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A Pediocin-Producing *Lactobacillus plantarum* Strain Inhibits *Listeria monocytogenes* in a Multispecies Cheese Surface Microbial Ripening Consortium

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The growth of *Listeria monocytogenes* WSLC 1364, originating from a cheese-borne outbreak, was examined in the presence and in the absence of a pediocin AcH-producing *Lactobacillus plantarum* strain on red smear cheese. Nearly complete inhibition was observed at 10^2 CFU of *L. monocytogenes* per ml of salt brine solution, while contamination with *Listeria* mutants resistant to pediocin resulted in high cell counts of the pathogen on the cheese surface. The inhibition was due to pediocin AcH added together with the *L. plantarum* culture to the brine solution but not to bacteriocin production in situ on cheese. Pediocin resistance developed in vitro at different but high frequencies in all 12 *L. monocytogenes* strains investigated, and a resistant mutant remained stable in a microbial surface ripening consortium over a 4-month production process in the absence of selection pressure. In conclusion, the addition of a *L. plantarum* culture is a potent measure for combating *Listeria* in a contaminated production line, but because of the potential development of resistance, it should not be used continuously over a long time in a production line.

Listeria monocytogenes, the causative agent of listeriosis, has resulted in numerous major food-borne outbreaks worldwide. Red smear cheeses are particularly sensitive to colonization with this pathogen (17, 27); 21 of 329 cheese samples have been found to contain *L. monocytogenes*, in one case more that 10^4 CFU per cm^2 of cheese surface (24). A recall of 80 tons of *L. monocytogenes*-contaminated soft and semisoft red smear cheeses in Germany in March 2000 prompted renewed concern about the presence of this bacterium in red smear cheese. It has been shown that contamination with *L. monocytogenes* and other species of *Listeria* occurs frequently in red smear cheese, even when pasteurized milk has been used for cheese making (24). Most likely, this is due to postprocess contamination during the traditional method of "old-young smearing," which includes frequent handlings and washes required for proper development of the complex, undefined microbial ripening consortium. Inhibition of *L. monocytogenes* after application of bacteriocinogenic cheese smear coryneform bacteria (5, 11) and *Staphylococcus equorum* (6) in situ has been reported and is of considerable interest in order to enhance the hygienic quality of these products.

Many lactic acid bacteria, including members of the genera *Lactococcus*, *Lactobacillus*, *Carnobacterium*, *Enterococcus*, and *Pediococcus*, are known to secrete small, ribosomally synthesized antimicrobial peptides called bacteriocins (1, 14, 16, 21), many of which inhibit *Listeria* (7, 15, 20). Some bacteriocins have been used to inhibit this pathogen in food, either through bacteriocin-producing cultures (20, 29) or by the addition of pure or semipure bacteriocin preparations (8, 20, 28). *Lactobacillus plantarum* ALC 01 was reported to secrete the bacteriocin pediocin AcH (9), which is also produced by *Pediococcus acidilactici* (13, 19, 25). The activity spectrum of pediocin AcH is relatively wide, and it exhibits a bactericidal mode of action leading to lysis of cells (18) in three steps: (i) binding to the cytoplasmic membrane, (ii) insertion of bacteriocin molecules in the membrane, and (iii) formation of a poration complex which leads to dissipation of the proton motive force. A review on pediocin was recently published by Rodriguez et al. (23). The antilisterial action of *L. plantarum* ALC 01, which is commercially available (Danisco, Niebüll, Germany), was investigated on red smear cheese by using either complex wash-off cultures from commercial cheeses or a defined ripening culture distributed by a culture supplier.

