Growth Reduction of *Listeria* spp. Caused by Undefined Industrial Red Smear Cheese Cultures and Bacteriocin-Producing *Brevibacterium linens* as Evaluated In Situ on Soft Cheese

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The undefined microbial floras derived from the surface of ripe cheeses which are used for the ripening of commercial red smear cheeses have a strong impact on the growth of *Listeria* spp. In some cases, these microbial consortia inhibit *Listeria* almost completely. From such undefined industrial cheese-ripening floras, linocin M18-producing (*lin*⁺) (N. Valdés-Stauber and S. Scherer, Appl. Environ. Microbiol. 60:3809–3814, 1994) and -nonproducing *Brevibacterium linens* strains were isolated and used as single-strain starter cultures on model red smear cheeses to evaluate their potential inhibitory effects on *Listeria strains in situ*. On cheeses ripened with *lin*⁺ strains, a growth reduction of *L. ivanovii* and *L. monocytogenes* of 1 to 2 log units was observed compared to cheeses ripened with *lin*⁻ strains. Linocin M18 activity was detected in cheeses ripened with *lin*⁺ strains but was not found in those ripened with *lin*⁻ strains. We suggest that production of linocin M18 contributes to the growth reduction of *Listeria* observed on model red smear cheeses but is insufficient to explain the almost complete inhibition of *Listeria* caused by some undefined microbial floras derived from the surface of ripe cheeses.

Red smear cheeses are economically important in Austria, Germany, France, Scandinavia, and Switzerland. Surveys on the occurrence of *Listeria* spp. in dairy products have revealed that red smear cheeses are more frequently contaminated with *Listeria* than are any other soft cheeses (3, 35). This is due to the production method. To achieve red smear cheeses of high quality, unripened cheeses are inoculated with undefined ripening floras from different dairies and are incubated with undefined wash-off microfloras derived from the surface of ripe cheeses. This procedure results in a continuous spreading of pathogenic contaminants such as *L. monocytogenes*. Since defined ripening cultures for red smear cheeses are unknown, the contamination circle cannot be interrupted easily. It is, therefore, important to develop defined ripening starters which inhibit *Listeria* efficiently.

The composition of microbial populations on the surface of red smear cheeses is exceedingly complex. Yeasts such as *Debaryomyces Hansenii*, *Candida intermedia*, and *Galactomyces geotrichum* metabolize lactic acid, thus raising the pH of the surface from approximately 5 to 7. This creates a habitat for a salt-tolerant bacterial consortium consisting of coryneform bacteria, micrococci, and gram-negative bacteria (5, 7, 8, 20). The surface microflora of red smear cheeses from six German dairies was found to be composed of *Arthrobacter citreus*, *Arthrobacter nicotianae*, *Brevibacterium fermentans*, *Brevibacterium linens*, *Brevibacterium oxydans*, *Corynebacterium ammonigenes*, *Corynebacterium variabilis*, *Microbacterium imperiale*, and *Rhodococcus fascians* (39). A variety of isolates could not even be identified. The microbial compositions of the smears from different dairies vary widely (39). The interaction of the microorganisms on such cheeses during ripening is unknown and, most probably, very complex. Salt content, pH, and aroma compounds produced by coryneform bacteria (6, 9, 19), as well as bacteriocin production, are examples of important parameters. One aim of this study was to investigate whether the antilisterial activity of complex industrial red smear cheese-ripening floras from different dairies also varies.

It has been reported that microorganisms isolated from the surface flora of red smear cheese display antagonistic effects against *Listeria* (15, 24, 25, 31, 36). In one study, 187 strains of coryneform bacteria were isolated from red smear cheese and screened for inhibitory effects against *Listeria spp.* (36). Among these isolates, *B. linens* M18 was found to produce the bacteriocin linocin M18 (37), which consists of a single protein subunit with a molecular mass of 28.5 kDa. Native linocin M18 forms aggregates of extremely high molecular masses (>2,000 kDa) (37) as do some other bacteriocins such as staphylococcin 414 (11), staphylococcin 1580 (17), lactacin F (28), and helveticin J (18). The gene *lin* has been cloned and its wide taxonomical distribution within coryneform bacteria has been demonstrated (38). Based on this strain collection, the second aim of this study was to compare, in situ, the antilisterial activities of linocin-negative and linocin-positive strains of *B. linens* on the surface of red smear cheese. Does bacteriocin production contribute to, or is it even sufficient to explain, the inhibition of *Listeria* on some commercially available cheeses?

