Potent antilisterial cell-free supernatants produced by complex red-smear cheese microbial consortia

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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 obtained from consortia grown for 8 h in liquid medium, a strong bactericidal activity was observed in several cases. The cell-free supernatants of 2 of these consortia (I and II) reduced an initial *L. monocytogenes* inoculum of 5×10^7 cfu/mL to zero after 24 h of incubation. No inhibitory substances could be washed off the complex consortia when incubated for a 10-min period. A taxonomical analysis of the antilisterial consortia I and II using Fourier transform infrared spectroscopy yielded a considerable species diversity, with lactic acid bacteria increasing strongly during the 8-h cultivation. Therefore, 23 lactic acid bacteria bacteriocin genes were assayed using specific PCR primers, identifying 3 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 lactic acid bacteria isolates of consortia I and II displayed no or only weak inhibition of *L. monocytogenes* on solid medium. The complex cell-free supernatants I and II, in contrast, exhibited an unusually broad inhibitory spectrum, killing *L. monocytogenes* ssp., *Bacillus* spp., *Staphylococcus aureus*, as well as gram-negative bacteria such as *Escherichia coli* DH5 α and *Salmonella enterica* serovar Typhimurium. Inhibition could not be abolished by heating to 100°C or by proteinase K treatment. Initial purification of an inhibitory substance from consortium I by solid-phase extraction and HPLC indicates the presence of rather small, extremely stable compounds, which, most probably, are not bacteriocins.

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

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 and Donachie, 1997). Its psychrophilic, acid-tolerant 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 a saprophytic to an intracellular lifestyle. Its ability to spread from cell to cell and thus avoid 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 to 30% among persons developing disease (de Valk et al., 2005).

The food-borne nature of *L. monocytogenes* was not recognized before the 1980s, when several listeriosis outbreaks were linked to the consumption of contaminated soft cheeses (Farber and 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, further complicates the control of *Listeria*. 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). Undefined cheese-surface consortia with antilisterial properties have been described in the past, sometimes containing bacteria producing bacteriocins (Valdes-Stauber and Scherer, 1994; Saubusse et al., 2007). Bacteriocins are small, ribosomally synthesized peptides often acting on the

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cytoplasmic membrane of target bacteria, thereby causing dissipation of the proton motive force, leakage of small molecules, and subsequent cell lysis (for review, see Jack et al., 1995; Cotter et al., 2005). 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 because they have to compete with the resident, often very stable house flora of a certain ripening cellar (Feurer et al., 2004; 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 that 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 antilisterial cheese-surface consortia, ii) to analyze their microbial composition in terms of species composition and diversity, and iii) to characterize the substances produced by the complex microbial populations.

MATERIALS AND METHODS

Collection of Microbial Consortia

Forty-nine 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 (Seward Ltd, Worthing, UK). Suspensions were centrifuged at $6,600 \times g$ for 25 min at 4°C and washed twice in saline solution to remove excessive cheese fat. After addition of glycerol to a final concentration of 15% and thorough mixing, aliquots of 2 mL each were frozen at -80°C (glycerol culture). All samples underwent routine analysis according to DIN EN ISO 11290-1 (2006) to detect natural contamination with *Listeria* sp.

Supernatant Preparation

Five hundred microliters of the glycerol culture was used to inoculate 15 mL of plate count broth supplemented with 3% sodium chloride (PC3+ broth) to adjust for the salty conditions of cheese brines (Valdes-Stauber et al., 1991). The flasks were incubated for 10 min or 8 h at 30°C with shaking (180 rpm). The cells were pelleted by centrifugation at $12,000 \times g$ for 10 min

at 4°C. The supernatants were filter sterilized (Millex GP 0.2- μ m filter units, Millipore, Schwalbach, Germany) and stored at -20°C until use. The preparation of cell-free supernatants (CFS) was carried out at least 3 times independently. The pH was measured after thawing using universal indicator strips (Macherey-Nagel, Düren, Germany); the contents of lactic acid and hydrogen peroxide were 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 on *Listeria* in poured plates using tryptic soy softagar (17.0 g/L of tryptone, 6.0 g/L of yeast extract, 3.0 g/L of soy peptone, 5.0 g/L of NaCl, 2.5 g/L of D-glucose, 2.5 g/L of K₂HPO₄, and 7.5 g/L of agar-agar).

