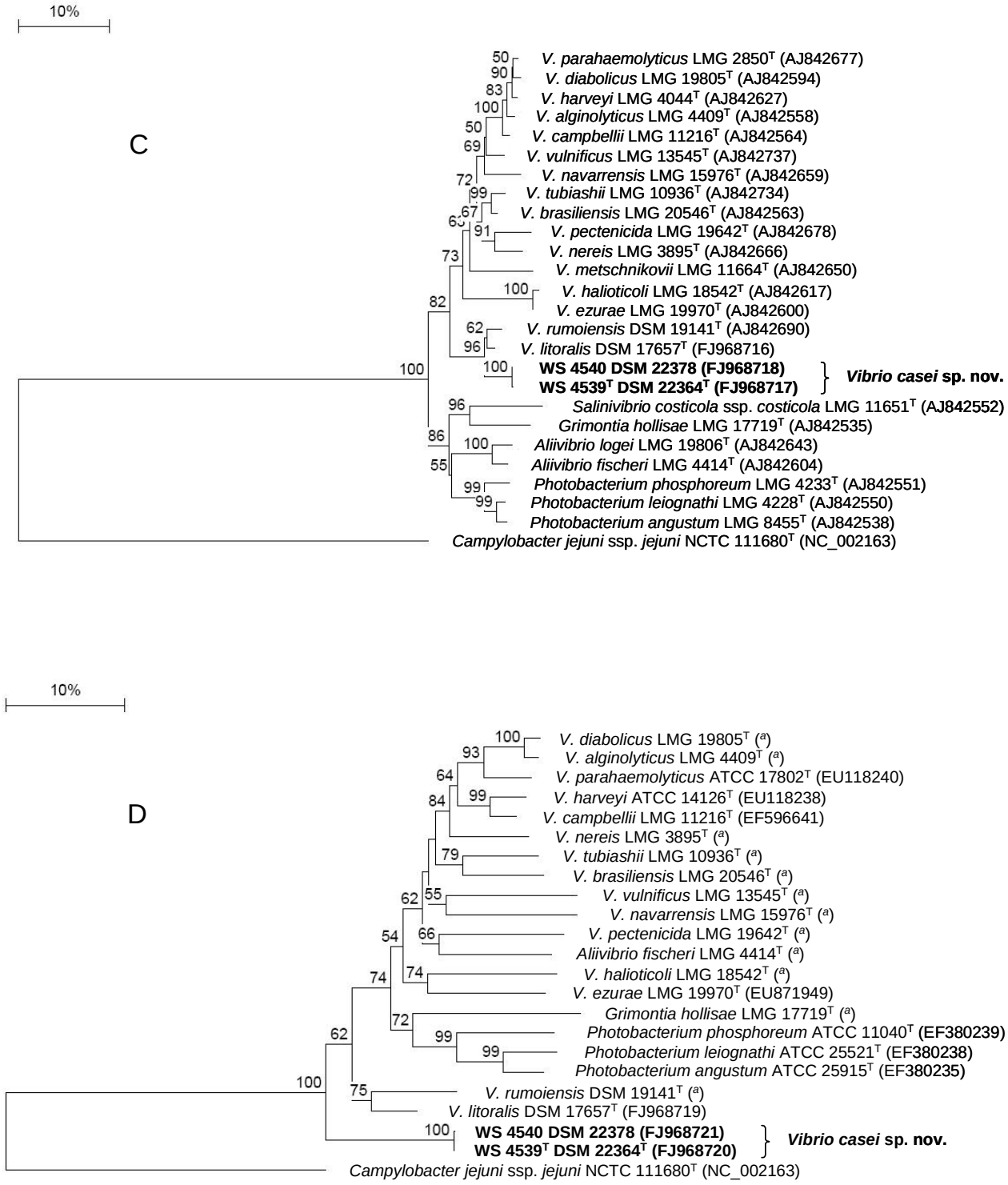

Figure S1. Neighbour-joining trees, based on comparison of selected house-keeping genes. As an outgroup, *Shewanella oneidensis* LMG 19005T was chosen for tree based on *atpA* sequences (A) and *Campylobacter jejuni* ssp. *jejuni* NCTC 11168T for trees based on *recA* (B), *rpoA* (C) and *pyrH* (D) sequences. Bootstrap values, expressed as percentage of 1000 replications, are given at branching points. The bar indicates a sequence divergence of 10 %. Published sequences were retrieved from the NCBI database or *(a)* from the Taxonomy of the *Vibrios* website (http://www.taxvibrio.lncc.br; no accession numbers available).

