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Use of a Genetically Enhanced, Pediocin-Producing Starter Culture, Lactococcus lactis subsp. lactis MM217, To Control Listeria monocytogenes in Cheddar Cheese

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Cheddar cheese was prepared with Lactococcus lactis subsp. lactis MM217, a starter culture which contains pMC117 coding for pediocin PA-I. About 75 liters of pasteurized milk (containing ca. 3.6% fat) was inoculated with strain MM217 (ca. 10⁶ CFU per ml) and a mixture of three Listeria monocytogenes strains (ca. 10³ CFU per ml). The viability of the pathogen and the activity of pediocin in the cheese were monitored at appropriate intervals throughout the manufacturing process and during ripening at 8°C for 6 months. In control cheese made with the isogenic, non-pediocin-producing starter culture L. lactis subsp. lactis MM210, the counts of the pathogen increased to about 10⁷ CFU per g after 2 weeks of ripening and then gradually decreased to about 10⁵ CFU per g after 6 months. In the experimental cheese made with strain MM217, the counts of L. monocytogenes decreased to 10⁴ CFU per g within 1 week of ripening and then decreased to about 10 CFU per g within 3 months. The average titer of pediocin in the experimental cheese decreased from approximately 64,000 arbitrary units (AU) per g after 1 day to 2,000 AU per g after 6 months. No pediocin activity (<200 AU per g) was detected in the control cheese. Also, the presence of pMC117 in strain MM217 did not alter the cheese-making quality of the starter culture, as the rates of acid production, the pH values, and the levels of moisture, NaCl, and fat of the control cheese and the experimental cheese were similar. Our data revealed that pediocin-producing starter cultures have significant potential for protecting natural cheese against L. monocytogenes.

Advances in genetic technologies have made it possible to develop lactic acid bacteria (LAB) starter cultures or adjuncts with enhanced fermentation characteristics, such as increased phage resistance, improved proteolytic activity, and faster acid production (16, 25). Genetically enhanced dairy cultures may extend the shelf life and/or improve the safety of the resulting cheese through the production of various antimicrobials, notably bacteriocins. At present, nisin, which is produced by Lactococcus lactis subsp. lactis, is the only bacteriocin approved for direct incorporation into cheese (6). However, compared to non-nisin-producing starter cultures, some nisin-producing starter cultures display slower lactose metabolism, less proteolytic activity, lower heat resistance, and greater sensitivity to phage attacks (18–20). Nisin and other bacteriocins may also be antagonistic towards mesophilic lactococci (non-bacteriocin-producing strains) used in mixed-strain starters or towards nonstarter lactic acid bacteria (NSLAB) important in flavor development. Thus, there has been continued interest in developing commercial starter cultures with the ability to produce bacteriocins to ensure adequate performance of the starter and enhance the quality and safety of the resulting cheese.

Several investigators have evaluated the potential of bacteriocin-producing starters or starter adjuncts, especially lactococci, enterococci, and lactobacilli, to enhance performance and/or control undesirable microbes in cheese. Cheddar cheese made with a paired nisin-producing Lactococcus starter system has been used successfully as an antimicrobial agent in pasteurized process cheese and cheese spreads to control Clostridium sporogenes, Listeria monocytogenes, and Staphylococcus aureus (34). Sulzer and Busse (31) used lactococci, enterococci, or lactobacilli that were inhibitory to L. monocytogenes, alone or in combination with commercial starter cultures, to control this pathogen in Camembert cheese. Listeriae were suppressed only when the inhibitory strain was the sole starter and when contamination with Listeria spp. occurred early during ripening. Appreciable inhibition of L. monocytogenes in Camembert cheese was also observed when a nisin-producing paired lactococcal starter system was used (20). In the study of Ryan et al. (28), sufficient lacticin 3147 was produced during cheese manufacturing and ripening to have an impact on the resident microflora; however, the full potential of the bacteriocin was not substantiated against a food-borne pathogen, such as L. monocytogenes, in cheese.