Cultivation Assay for Detection of Antilisterial Activity

In the cultivation assay, *L. monocytogenes* EGDe (serovar 1/2a) was grown overnight in brain heart infusion broth (Merck) and inoculated 1:20 in PC3+ broth. After 2 h of incubation, cells were diluted to obtain a cell density of 5×10^8 cfu/mL. Five milliliters of the 49 CFS was inoculated with 0.5 mL of the *Listeria* suspension and incubated for 24 h at 30°C and 180 rpm. Samples of 100 μ L were serially diluted and plated onto PC3+ agar in duplicate. Viable cells of the indicator strain were counted after 2 d of aerobic incubation at 30°C. Bacteriostatic activity was assumed when *Listeria* was not able to proliferate in the CFS but did not die; bactericidal activity was defined as a reduction of the initial inoculum of at least 2 log units.

Initial Characterization of Antilisterial Substances

Consortia I and II, representing 2 different Munster cheeses, displayed a high inhibitory capacity among 8 antagonistic CFS. The complex CFS I and II were characterized further using 1×10^3 cfu/mL *L. monocytogenes* EGDe. Both CFS were exposed to 100°C for 1 min and to 121°C for 15 min before inoculation. 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 (3,800 U/mg, Sigma-Aldrich, Hamburg, Germany). In a combined approach, proteinase K was added after the reaction of catalase was completed. Both CFS were subjected to serial 2-fold dilutions up to 1:16 in PC3+ broth. The inhibitory spectrum was determined in a cultiva-

tion assay (see above) using 1×10^3 cfu/mL each of the alternative indicator strains listed in Supplemental Table S1 (<http://www.journalofdairyscience.org/>). All experiments were conducted at least twice.

Isolation of Inhibitory Lactic Acid Bacteria

Eight-hour liquid cultures of consortia I and II were plated onto All Purpose Tween 80 agar plates (Merck) and incubated for 2 d at 34°C in an anaerobic jar containing the catalyst Anaerocult IS (Merck). Several hundred lactic acid bacteria (**LAB**) were randomly picked and transferred to new All Purpose Tween 80 agar plates. A 2-d period for the production of inhibitory substances under anaerobic conditions was given. Overnight cultures of the indicator strains listed in Supplemental Table S1 were used to inoculate tryptic soy softagar at 1:100. Ten milliliters of the seeded softagar was poured over agar plates containing picks or streaks of the LAB (overlay assay). After an overnight incubation at 30°C under aerobic conditions, antagonistic acting colonies were identified on the basis of clearing zones they caused in the confluent lawn of the indicator strains. Inhibitory strains were recovered from the plates, streaked to demonstrate purity, and applied to the assay a second time.

Biodiversity Analysis by Fourier Transform Infrared Spectroscopy

The composition of both the original and the 8-h propagated consortia I and II were studied in detail using Fourier transform infrared spectroscopy. One hundred bacterial colonies were picked randomly from PC3+ agar plates after 3 to 5 d of aerobic incubation at 30°C. Lactic acid bacteria were cultivated anaerobically on All Purpose Tween 80 agar at 34°C (see above). The sample preparation to obtain reliable infrared spectra was carried out as described (Kümmerle et al., 1998; Oberreuter et al., 2002). For identification purposes, the spectra recorded were compared with a reference database made up of spectra of known strains grown under the same conditions as above. Two independent measurements were conducted using an HTS-XT Fourier transform infrared spectrometer (Bruker, Karlsruhe, Germany). For data processing, OPUS software version 6 (Bruker) was used. The spectral distances of the isolates, reflecting the similarity of 2 spectra by comparing the size of nonoverlapping areas, were used to discriminate the isolates at strain level. Cluster analysis was performed using the average linkage algorithm (Tindall et al., 2000), visualizing the abundance ratio of strains in a dendrogram.

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. Five hundred microliters of glycerol culture was mixed with 1 mL of Rotiphenol. After proteinase K treatment for digestion of disturbing proteins, the enzyme was heat inactivated. The PCR were performed using specific primer pairs for 23 different bacteriocin genes, listed in Supplemental Table S2 (<http://www.journalofdairyscience.org/>; oligonucleotide primers purchased from Invitrogen, Karlsruhe, Germany). The PCR amplification was carried out in a 50- μ L reaction mixture containing 1 pmol of specific primers, 200 μ M deoxynucleotide triphosphates mixture (Thermo Scientific, Hamburg, Germany), 1.5 mM MgCl₂, 1 U of Thermoprime Plus Polymerase (Thermo Scientific), and 100 ng of template. The PCR conditions were as described recently (see Supplemental Table S2). The DNA extracts of bacteriocin-producing strains, listed in Supplemental 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.