**MATERIALS AND METHODS**

**Ripening starters and bacterial strains.** Undefined, complex, ripening microfloras were obtained from three commercial cheese dairies producing Weinkäse (dairy A), Romadur (dairy B), and Limburger (dairy C) cheeses. All three cheese types are red smear soft cheeses which differ mainly in their forms but not in the cheesemaking parameters used. *B. linens* M18, D2, D7, and D11 were isolated from red smear soft cheeses. They were characterized by using standard taxonomical markers such as sugar fermentation pattern or fatty acid analysis, which clearly showed that these strains were different (data not shown). *B. linens* M18 is known to produce the bacteriocin linocin M18, which has been shown to inhibit *Listeria* (37, 38). *B. linens* D11 also inhibits *Listeria*. The antilisterial substance produced by this strain was concentrated from liquid culture by ultrafiltration.
purified by gel filtration chromatography, and tested for its enzyme sensitivity as described previously for lincom M18 from L. linens M18 (37). The antilisterial substance produced by L. linens D11 in liquid medium showed the same properties as lincom M18 concerning molecular mass, enzyme and temperature sensitivity, and activity spectrum. Furthermore, the result indicated that lincom M18 was detected in L. linens D11 by PCR as described previously (38). Neither did strains D2 and D7 have antilisterial activity nor was it possible to amplify the bacteriocin structural gene from their DNA. They were used as bacteriocin-negative controls.

L. innocua WSLC 2011 (ATCC 33090) and WSLC 2012 (ATCC 33091), L. ivanovii WSLC 3061, and L. monocytogenes WSLC 1019 (ATCC 19116) and WSLC 1364 from Vacherin Mont d’Or cheese (2) were used for contamination of the unripened cheeses during an early ripening phase by smearing. Usually, 3 days. Cheeses were dipped into the smear water in order to moisten the entire surface of the cheese. Sterile gloves were used to manually rub the liquid over the surface of the cheese. Sterile membranes with a 50-kDa cutoff (Sigma) were placed between the soft agar and the cheese floras.

L. ivanovii WSLC 2011 or 2012 was replaced with L. monocytogenes WSLC 3061 (37). The contaminating microbe L. innocua WSLC 3061 (37). In trial 3, L. ivanovii WSLC 3061 was replaced with the pathogen L. monocytogenes WSLC 1919. In the case of a smear-making process being contaminated with Listeria, the pathogens are transferred to the unripened cheeses during an early ripening phase by smearing. Usually, contaminations by L. monocytogenes were not exceedingly high. Therefore, to approximate the contamination conditions which may occur in the dairy process, inoculation was performed at day 3 with 10 CFU/cm² in trial 4.

Cell count evaluation and determination of Listeria. The pH of the cheese surface was measured five times per cheese with a surface electrode (Ingold, Steinweg, Germany). The means of five measurements are shown. For the determination of cell counts, the two main surfaces of 5-mm-thick slices of cheese (45 cm²) were homogenized in 180 ml of 1.75% trisodium citrate–dihydrate solution (Na₃C₆H₅O₇·H₂O; Merck). Serial 10-fold dilutions of these suspensions were plated either on PC¹ agar for aerobic plate counts (30°C, 72 h) or on yeast extract-glucose-chloramphenicol-bromphenol blue agar (YGCGB agar; Merck) for yeast counts (25°C, 72 h) or on Oxford agar (Oxoid, Hampshire, England) for listerial counts (30°C, 48 h). Cell counts were calculated per cm². In experiments performed with single-strain L. linens cultures, PC¹ agar plates were exposed to daylight after incubation for 3 days, and orange-pigmented L. linens colonies, which were clearly different from those of the indigenous microflora present on the unripened soft cheeses, were counted separately.

In all experiments, Listeria cells grown on the cheeses were isolated and phase typed (21) to make sure that there were no other Listeria strains present, originating possibly from contaminated material taken from the dairy.

Significance of cell counts. In order to detect variations in cell counts resulting from dilution and plating on different agar media, one cheese sample was diluted in parallel four times and plated on the different media. Means and standard deviations of the cell counts were calculated. The cell counts were 4.2 ± 10⁹/cm² ± 17% on Oxford agar, 1.2 ± 10⁷/cm² ± 17% on YGCGB agar, and 1.2 ± 10⁶/cm² ± 5% on PC¹ agar. Since not enough cultures could be ripened in our laboratory at one time in order to add error bars to the figures, entire experiments were repeated. In different experiments, however, due to the complexity of the cheese-ripening system, bacterial counts were somewhat different while the trends of the results were always identical. For these reasons, typical experiments are shown depicting trends which are clearly significant in all cases.