In studies of dairy applications of other bacteriocinogenic cultures, Eppert et al. (8) used a linocin-producing Brevibacterium linens strain to obtain a 1- to 2-log₁₀ reduction in the amount of L. monocytogenes in soft, smear-ripened cheese, and Joosten and Nunez (15) used bacteriocin-producing enterococci and nisin-producing L. lactis strains separately to inhibit the growth and production of histamine by Lactobacillus buchneri St2A in Manchego cheese. In Taleggio cheese, an enterocin-producing starter adjunct resulted in an appreciable decrease in the number of L. monocytogenes cells compared to control cheese (11), while an enterocin-producing adjunct culture, Enterococcus faecalis INIA 4, accelerated flavor formation in semihard cheese (10). It should be noted, however, that enterococci may not be desirable for routine application in cheese making because some isolates (i) may produce tyramine, (ii) may be responsible for clinical infections, (iii) may...
inhibit NSLAB involved in cheese ripening, and/or (iv) may be used as indicators of fecal contamination (1).

Uljas and Luchansky (32) reported that lactin 99, which was produced by the starter adjunct Lactobacillus plantarum JBL2132, inhibited NSLAB that form calcium lactate crystals in Cheddar cheese. Other investigators reported that using a commercial bacteriocinogenic mixture of L. plantarum strains resulted in a 2-log reduction in the number of S. aureus cells in 10 days during Montasio cheese ripening at 12°C compared to the control, in which the number of pathogen cells increased slightly (30). In addition, isolation from cheese of L. plantarum WHE 92, which produces a pediocin, may provide additional opportunities to use bacteriocinogenic lactobacilli as starters or starter adjuncts in cheese (7). Lactobacilli can play a significant role in flavor development in cheese, and unlike hemolytic or cytolytic enterococci, lactobacilli do not pose a significant threat to human health. Thus, identification of bacteriocinogenic lactobacilli and optimization of these organisms as adjunct cultures could have an appreciable impact on the quality and safety of several varieties of natural cheese.

With the possible exceptions of nisin and lactococci, pediocins and/or pediococci have been used more extensively to control undesirable microbes in foods than any other biopreservatives (23, 29). Pediococcus spp. typically ferment lactose poorly and are not known for their proteolytic ability in milk; thus, they are used primarily as starter cultures for meat fermentations (4). The genes coding for pediocin PA-1 (also known as pediocin AcH), a class II, heat-labile, small, peptid bacteriocin produced by Pediococcus acidilactici, have been sequenced and subcloned into vectors suitable for expression in other bacterial hosts, including lactococci (3, 5, 14, 22, 33). Pediocin PA-1 was expressed under the control of the strong lactococcal promoter P32 on pMC117 by L. lactis in microbiological media (5). To more fully exploit the commercial potential of a pediocin-producing lactococcal starter culture, in the present study we evaluated the efficacy of L. lactis subsp. lactis MM217 (containing pMC117) for controlling L. monocytogenes during the manufacture and ripening of Cheddar cheese.

MATERIALS AND METHODS

Bacterial strains. L. lactis subsp. lactis MM210 (Lac′ Prt+; Rhone-Poulenc Marshall Products, Madison, Wis.) was passaged twice in 11% nonfat dry milk (Carnation; Nestle Food Co., Glendale, Calif.) overnight at 32°C prior to inoculation into M17 (Difco Laboratories Inc., Detroit, Mich.) broth containing 0.5% lactose (LM17 broth). L. lactis LL108 (Lac′ Prt− RepA−) (17), the source of plasmid pMC117 (Emr; 7.1 kb) carrying the pediocin PA-1 operon (ped) (5), was passaged twice in M17 broth containing 0.5% glucose and 5 μg of erythromycin (Sigma Chemical Co., St. Louis, Mo.) per ml at 30°C overnight prior to use. L. monocytogenes JBL1003 (serotype 1/2a; meat isolate), JBL1181 (serotype 4b; cheese isolate), and JBL1180 (serotype 4b; cheese isolate), which were obtained from the University of Wisconsin Food Research Institute, were each passaged twice in brain heart infusion (Difco) broth at 37°C overnight prior to use. To prepare a mixture of the three L. monocytogenes strains, 1-ml portions of the three freshly grown L. monocytogenes strains were mixed together in a sterile test tube.