Bacterial strains and determination of their inhibitory activity. *L. plantarum* ALC 01, a pediocin AcH producer isolated from Munster cheese (10), and *L. plantarum* ATCC 14917, a bacteriocin-negative strain, were used as test bacteria to demonstrate bacteriocin-mediated antilisterial activity. Both strains were cultured for 14 h at 37°C in a special culture medium (VisStart TW ALC01; Danisco) supplied by the manufacturer of the ALC 01 strain, to reach a final pH of 3.9 and a maximum pediocin AcH activity. A total of 12 different *L. monocytogenes* strains (Table 1), isolated from various foods, were used as indicator strains. For detection of pediocin AcH released into the growth medium, a sample of *L. plantarum* ALC 01 was centrifuged (10,000 × *g*, 10 min, 4°C). The supernatant was neutralized, filtered through a 0.45-μm-pore-size membrane filter, and used in a "spot-on-the-lawn" assay (2) by spotting 10 μl of the samples onto a lawn of *L. monocytogenes* indicator cells, as specified in Table 1. Indicator plates contained 7 ml of 0.8% tryptose-soft agar (TB, with 8 g of agar/liter; Merck, Darmstadt, Germany) and 100 μl of an overnight culture of the *L. monocytogenes* indicator strains. Activity was quantified by

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TABLE 1. Sensitivity and resistance of various *L. monocytogenes* indicator strains against pediocin AcH produced by *L. plantarum* ALC 01

L. monocytogenes strain	Serovar	Origin (strain)	Inhibition zone diam (mm)	Frequency of resistant strains[a]
WS 3550	4b	Vacherin Mont d'Or cheese	13	1×10^{-6}
WS 3545	4b	Massachusetts milk outbreak (Scott A)	13	4×10^{-3}
WS 2823	4b	Cheese	10	ND
WS 2824	1/2c	Cheese	12	7×10^{-6}
WS 2825	1/2b	Cheese	12	1×10^{-5}
WS 2826	1/2a	Cheese	13	6×10^{-5}
WS 2827	1/2c	Poultry	10	5×10^{-6}
WS 2828	1/2a	Milk	10	ND
WS 2829	1/2a	Red smear cheese	12	5×10^{-4}
WS 2830	4b	Red smear cheese	11	4×10^{-5}
WS 2831	1/2b	Red smear cheese	11	5×10^{-5}
WS 2832	1/2a	Cheese	11	2×10^{-6}

[a] ND, not determined.

serial twofold dilutions (2) and expressed in activity units (AU) per milliliter. Sensitivity tests were repeated twice. ALC 01 produced clear zones of inhibition on solid media against all *L. monocytogenes* indicator strains (Table 1), whereas the control strain, *L. plantarum* ATCC 14917, showed no inhibition. *L. monocytogenes* WSLC 1364 (serovar 4b), isolated from Vacherin Mont d'Or cheese (3), was used for contamination experiments due to its origin from a listeriosis outbreak caused by red smear soft cheese. A pediocin-resistant mutant was derived from this strain (WSLC 1364R) by growing the wild type in the presence of approximately 25,000 AU of pediocin per ml. After 24 h, inhibition zones were examined under Henry's illumination for pinpoint colonies, indicating resistant mutants, which were purified on Palcam agar.

Inhibition of *L. monocytogenes* on cheese. To evaluate the antilisterial potential of the *L. plantarum* ALC 01 strain in situ on soft cheese, ripening experiments of model cheese were performed. An undefined wash-off flora from commercially produced cheese and a defined commercial ripening culture containing *Brevibacterium linens*, *Geotrichum candidum*, and *Debaryomyces hansenii* (OFR 9 and DH 2; Danisco) was used as described by Eppert et al. (11) under laboratory conditions in glass desiccators. Smearing was applied five times at intervals of 2 or 3 days under sterile conditions. The smear brine finally contained approximately 10^8 CFU/ml of the ripening flora. For contamination, an aliquot of a diluted overnight *Listeria* culture (see below) was added to the smear brine just before smearing on day 1. For determination of *Listeria* and *Lactobacillus* cell counts, two slices 3 to 4 mm thick were removed from the flat surfaces of a round cheese (approximately 20 g, corresponding to roughly 45 cm^2), homogenized in 180 ml of 1.75% trisodium citrate-dihydrate solution with a stomacher, diluted, and plated on Palcam and MRS agars (Merck). Cell counts were calculated per square centimeter of cheese surface. When *Listeria* cell counts were expected to fall below 100 CFU/cm^2, 25 g of the cheese surface was examined by an enrichment procedure according to International Standard ISO 11290-1. To ensure that the ripening processes in the laboratory were typical for red smear cheeses produced in dairies, pH, aerobic plate counts, and yeast counts on the cheese surface were determined throughout. In all experiments, the development of these parameters was typical for the ripening of industrial red smear cheese (11) (data not shown).