Detection of lincom M18 on the surface. To quantify lincom M18 activity on the surface of the cheeses, 1.5 cm² from the surface of each cheese (0.4 g) was homogenized in 1.5 ml of sodium phosphate buffer (500 mM, pH 7) to standardize the pH of the preparation, because cheese ripening starts at approximately pH 5. For quantification of the antilisterial substance serial two-fold dilutions in the same buffer were placed on agar plates with the indicator strain L. ivanovii WSLC 3061 (37). Sterile cellulose-acetate membranes (0.2-µm pore size; Sartorius, Göttingen, Germany) were placed on the agar plates, and 20 µl of each dilution was pipetted onto these membranes. The plates were incubated at 6°C for 24 h, and the membranes were removed after diffusion. The agar plates were then incubated at 30°C for 24 h, and inhibition was detected by using Heidenhain’s medium. Bacteriocin activity was quantified by the critical dilution method (14).

The inhibitory substance extracted from cheese was tested for enzyme sensitivity. Protease VIII (7 to 15 U/mg), pronase E (4 U/mg), ficin (0.25 to 0.75 U/mg), papain (10 to 20 U/mg), proteinase K (10 to 20 U/mg), α-chymotrypsin (40 to 60 U/mg), plasmin (2 to 4 U/mg), catalase (40,000 to 60,000 U/mg), and rennet (20 U/mg) were all purchased from Sigma, Deisenhofen, Germany. Enzyme solutions with a concentration of 10 mg/ml were prepared in sodium phosphate buffer (30 mM, pH 7.0). A total of 20 µl of enzyme solution was pipetted on the agar plates with the indicator strain after diffusion of the antilisterial substance at 6°C before incubation at 30°C. Enzyme solutions were spotted as controls. In some experiments, sterile dialysis membranes (10,000 to 10,000 M dalton, Sigma) with a 50-kDa cutoff were placed on the agar plates with the cellulose-acetate membrane to evaluate the apparent molecular size of the inhibitory substance. In addition, parts of the cheese surface were placed on the membranes with the smear downside. Diffusion and incubation of these plates were performed as described above.

RESULTS

Inhibition of Listeria by undefined industrial red smear cheese floras. Ripening of cheeses with three different undefined starters led to similar developments of pH and cell counts of yeasts and bacteria (Fig. 1A and B). In contrast, development of inoculated Listeria was dependent on the culture used for ripening (Fig. 1C). Ripening starter from dairy C proved to be most competitive against Listeria. There was little growth of L. innocua between days 4 and 8. Afterwards, Listeria counts declined slightly. Ripening starters from dairies A and B had a less distinct effect on the multiplication of the contaminant. Final counts of Listeria ranged between 10³ and 10⁴ CFU/cm², which corresponds to an increase of 2 to 3 log units. Dairies B and C were chosen for the (successful) reproduction of results three times over a 1-year period with different batches of cheeses. Inoculation with L. innocua WSLC 2011 or 2012 was done at day 7 with 5 CFU/cm². In all cases, the same trend as is shown in Fig. 1 was observed. It was also demonstrated that ripening culture from dairy C inhibited different L. monocytogenes strains on cheese (12a).

Cheese ripening with single-strain cultures of L. linens. To ensure that (i) the ripening processes in the laboratory were typical for red smear soft cheeses produced in dairies and (ii) ripening was comparable to that and control cheeses with L. linens single-strain cultures, pH, aerobic plate counts, and yeast counts on the cheese surface were determined throughout.

The development of pH on the cheese surface is shown in Fig. 2A. In all trials the development of pH was typical for the ripening of industrial red smear cheeses (4, 32). The pH rose from 5 to

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approximately 7 at day 10 and remained nearly constant until the end of ripening (compare Fig. 1).