5.4.2 RNA preparation and reverse transcription

Total RNA of *V. casei* sp. nov. was extracted using Trizol (Invitrogen, Karlsruhe, Germany), following the instructions of the manufacturer. Reverse transcription was performed on extracts standardised to 1 µg RNA by spectroscopic measurements at 280 nm (Nanodrop ND-1000, Peqlab, Erlangen, Germany). The reaction volume of 20 µl contained DNase digested RNA (TURBO DNase, Ambion, Darmstadt, Germany), dNTP mixture (500 µM), DDT (5 mM), RNase OUT (40 U, Invitrogen) and first strand buffer. 1 pmol of insertion-specific primer 188r (5´-GCT TTR CTT CTT TCC TTT AA-3´, consisting of 10 nucleotides insertional region and 10 nucleotides downstream of insertion) was added. Samples were completed by the addition of Superscript III (200 U, Invitrogen); for the blanks ultrapure water was added instead. As positive control, a second approach was reversely transcribed using the internal primer 198r (1 pmol, 5´-AAG GTC CCC CGC TTT RCT TC-3´) which covers only the region downstream of the insertion. The incubation conditions for the RT reaction were as follows: 25 ℃ for 5 min, 50 ℃ for 90 min and 70 ℃ for 15 min. PCR amplification of cDNA was carried out in a 50 µl reaction mixture containing 1 pmol of primers 27f and 188r, 200 µM dNTP mixture, 1 U Thermoprime Plus Polymerase (Thermo Scientific) and 20 ng of template. PCR conditions were as follows: 95 ℃ for 2 min, 30 cycles of 95 ℃ for 20 sec, 45 ℃ for 30 sec, and 72 ℃ for 15 sec. The size of the resulting fragment was in accordance with the position of the 14-bp insertion in the 16S rRNA gene and could be further proven by sequencing. No PCR amplification could be achieved when RNA samples did not undergo reverse transcription (blank samples) or when water was added instead of template (blind samples).