For ripening experiment A (Fig. 1A), a stock culture of *L. plantarum* (containing approximately 50,000 AU of pediocin AcH/ml) was mixed 1:1 with 10% NaCl solution to reach a final NaCl concentration of 5%. The commercial undefined wash-off flora was added, yielding *L. plantarum* cell counts of 5×10^8 CFU/ml and *L. monocytogenes* cell counts of 2×10^2 and 4×10^3 CFU/ml. In this experiment, inhibition of the growth of *L. monocytogenes* was observed compared to control cheeses ripened with the bacteriocin-negative strain ATCC 14917 (Fig. 1A). The effect was dependent on the contamination level: when cheeses were challenged with 4×10^3 CFU of *Listeria*/ml, an inhibition of 1 to 2 log cycles could be demonstrated during the whole ripening period, whereas pronounced inhibition could be achieved with low initial contamination levels (2×10^2 CFU/ml of brine). Until day 14, no *Listeria* cells could be detected on the cheese surface. Between days 25 and 35, *Listeria* cells grew to approximately 3×10^3 CFU/cm^2 on cheeses ripened with the addition of ALC 01 and to 6×10^5 CFU/cm^2 on control cheese ripened with the bacteriocin-negative control strain. Although the control experiment using a pediocin-negative *L. plantarum* strain is in favor of the hypothesis that it is pediocin which inhibits *L. monocytogenes*, other inhibitory factors cannot be excluded, because the *L. plantarum* strains were not isogenic. In order to gain further data, a pediocin-resistant *L. monocytogenes* mutant, WSLC 1364R, was also used (Fig. 1B). As expected, this mutant was not inhibited at all by the pediocin-producing *L. plantarum* strain.

For ripening experiments using either the supernatant or the cell pellet from an *L. plantarum* culture (Fig. 1C), cells were harvested by centrifugation (10,000 $\times$ *g*, 20 min, 4°C) and the supernatant was collected. Cells were washed twice by centrifugation and resuspended in fresh culture medium. A 10% NaCl solution was added as described above to either the resuspended cell pellet or the filter-sterilized supernatant, and the defined ripening culture was added. *Listeria* cells on cheeses inoculated with the resuspended pellet reached final counts of approximately 4×10^3 CFU/cm^2, and the qualitative determination of *Listeria* on the cheese surface was possible at days 7 and 24 of ripening. Partial inhibition by the *L. plantarum* cell pellet may be due to pediocin produced before addition of the cells, to leakage of intracellular pediocin from the producer cells, or to pediocin produced in situ. On cheeses challenged with the filter-sterilized supernatant of the culture containing