RNA Extraction and Reverse Transcription

Total RNA of consortia I and II was extracted directly from the thawed consortium using Trizol (Invitrogen), following the instructions of the manufacturer. Reverse transcription was performed on extracts standardized to 1 μ g of RNA by spectroscopic measurements at 260 nm (Nanodrop ND-1000, Peqlab, Erlangen, Germany). The reaction volume of 20 μ L contained DNase digested RNA (TURBO DNase, Ambion, Darmstadt, Germany), 500 μ M dNTP mixture, 5 mM dithiothreitol (Invitrogen), 40 U of RNase OUT (Invitrogen), and first strand buffer. One picomole of reverse primers (specific for lactococcin G, helveticin J, curvacin A, acidocin B, and cytolysin) was added for specific reverse transcription. Samples were completed by the addition of 200 U of Superscript III (Invitrogen); in blank samples no enzyme was added. The incubation conditions for the reverse transcription reaction were as follows: 25°C for 5 min, 50°C for 90 min, and 70°C for 15 min. The amplification reactions using cDNA as template were carried out as described for the genomic DNA 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).

Figure 1. High performance liquid chromatography run of solid-phase extraction fraction eluted with 20% methanol (S2). Fraction 8 (H8) showed the greatest bioactivity in the cultivation assay, reducing 1×10^3 cfu/mL of *Listeria monocytogenes* EGDe to 2×10^2 cfu/mL after 24 h of incubation.

Fractionation of Complex Supernatant I by Solid-Phase Extraction

Aliquots (2.5 mL each) of CFS I were applied onto the top of a C18- SPE (solid-phase extraction) cartridge (6 mL, 1 g, 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), and solvents used were of HPLC grade (Merck). 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, vol/vol; 6 mL; fraction S2) in 20% steps (S3–S5, 6 mL each), and methanol (10 mL; fraction S6). The fractions S1 through S6 were concentrated in a vacuum (40°C) and, after freeze-drying, resuspended in 1 mL of water. Bioactivity of individual fractions was determined in the cultivation assay.

HPLC of CFS I: Separation of SPE Fractions S2 and S3

Fractions active against *L. monocytogenes* EGDe were suspended in 1 mL of water, and aliquots (250 μ L) were separated by means of semipreparative HPLC. The HPLC system consisted of an HPLC pump system PU 2087; a high-pressure gradient unit; and a PU-2075 UV detector using a semipreparative reversed-phase (RP) column, Luna Phenyl Hexyl, 10.0 $\times$ 250 mm,

5 μ m (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, 10 subfractions each of S2 and S3 (S2/H1–H10 and S3/H1–H10, respectively; Figure 1) were suspended in 10 mL of water and freeze-dried to determine the bioactivity of the individual fractions (see above).

RESULTS AND DISCUSSION

Antilisterial 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. If antilisterial substances are already present in the consortium, they should be washed off the cells. However, no antilisterial effect was detected in these supernatants. In contrast, growth of *Listeria* was often stimulated and reached levels of 3×10^9 cfu/mL (growth in PC3+ broth reached 1×10^9 cfu/mL). This difference was possibly due to the glycerol added to the consortia for storage. It is concluded that, in the microbial consortia directly isolated from cheeses, no or only negligible amounts of antagonistic substances are present, which can be washed off the

Figure 2. Effect of cell-free culture supernatants (CFS) from consortium I (squares) and consortium II (triangles). Two different experiments are shown. 1) Filled symbols: 10^7 cfu/mL of *Listeria monocytogenes* EGDe was inoculated in each CFS and sampled every hour. 2) Open symbols: 10^3 cfu/mL of *L. monocytogenes* was inoculated in serial 2-fold dilutions of each CFS and sampled once after 24 h. PC3+ = plate count broth supplemented with 3% sodium chloride.