Isolation, transfer, and digestion of plasmid DNA. Plasmid DNA was isolated from lactococci by the method of O’Sullivan and Klaenhammer (24). Restriction enzymes were purchased from Promega Corporation (Madison, Wis.) and were used as recommended by the manufacturer. Electrotansformation of lactococci was performed essentially as described by Holo and Nes (13). The electroporated cells (40 μl) were immediately mixed with 960 μl of ice-cold expression broth (LM17 containing 0.5 M sucrose, 20 mM MgCl2, and 2 mM CaCl2) and then incubated at 25°C for 1 to 3 h. Cells were spread plated onto selective streptococcal regeneration medium (13) agar plates containing 5 μg of erythromycin per ml and incubated at 37°C. Electrotansformed cells were transferred with sterile toothpicks onto LM17 agar plates without erythromycin and incubated overnight at 32°C, and then the resulting colonies were overlaid with 8 ml of tryptose phosphate (Difco) soft agar (containing 0.8% agar) seeded with 1 ml of the thoracic or L. monocytogenes mixture per ml. After overnight incubation at 37°C, colonies exhibiting a zone of inhibition were streak plated onto LM17 agar plates without erythromycin.

RESULTS

Expression of the pediocin operon in L. lactis subsp. lactis MM210. L. lactis subsp. lactis MM210 was electrotansformed with pMC117 to generate a pediocin-producing lactococcal starter culture. When the resulting Emr cells of strain MM210 were overlaid with the three-strain L. monocytogenes mixture, inhibition zones were observed around some of the colonies. Inhibition zones were not observed when 5 μl of proteinase K (0.1 mg/ml) was spotted next to the colonies, indicating that the inhibitory substance was proteinaceous. Also, inhibition zones were not observed when untransformed colonies of L. lactis subsp. lactis MM210 were overlaid with the three-strain L. monocytogenes mixture. Analyses of plasmid DNA isolated from the Emr electrotansformants that exhibited inhibition zones revealed the presence of a 7.1-kb plasmid which contained a 3.5-kb SmaI-BamHI fragment, as expected for pMC117 (data not shown). A representative electrotansformant, designated L. lactis subsp. lactis MM217, was used for further analyses.

Survival of L. monocytogenes in Cheddar cheese made with L. lactis subsp. lactis MM210 or MM217. In experimental cheese made with L. lactis subsp. lactis MM217, the number of
L. monocytogenes cells steadily decreased from the initial level, 3.5 log$_{10}$ CFU per g, to about 2.0 log$_{10}$ CFU per g within 7 days and to about 1.0 log$_{10}$ CFU per g after 184 days of ripening (Table 1). In contrast, in cheese made with the isogenic nonbacteriocinogenic starter culture, L. lactis subsp. lactis MM210, the number of L. monocytogenes cells increased in the first 7 days of ripening, after which the number of pathogen cells gradually decreased to about 4.0 log$_{10}$ CFU per g after 184 days of ripening. In general, after 184 days of ripening at 8°C, the number of L. monocytogenes cells in the experimental cheese made with the bacteriocinogenic starter culture was at least 3.0 log$_{10}$ lower than the number of such cells in control cheese made with the nonbacteriocinogenic starter culture. There was no difference between the number of LAB on M17 agar plates (all LAB, including strain MM210 or MM217) and the number of LAB on M17 agar containing erythromycin, indicating that the inhibitory activity produced by strain MM217 was not self-inhibitory or inhibitory to other NSLAB (data not shown).

**Recovery of pediocin activity from Cheddar cheese.** When the bacteriocinogenic starter L. lactis subsp. lactis MM217 was added to cheese milk, the level of inhibitory activity detected was 200 AU per ml. Although some inhibitory activity was lost in the whey, a considerable amount remained with the cheese after 1 day of ripening (Table 1). The level of inhibitory activity remained at 64,000 AU per g during the first 14 days of ripening and then gradually decreased. The decrease in the level of recoverable inhibitory activity corresponded to the decrease in the pathogen counts. In all cases, the level of activity decreased when protease K (0.1 mg/ml) was added to the test mixture, indicating that the inhibitory substance was proteinaceous. As inhibitory activity was not detected (the detection limit was 200 AU per ml or 200 AU per g) during the manufacturing or ripening of the control cheese and as the strains used in this study are isogenic (that is, they differ only in the presence of the ped operon), we concluded that pediocin was the inhibitory substance.

**Analyses of Cheddar cheese composition.** Proximate analyses of each cheese within 1 week after ripening at 8°C showed that it conformed to the standard of identity for Cheddar cheese (2) and that there were no appreciable differences between the composition of the control cheese (32% moisture, 36% fat, 1.3% salt, pH 5.2; n = 1) and the composition of the experimental cheese (31.3% ± 1.25% moisture, 36.3% ± 0.94% fat, 1.26% ± 0.05% salt, pH 5.1 ± 0.05; n = 3).