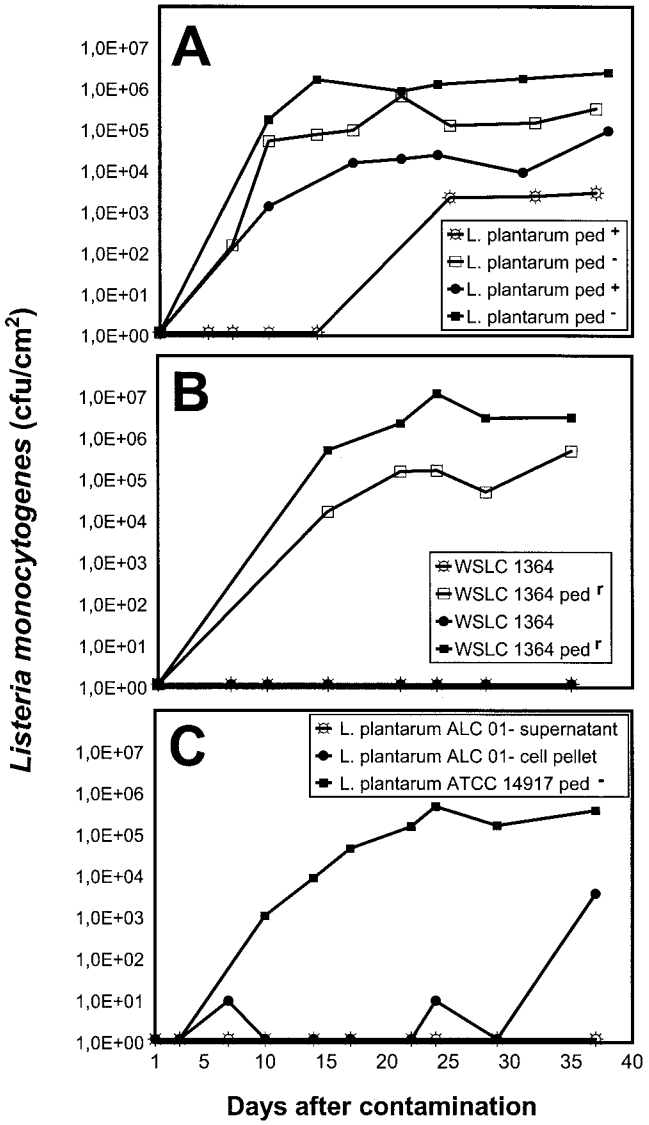

FIG. 1. Inhibition of growth of *L. monocytogenes* WSLC 1364 by *L. plantarum* ALC 01. Ripening experiments were performed on soft cheese using a commercial, undefined multispecies microbial consortium. (A) *Listeria* cell counts on the cheese surface after contamination at day 1 with 2×10^2 CFU (open symbols) and 4×10^3 CFU (solid symbols) per ml of brine solution. Control cheeses were ripened with the pediocin AcH-negative type strain *L. plantarum* ATCC 14917. (B) Contamination with 10^2 CFU (open symbols) or 10^3 CFU (solid symbols) per ml of brine solution. Control cheeses were contaminated with the resistant mutant WSLC 1364R (ped^r). (C) *Listeria* contamination with 7×10^2 CFU/ml of brine solution. In this case, cheese ripening was performed with a commercial, defined ripening culture. Either the supernatant or the pellet of the 14-h culture in VisStart TW ALC01 medium was used. Control cheeses were ripened with the addition of a 14-h culture of the pediocin AcH-negative type strain *L. plantarum* ATCC 14917, cultivated in VisStart TW ALC01.

the bacteriocin, listeriae appeared to be eradicated from the cheeses. It is concluded from this experiment that no growth or in situ production of pediocin is necessary to achieve inhibition of *L. monocytogenes*. This is in agreement with the observation that *Lactobacillus* cell counts on the cheese surface were approximately 4×10^7 CFU/cm^2 in all experiments and no significant growth of this strain during cheese ripening could be observed.

Formation and stability of pediocin-resistant mutants. Bacteriocins such as pediocin AcH act by means of a single-hit mechanism (26) and become inactivated at some step after binding to the target cell. The amount of pediocin AcH added to the brine solution is therefore insufficient to eliminate extremely high initial levels of *L. monocytogenes*, which will lead to growth of the pathogen due to pediocin-sensitive survivors (Fig. 1). Alternatively, one could assume that pediocin-resistant mutants would preferentially multiply during the ripening of the cheeses. First, we determined the frequency of the appearance of resistance in 12 different *L. monocytogenes* strains. For determination of the mutation frequency, a log-phase culture of the *Listeria* strain was incubated with pediocin AcH (approximately 25,000 AU/ml) in a 5% sodium chloride solution at 11°C. After 1 h, 100 μl was spread on pediocin AcH-containing PC agar plates (approximately 25,000 AU/plate). For calculation of the mutation frequency, the number of CFU in the log-phase culture was related to number of CFU on pediocin AcH-containing PC agar plates. It was found that the frequency of pediocin-resistant mutant was strongly strain dependent and varied between 4×10^{-3} and 1×10^{-6}. This is in agreement with a recent study of 20 strains of *L. monocytogenes* reporting pediocin resistance frequencies of 10^{-4} to 10^{-6} (reference 12 and references therein).