cells. Therefore, a longer incubation period of 8 h at a high initial cell density (approximately 3×10^8 cfu/mL) was applied. A high initial cell density was chosen to trigger the production of antagonistic substances, which might be linked to quorum-sensing signaling cascades (Kleerebezem, 2004; Navarro et al., 2008). The incubation was performed at 30°C to fit most physiological groups constituting a cheese ripening consortium, enabling greater metabolic rates. When 5×10^7 cfu/mL of *L. monocytogenes* EGDe (serovar 1/2a) was inoculated in 8-h CFS, 8 of 49 supernatants displayed a bactericidal effect, sometimes reducing the inoculated *L. monocytogenes* to zero after 24 h, and 5 CFS acted bacteriostatically. The strongly inhibitory supernatants from consortia I and II, 2 different Munster cheeses from the French retail market, were chosen for further studies. Both CFS had a content of lactic acid of 4.4 mM and a content of H₂O₂ of less than 15 μ M; pH values were 6.0. The minimal inhibitory concentration of H₂O₂ in *L. monocytogenes* lies between 0.26 and 2.2 mM (Romanova et al., 2002); the minimal inhibitory concentration of lactic acid has been determined to be around 220 mM at refrigeration temperatures (González-Fandos and Dominguez, 2006). Obviously, the actively growing consortia, in which at the same time a biodiversity shift took place (see below), produced soluble antilisterial substances other than lactic acid or hydrogen peroxide.

Table 1. Characteristics of supernatants I and II and viable cell counts after 24 h when 10^3 cfu/mL of *Listeria monocytogenes* EGDe was cultured in untreated, heat-treated, and enzyme-treated cell-free supernatants (CFS)¹

Characteristic or treatment of CFS	Viable <i>L. monocytogenes</i> EGDe after 24 h of incubation in	
	CFS I	CFS II
Untreated	0	0
1 min at 100°C	0	1.1×10^4
15 min at 121°C	1.8×10^3	2.3×10^6
Proteinase K	0	9.0×10^2

¹Growth of *L. monocytogenes* EGDe in plate count broth supplemented with 3% sodium chloride with the addition of enzymes resulted in 10^9 cfu/mL (data not shown). Results of a representative experiment are given.

The serially 2-fold diluted CFS of consortia I and II, inoculated with 10^3 cfu/mL of *Listeria*, exhibited different bactericidal potentials, with CFS I clearly being more active (Figure 2, open symbols). Also, CFS I was more active when a kinetics of inhibition of 10^7 cfu/mL of *Listeria* was measured (Figure 2, filled symbols). In addition, supernatants I and II differed in other respects. Whereas boiling did not affect the antilisterial potential of CFS I, it abolished the bactericidal, but not the bacteriostatic, effect of CFS II, suggesting a mixture of active components. Upon autoclaving, CFS I maintained a bacteriostatic action, whereas in CFS II, inhibition of listerial growth was almost completely abolished. A treatment with proteinase K left CFS I unaffected, indicating the involvement of nonpeptide molecules, whereas CFS II was partially susceptible to proteinase K, indicating the participation of a proteinaceous compound (results summarized in Table 1).

The inhibitory spectrum of both consortia was unexpectedly broad (Table 2), comprising *L. monocytogenes* isolates of 4 different serovars (1/2a, 1/2b, 3a, and 4b), *Bacillus* spp., *Staphylococcus aureus*, as well as the gram-negative bacteria *Salmonella enterica* Typhimurium and *Escherichia coli* DH5 α . Whereas 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.

Distinct Shifts of Biodiversity upon Cultivation in Liquid Medium

A taxonomic analysis of both consortia was conducted using Fourier transform infrared spectroscopy (Wenning et al., 2006). Both consortia grown on the cheese surface showed a high biodiversity (Figure 3, gray bars). Forty-

Figure 3. Biodiversity of consortia I and II. Gray bars indicate the original species composition, and black bars reflect the composition after 8 h of incubation in broth. The identities of 100 isolates per consortium and per time point were analyzed. The insets show the composition of the lactic acid bacteria.

eight strains of different physiological groups could be distinguished in consortium I, with 45% belonging to the LAB, 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 LAB, and 8% to the gram-negative bacteria. *Arthrobacter*, *Corynebacterium*, and *Staphylococcus* species were represented in high numbers. Gram-negative bacteria have been shown to constitute an important group in

ripening consortia of Irish and French smear cheeses (Feurer et al., 2004; Mounier et al., 2005). Compared with recent biodiversity studies (Maoz et al., 2003; Rea et al., 2007), the high number of LAB in the surface consortium is unusual. This may be due to some carryover of LAB from the cheese matrix during sampling of microbial consortia.