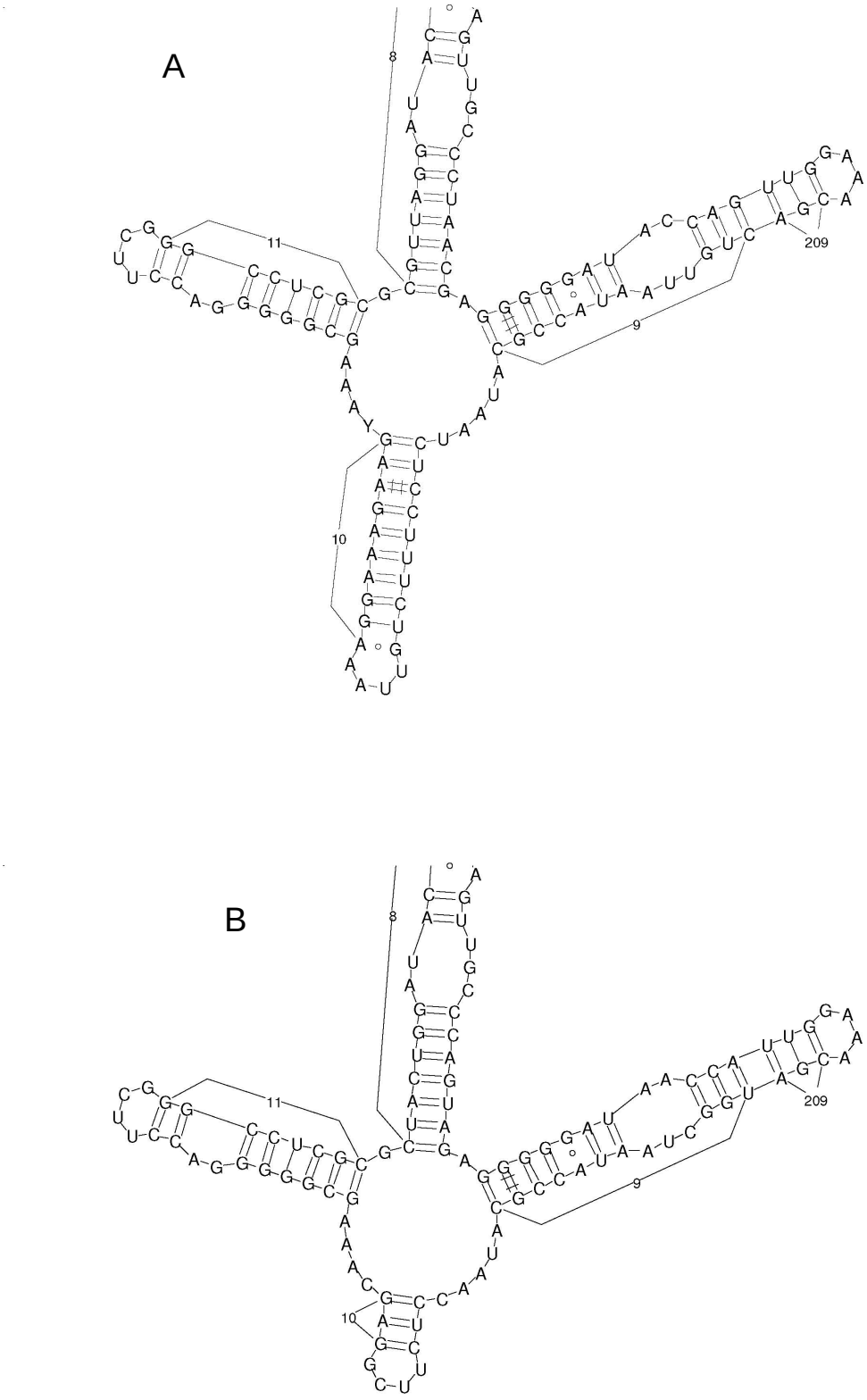

Figure S2. Model of secondary structure of mature 16S ribosomal RNA molecule, A) short helix 10 as calculated for *V. rumoiensis* and B) elongated helix 10 as calculated for *V. casei* sp. nov. Models were constructed using the ARB software and kindly provided by Dr. Wolfgang Ludwig, Chair of Microbiology, Technical University of Munich.

6 General discussion

6.1 Determination of anti-listerial activity- limitations and opportunities

The first aim of the current study was to set up standardized methods for the detection and quantification of the anti-listerial potential of undefined, complex cheese ripening consortia. For this purpose, four different methods were established and compared using Pearson's analysis (see Chapter 2). Significant correlations were obtained between the two approaches working with the original ripening flora which was stored at -80 °C, and the two methods including successive propagation steps on a model cheese. After such propagation, a stable anti-listerial activity could be reported which, in most cases, was increasing during the course of the experiment. Obviously, the measurement of anti-listerial activity is less influenced by the physiological state of the inoculated cells when successive ripenings steps have been performed.

However, this procedure led to a dramatic microbial succession, in general favouring Gram negative and lactic acid bacteria. Coryneform species, which constitute the typical ripening flora of red smear cheeses, were not competitive under the experimental conditions. The diversity of strains, as evaluated using FT-IR spectroscopy, was also decreasing upon each ripening step. A deduction of species combinations for *Listeria* reduction on cheeses is therefore limited.

Furthermore, it was not possible to recover, to store and to process microfloras from cheese samples without modifying important properties such as the balance between the strains or their physiological state. Therefore, the standardized procedures of an anti-listerial activity measurement may not fully reflect the conditions that occurred during the production of the cheeses on which the microfloras developed.