In order to check the stability of resistance of *L. monocytogenes* WSLC 1364R, a mutant was grown in brain heart infusion broth (BHI; Merck) for 24 h at 30°C. Subsequently, 10 μl of the culture was transferred to fresh, pediocin-free BHI broth for a growth cycle of 24 h. This procedure was repeated for a total of 10 transfers. At day 10, a sample was taken, diluted, and plated on BHI agar, and 50 colonies were picked randomly and tested for resistance to pediocin AcH. It was observed that after 10 transfers (approximately 100 generations), all isolates still were resistant to pediocin AcH. The high stability of pediocin resistance has also been reported by Rekhif et al. (22) and Duffes et al. (9).

In addition, a long-term ripening experiment was carried out to determine the stability of the pediocin-resistant mutant WSLC 1364R (which could grow in the presence of approximately 25,000 AU/ml) over a period of 16 weeks of ripening of the cheeses. For this experiment, the traditional method of "old-young smearing" was imitated under laboratory conditions, by transferring the ripening flora (including the listerial contamination) from batch 1 to batch 2 after 15 days of ripening and again from batch 2 to batch 3 after an additional 14 days of ripening (day 29). This procedure was continued until day 113 of ripening (day 15 of batch 7). Infection with *L. monocytogenes* WSLC 1364R (1.5×10^2 CFU/ml of brine solution) was applied only once, at day 1 of the first batch. *Listeria* cell counts on the cheese surface were found to exceed 10^8 CFU/cm^2 (day 29 to 43) and then slightly decreased (approximately 10^7 CFU/cm^2 at day 113) until the end of this ripening experiment. At various times, homogenized parts of the cheese surface were diluted and plated on Palcam agar, and 50 colonies were picked randomly and tested for resistance to pediocin AcH as described above. As seen in the in vitro serial transfer experiment, resistance was also stable in the

complex microbial consortium until the end of the experiment (day 113).

Is resistance likely to occur in a production line? Under our small-scale laboratory conditions, we found pediocin-resistant mutants after applying pediocin to cheese infected with high *L. monocytogenes* cell counts, 10^5 CFU/ml of brine solution, in one of four experiments (data not shown). It is, of course, not possible to perform a contamination experiment in a real production line. Therefore, some estimates may help give an idea of the potential occurrence of resistant mutants in a dairy. If one assumes a titer of *Listeria* cells in a dairy of 10 cells/ml of brine solution and a frequency of 10^{-4} for the emergence of pediocin resistance, one would expect one mutant cell per liter. From the brine solution, less than 1 ml is transferred to the surface of an individual cheese. Even if a resistant mutant is transferred to 1 out of 1,000 cheeses, this single mutant cell needs to successfully compete with the microbial cheese-ripening consortium. It has been reported that the fitness cost of pediocin resistance of *Listeria* can reduce the maximum specific growth rate to 44% (12). Therefore, we expect that the establishment of a resistant cell line would be a rare event in a cheese-making environment with a low average titer of *Listeria* in the brine solution. However, it cannot be excluded that such an event may happen, and resistance to class II bacteriocins certainly is a potential obstacle to their application as food preservatives (23).

Conclusions. Contamination levels of 10^2 CFU of *L. monocytogenes* per ml of brine solution are rather high compared to those found in brine solutions of red smear cheese dairies. Nevertheless, complete eradication of *L. monocytogenes* was observed in our experiments. Therefore, the supplementary use of pediocin-producing *L. plantarum* strains appears to be a promising measure to combat *L. monocytogenes* in an infected production line. However, resistant mutants are frequently found in all *Listeria* strains, multiply easily within a food model system like soft cheese, and remain resistant over a long period. Therefore, the continuous use of pediocin AcH appears not to be suitable as a primary means of food preservation (4). We recommend restricting its use to cases of acute contamination of a dairy with *L. monocytogenes*. Combination of pediocin AcH with other bacteriocins as part of a hurdle concept may well constitute an approach to avoid the outgrowth of resistant cells, especially if bacteriocins such as nisin and lactacin, which have a completely different structure from that of pediocin, are used (23).

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