The typical development of yeast counts and aerobic plate counts during ripening and storage is shown in Fig. 2B. Aerobic plate counts of the surface flora right before the first smearing amounted to about $5 \times 10^6$ CFU/cm². The increase in aerobic plate counts from days 1 to 3 comprises inoculation by smearing and microbial growth. Between days 1 and 5 of the ripening process, a pronounced growth of yeast from $5 \times 10^3$ to $5 \times 10^7$ CFU/cm² was observed. From day 10, aerobic plate counts were at least 1 log unit higher than those of the yeasts. Accordingly, in the later phase of ripening and during storage, aerobic plate counts mainly reflect bacterial counts. After the first smearing at day 1, *B. linens* accounted for 40% of the aerobic plate counts. The percentages of both *B. linens* M18 and D2 increased to approximately 90% at day 10 (Fig. 2C), demonstrating the strong predominance of *B. linens* in the smear flora. During the ripening process, aerobic plate counts reached approximately $5 \times 10^9$ CFU/cm² between days 14 and 21 and then remained constant. These cell counts are typical for surface-ripened Weinkäsere cheeses. The cell count developments were similar for all cheeses ripened with the four *B.
*B. linens* strains selected. This is a fundamental requirement for using *B. linens* D2 and D7 as appropriate bacteriocin-negative controls versus *B. linens* M18 and D11. At the day of packaging, a thin orange smear had developed on all cheeses and the cheese matrix was uniformly ripened from the surface to the core.

**Growth of *Listeria* on single-strain-ripened cheeses.** In trials 1 and 2, the cheeses were contaminated with *L. ivanovii* on day 7 or 8 at a level of 10^3 CFU/cm^2. The *Listeria* cells grew to approximately 5 × 10^7 CFU/cm^2 on the control cheeses (*B. linens* D2 and D7). On cheeses inoculated with *B. linens* M18 or D11 (both *lin^−*), the detectable *Listeria* counts first fell below the detection limit of ≥10 CFU/cm^2 and then increased to 10^5 CFU/cm^2 (Fig. 3A and B). In trial 3, contamination was performed with *L. monocytogenes* WSLC 1019 at a concentration of 10^3 CFU/cm^2 at day 3 or 10^2 CFU/cm^2 at day 5. Growth of *Listeria* was retarded on the test cheeses ripened with *lin^−* *B. linens* M18. Listerial cell counts reached 5 × 10^6 CFU/cm^2 on the test cheeses and 5 × 10^7 CFU/cm^2 on the control cheeses at day 37 (Fig. 3C). In trial 4 (Fig. 3D), low-level contamination at a concentration of 10 CFU/cm^2 was performed at day 3 with *L. monocytogenes* WSLC 1364. *Listeria* cells did not grow between days 3 and 7. The detection limit was ≥4 CFU/cm^2 in comparison to ≥10 CFU/cm^2 in trials 1 and 2 because of a slightly modified sample preparation. *Listeria* counts were lower on test cheeses than on control cheeses, the difference being about 2 log units at day 10 and 1 log unit at day 56. A similar growth reduction on the test cheeses was observed when *L. monocytogenes* WSLC 1019 was used for contamination at day 1 or day 3 at a concentration of 10 CFU/cm^2 (data not shown).

**Linocin M18 activity on single-strain-ripened cheeses.** In order to evaluate our results, it was important to know whether bacteriocins were actually produced on the cheeses. The aqueous extraction of inhibitory substances from the surface of test and control cheeses yielded similar results from days 3 to 7 (Fig. 4). After approximately 2 weeks, a strong increase in an antilisterial substance was detected on the test cheeses (*lin^−*) but not on the control cheeses (*lin^+*). These large amounts of antilisterial substance remained active until the end of storage. This result could be reproduced in further experiments (data not shown). Detection of the antilisterial substance(s) was also possible with parts of the cheese surfaces placed on membranes lying on an agar surface (Fig. 4, inset A). The antilisterial substance produced on the test cheeses was retained by a dialysis membrane with a 50-kDa cutoff (Fig. 4, inset B) and was as sensitive to protease VIII, pronase E, trypsin, proteinase K, and α-chymotrypsin as was linocin M18. Ficin, papain, rennet, plasmin, and catalase affected neither linocin M18 activity nor the activity of the extracted substance. The same amount of linocin M18 activity could be extracted from the cheeses ripened with *B. linens* D11 (data not shown). We suggest, therefore, that linocin M18 was produced on cheeses ripened with either *B. linens* M18 or D11.