The initial composition of the consortia changed considerably upon the 8-h cultivation in broth (Figure 3, black bars). The coryneform bacteria, which constituted

Table 2. Viable cell counts after 24 h of gram-positive and gram-negative indicator strains cultured in cell-free supernatants (CFS) I and II¹

Indicator strain	Serovar	CFS I	CFS II	PC3+ broth ²
<i>Listeria monocytogenes</i> EGDe	1/2a	0	0	1.1×10^9
<i>L. monocytogenes</i> WSLC 1416	1/2a	0	7.1×10^2	1.6×10^8
<i>L. monocytogenes</i> WSLC 1499	1/2b	0	1.2×10^3	3.4×10^8
<i>L. monocytogenes</i> WSLC 11149	3a	0	7.6×10^2	5.3×10^8
<i>L. monocytogenes</i> WSLC 1364	4b	0	1.8×10^3	5.5×10^8
<i>Staphylococcus aureus</i> ATCC 12600		0	0	2.1×10^9
<i>Bacillus megaterium</i> WS 1537		0	0	1.1×10^9
<i>Bacillus cereus</i> F4810/72	Emetic	0	2.3×10^1	3.9×10^8
<i>Bacillus cereus</i> ATCC 10987	Nonemetic	0	0	3.7×10^8
<i>Salmonella enterica</i> ATCC 14028	Typhimurium	0	0	2.5×10^9
<i>Escherichia coli</i> DH5 α ATCC PTA-1798		0	0	1.1×10^8

¹Results of a representative experiment are given.

²Plate count broth supplemented with 3% sodium chloride.

the predominant group in the smear, did not multiply or even disappeared, whereas the growth of gram-negative bacteria and LAB was favored, resulting in a doubling of their numbers. Taking into account that all isolates with less than 1% of the total cell count are below the detection limit of the method, the biodiversity in broth after an 8-h incubation is very different compared with the original diversity in the smear. 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 antilisterial potential was reported after repeated inoculation of the consortia on the cheese, concomitant with an increase of LAB and gram-negative cell counts. Therefore, we suggest that coryneform bacteria did not produce the inhibitory substances. Rather, the inhibition observed was thought to be due to bacteriocin production of the LAB.

Detection of Bacteriocin Genes by PCR and Isolation of Antilisterial LAB

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. Polymerase chain reactions for consortium I yielded positive results for genes encoding lactococcin G, helveticin J, and curvacin A. For consortium II, amplification of genes encoding 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 antilisterial LAB was performed using anaerobic incubation on all purpose tween agar. Among 776 LAB colonies of the 8-h propagated consortium I, only 25 antilisterial colonies were found, comprising 4 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 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 *L. lactis* or *L. 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 *L. helveticus* nor *Lactobacillus acidophilus* (producer of acidocin B) was 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., 2004; 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). It is possible that 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 to 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 to both heat and proteolytic enzymes (Joerger and Klaenhammer, 1986). Proteinase K and heating to 100°C decreased the inhibitory 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 from bacteriocins are involved in the strong listericidal effect. Therefore, supernatant I was further investigated.

Partial Purification of Inhibitory Fractions from CFS I

The complex supernatant of consortium I was separated into 6 fractions (S1–S6) using SPE on RP-18 columns. 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 1). Activity was recovered from fractions H3 and H8, with H8 having the greatest inhibitory potential against *L. monocytogenes* EGDe in the cultivation assay (Figure 1, inset). Compared with the native CFS I, fraction H8 displayed an antilisterial activity of only 90%, reducing the initial 10^3 cfu/mL to 10^2 cfu/mL after 24 h. The cause of the decrease in activity is unknown. We can possibly exclude denaturation of inhibitory proteins because the inhibitory potential of native CFS I was destroyed neither by addition of 30% methanol nor by freeze-drying and it was stable over a period of 3 yr at -20°C (data not shown). 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. 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. Short-chain fatty acids (Caplice and Fitzgerald, 1999) or antibacterial peptides (López-Expósito et al., 2006) might be involved in this inhibition.

CONCLUSIONS

Intrinsic microbial food protection, often mediated by bacteriocinogenic starter cultures, becomes more important with increasing consumer awareness of genetically modified organisms and questionable additives. In the present study, production of potent inhibitory substances by 2 cheese ripening consortia in an 8-h liquid culture was monitored. While culturing, the consortia underwent a significant microbial succession. However, the antagonistic potential of these substances 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 to identify these substances and their producers and to figure out a potential antagonistic function in situ in cheese microbial consortia.

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