However, a reproducible inhibition of a five strain cocktail of 10^4 cfu/g *L. innocua* was achieved using four different methods in each three independent runs. The comparison of both *in vitro* and *in situ* applications is of great interest to deepen our knowledge of complex microbial communities and their potential to prevent proliferation of pathogenic or spoilage bacteria. Since there is no standard procedure yet to determine the anti-listerial activity of cheese ripening consortia, the development of new methods will contribute to an improvement of future experimental setups.

6.2 Comparative flora analysis

Since only a fraction of the microbial consortia inhibited Listeria, the anti-listerial activity observed is not just a property of a complex microbial community due to general species competition, but depends critically on the species composition of a cheese microbial community. The analysis of flora composition was carried out using FT-IR spectroscopy, a technique suitable to distinguish microorganisms at strain level.

Four anti-listerial and two non-inhibitory consortia were investigated, revealing each flora having unique properties in terms of species composition and diversity (Chapter 3). Coryneform species, which constitute the main part of a ripening consortium, are represented between 43 and 91 %. An unexpectedly high fraction of lactic acid bacteria was detected in three of the four anti-listerial consortia investigated. While this may partly be due to some carry over of LAB from the cheese curd during sampling of the microbial consortia, it should be noted that the majority of the lactic acid bacteria detected are not members of starter cultures typically used for cheese production.

The composition of bacterial communities producing an anti-listerial supernatant was by no means stable. The coryneform bacteria did not multiply or even disappeared during the eight-hour cultivation in broth. The growth of Gram negative and lactic acid bacteria was favoured instead and they highly increased in numbers (Chapter 4). Again, a deduction of species combinations necessary to express an anti-listerial effect in CFS was not possible. In recent studies, flora analysis using strain typing methods revealed species being often present as single clones (Brennan *et al*., 2002; Mounier *et al*., 2005). This was not the case for the bacterial communities examined in this work.

In the course of flora analysis the novel species *Vibrio casei* was detected and validly published (Chapter 5). The incidence of *V. casei* is estimated to be less than 0.5 % in both consortia (Reflets de France Petit Livarot and Petit Munster Ermitage, respectively). The high salt concentration and the low ripening temperature in a cheese making facility may act as selective forces for *Vibrio*, which is known to occupy brackish and marine waters. It remains speculative if cheese brines are the major habitat of *V. casei* or if it is a secondary contamination which could successful establish itself in the ripening room.

6.3 Exploring inhibitory properties of single strains

A high-throughput screening was carried out on solid medium to test several hundred LAB colonies of two anti-listerial consortia for secretion of antagonistic compounds (Chapter 4). Ten strains of lactococci and four strains of enterococci displayed a degree of inhibition against *L. monocytogenes* EGDe. However, the size of clearing zones in a confluent *Listeria* lawn suggested a low diffusion potential of the substances since the inhibition halos were restricted to the growth zone of the test organisms. The alternative indicator strains *B. cereus* and *S. aureus* showed a higher sensitivity and did not grow in a 2-3 mm area surrounding the LAB colonies. The Gram negative organisms *S. enterica* Typhimurium and *E. coli* DH5α were less or not affected by these substances, respectively (data not published).