**DISCUSSION**

Ripening experiments done by inoculation of the cheese surface with wash-off cultures from commercial cheese showed that listerial growth was clearly dependent on the microbial composition of the undefined surface floras (Fig. 1). The rip-
contribution to the inhibition of Listeria grown on model red smear cheeses and found linocin M18 activity or the linocin-negative strain B. linens D2 (trial 4) by arbitrary activity units per cm² of cheese surface. Inset: A, antilisterial activity detected by diffusion from the cheese surface through a cellulose-acetate membrane with a pore size of 0.2 μm; B, retention of the antilisterial substance by a dialysis membrane with a 50-kDa cutoff.

FIG. 4. Detection of linocin M18 on cheese. Relative quantification of an inhibitory substance extracted from cheeses ripened with linocin M18-producing strain B. linens M18 or the linocin-negative strain B. linens D2 (trial 4) by arbitrary activity units per cm² of cheese surface. Inset: A, antilisterial activity detected by diffusion from the cheese surface through a cellulose-acetate membrane with a pore size of 0.2 μm; B, retention of the antilisterial substance by a dialysis membrane with a 50-kDa cutoff.

...ing starter from dairy C almost completely inhibited a variety of Listeria, including L. monocytogenes strains. It is interesting that the antagonistic behavior of undefined complex red smear cheese flora is a stable feature of these microbial consortia, since the experiment shown in Fig. 1 could be reproduced with cheese produced over a period of 1 year, indicating that the cheese microbial consortia of each dairy form a remarkably stable community.

The molecular basis of the effects observed is unknown. However, bacteriocin production of ripening cultures may have contributed to the inhibition of Listeria. Therefore, we used bacteriocin-producing B. linens strains (37, 38) for the production of model red smear cheeses and found linocin M18 activity on the cheese surface (Fig. 4). In contrast, no inhibitory substance larger than 50 kDa could be extracted from the cheeses ripened with B. linens D2. Linocin M18, apparently, was stable on the cheese surface. Stability of other bacteriocins has been reported for lactacin 3147 in cheddar cheese (30) and for an enterococcal bacteriocin in Taleggio cheesemaking (12) but not for nisin activity during the ripening of Camembert cheese (23).

A growth reduction of Listeria on cheeses ripened with lin+ B. linens strains was detected in comparison to control strains under different conditions of contamination (Fig. 3). In all experiments with single-strain cultures, Listeria cells were inhibited by 1 or 2 log units compared to control strains. Such a growth reduction is in accordance with reports dealing with the inhibition of Listeria by bacteriocinogenic lactic acid bacteria in situ (1, 10, 22, 33).

Based on these data, we suggest that linocin M18 caused the growth reduction of Listeria observed on our model cheeses. This idea is further supported by the fact that identical results emerged when different lin− and linocin-negative B. linens strains were used. However, only the use of an isogenic lin mutant could be considered final proof for this hypothesis. The application of isogenic bacteriocin-negative derivatives as control strains in food fermentation experiments has been described for lactic acid bacteria (10, 23, 33). The gene for linocin M18 is located on the chromosome (37), and therefore, it is necessary to construct an adequate vector system to obtain a lin mutant derivative of B. linens M18 by homologous recombina-

tion. Unfortunately, no plasmid vector is available for B. linens so far. Vectors which have been constructed for Corynebacterium glutamicum did not work in B. linens (data not shown).

In all experiments using single-strain cultures, Listeria grew to high densities on the cheese surface (Fig. 3). This could be due to the growth of bacteriocin-resistant mutants (13, 26, 29, 40). However, all Listeria cells isolated at the end of storage were of the same phage type as the initially introduced strain, and they were equally sensitive to linocin M18. Growth of Listeria in the presence of bacteriocin-producing strains, therefore, was not due to survival of resistant mutants. In contrast, the undefined wash-off cultures had much more pronounced inhibitory effects (Fig. 1). Although bacteriocin production may play a certain role in preventing the growth of Listeria within a multiple hurdle concept (27), its production observed on single-strain-ripened cheeses cannot explain the striking effects observed with industrial wash-off cultures (Fig. 1). Additional factors must be responsible for the inhibition of Listeria, e.g., the production of other inhibitory substances or unknown ecological interactions within the complex smear flora, such as competition and symbiotic relationships. The development of a defined surface-ripening culture probably will comprise a combination of reciprocally compatible strains with different bacteriocinogenic activity as well as suitable ripening, sensory, and antimycotic properties. Considering the complexity of the natural smear ecosystem, it is not likely that this goal will be reached easily.

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