The effect was unstable since the selected antagonistic colonies failed to reproduce inhibition when transferred from fully grown plates to new plates individually. A similar scenario was reported for a screening carried out on hydrophobic grid-membrane filters, where only 99 of 283 colonies maintained their inhibitory action after re-streaking (Carnio *et al*., 1999). Bacteriocin expression is often regulated via a quorum-sensing mechanism, mediated by peptide pheromones or the bacteriocin, which acts as an autoinducer. In recent studies, the early or increased bacteriocin production of LAB upon co-cultivation with different Gram positive bacteria in liquid media was demonstrated (Barefoot *et al*., 1994; Chang *et al*., 2007; Tabasco *et al*., 2009). In other cases bacteriocin production was totally dependent on the presence of inducing bacteria and did not occur when the producer was grown alone (Maldonado *et al*., 2004a; Rojo-Bezares *et al.,* 2007). The inducing factors of lactacin B and kimchicin GJ7 were shown to have a proteinaceous nature and are most probably located on the cell wall of sensitive bacteria (Barefoot *et al*., 1994; Chang *et al*., 2007). *Streptococcus macedonicus* produces macedocin only when grown in milk. It was demonstrated that the presence of specific casein fragments, resulting from milk degradation by the producing strain, are necessary for induction of gene transcription (Georgalaki *et al*., 2009). This elegant study adds further emphasis on the influence of environmental factors on bacteriocin gene expression.

A screening carried out on solid medium was obviously not suitable to identify strains responsible for the inhibition reported in liquid medium. For consortium FC12, which displayed a strong bactericidal activity both in liquid media and on a model cheese, supernatants of 41 strains constituting the “original” flora were obtained. Single strain CFS

were inoculated according to Method A (Chapter 2), resulting in viable counts of 10^9 cfu/ml *L. monocytogenes* EGDe after 24 h of incubation. Only a slight decrease in growth (1 log unit reduction) was observed when the indicator strain was cultured in the CFS of enterobacteria and staphylococci (data not published). This was most probably due to nutrient depletion since enterobacteria are rapid growing organisms and staphylococci are closely related to *Listeria*, thereby having similar substrate requirements (Jameson effect).

6.4 Genetic determinants of inhibition

The presence of 23 bacteriocin genes typically produced by *Lactobacillus*, *Lactococcus* and *Enterococcus* species was explored in a PCR based screening of 25 consortia (Chapter 3). The eight bacteriocins identified comprised classes I, IIa, IIb, IIc and III according to the classification of Drider *et al*. (2006). Amplification of lactococcin G in total RNA extracts of five (non-inhibitory) consortia was successful. Other bacteriocins are either not expressed on cheese surfaces or DNA from dead cells was amplified in the first approach. Eventually, mRNA species of bacteriocin genes detected in the DNA were degraded during the storage of the consortia at -80 ℃. To our knowledge, this is the first time that *in situ* transcription of bacteriocins on a cheese surface is demonstrated.

6.5 Partial purification of inhibitory molecules secreted in complex culture supernatants

For the production of potent anti-listerial CFS, microbial cheese ripening consortia were suspended in PC3+ broth and incubated for an eight-hour period. No anti-listerial effect was detected in supernatants prepared from consortia cultured for only 10 minutes. In contrast, growth of *Listeria* was often stimulated then and reached levels higher than in nutrient broth. It is concluded that actively growing consortia are necessary to produce the heat-stable, broadband antagonistic substances which are described in Chapter 4. The inhibitory principle of CFS I was neither destroyed by addition of 30 % methanol nor by freeze-drying. It was stable over a period of 3 years in aqueous solution at -20 ℃ and it was not degraded by proteinase K. The involvement of a proteinaceous compound in the anti-listerial effect therefore appears unlikely. *L. monocytogenes* isolates of four different serovars (1/2a, 1/2b, 3a and 4b), *Bacillus* sp., *and S. aureus* as well as the Gram negative bacteria *Salmonella enterica* Typhimurium and *E. coli* DH5α were often killed within 6 h after exposure to CFS I.

A partial purification was achieved in two consecutively reverse phase HPLC runs, yielding one active peak. When the active fraction was subjected to liquid chromatography-mass spectrometry (LC-MS), several mass peaks were detected using the positive or negative electrospray ionization (ESI) mode (data not published). At this state the supernatant was far too complex for direct peptide sequencing because a mixture of substances was present. Further research is needed to identify these substances and figure out a potential antagonistic function *in situ* on cheese surfaces. Besides bacteriocins, short-chain fatty acids (SFCA, Caplice & Fitzgerald, 1999) or other anti-bacterial peptides (Lopez-Exposito *et al*., 2006) may be involved in this inhibition.

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