**Table 1. Survival of L. monocytogenes and pediocin activities in Cheddar cheese made with the bacteriocinogenic starter culture L. lactis subsp. lactis MM217 (experimental cheese; n = 3) or the isogenic nonbacteriocinogenic starter culture L. lactis subsp. lactis MM210 (control cheese; n = 1)**

<table>
<thead>
<tr>
<th>Cheese-making procedure</th>
<th>Survival of L. monocytogenes in control cheese*</th>
<th>Pediocin activity</th>
<th>Survival of L. monocytogenes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td>3.32</td>
<td>200</td>
<td>3.51 ± 0.26</td>
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<tr>
<td>Curd after cooking</td>
<td>3.74</td>
<td>NS</td>
<td>4.01 ± 0.52</td>
</tr>
<tr>
<td>Whey when drained</td>
<td>NS</td>
<td>3.20</td>
<td>NS</td>
</tr>
<tr>
<td>Curd after pressing</td>
<td>4.41</td>
<td>NS</td>
<td>3.65 ± 0.33</td>
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<tr>
<td>Ripening</td>
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<td></td>
<td></td>
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<tr>
<td>1</td>
<td>4.75</td>
<td>64,000</td>
<td>3.22 ± 0.80</td>
</tr>
<tr>
<td>7</td>
<td>7.47</td>
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<td>1.90 ± 0.09</td>
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<tr>
<td>14</td>
<td>5.65</td>
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<tr>
<td>21</td>
<td>5.3</td>
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<tr>
<td>28</td>
<td>5.61</td>
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<tr>
<td>92</td>
<td>3.83</td>
<td>8,000</td>
<td>0.82 ± 0.10</td>
</tr>
<tr>
<td>184</td>
<td>4.1</td>
<td>2,000</td>
<td>0.92 ± 0.88</td>
</tr>
</tbody>
</table>

* Log$_{10}$ CFU per milliliter or log$_{10}$ CFU per gram.

**Discussion**

L. monocytogenes has caused numerous outbreaks and sporadic cases of listeriosis worldwide, and some of these have been attributed to dairy products (9). A national survey conducted in England and Wales showed that although most soft-ripened, unripened, or hard cheeses made from pasteurized or unpasteurized milk contained <10 CFU of Listeria spp. per g, 12% of the samples contained >10$^5$ L. monocytogenes CFU per g (12). The threat of listeriosis may be increased by the ability of L. monocytogenes to persist in certain cheeses, sometimes for extended periods of time (9). Thus, the purpose of the present study was to construct a pediocin-producing lactococcal starter culture and evaluate its antilisterial potential in Cheddar cheese. Advantageous characteristics, such as heat stability, as well as retention of activity at pH 5.1 and/or at refrigeration temperatures, extend the utility of pediocin as a biopreservative for cheese.

The pediocin-producing plasmid pMC117 was originally cloned into L. lactis LL108; however, this strain is both proteinase negative and lacticase negative and is incapable of adequate growth and acid production in milk (5). In the present study, L. lactis subsp. lactis MM210 was selected as an alternate host for pMC117, as this strain is well characterized genetically, has a weakened but active host restriction and modification system, and has previously been used in Cheddar cheese manufacturing (27). The electrotransformed derivative of strain MM210 containing pMC117, designated L. lactis subsp. lactis MM217, had the same growth characteristics and acid-producing capability as the isogenic parental strain, L. lactis subsp. lactis MM210 (data not shown). Also, the behavior of strain MM217 was physiologically similar to the behavior of strain MM210 during cheese manufacturing, indicating that the presence of pMC117 did not alter the cheese-making properties of the original starter strain. More important, the level of survival of L. monocytogenes in Cheddar cheese made with L. lactis subsp. lactis MM217 was considerably lower than the level of survival of the pathogen in the control cheese. Since no inhibitory activity was detected in the control cheese, in situ production of pediocin by strain MM217 in the experimental cheese was responsible for the appreciable decline in the number of L. monocytogenes cells. Although other investigators established that preformed pediocin added to foods, including cheese, was inhibitory to L. monocytogenes (23, 29), inhibition of L. monocytogenes by in situ production of pediocin in Cheddar cheese by a lactococcal starter culture has not been reported previously.

After 6 months of ripening at 8°C, the level of recoverable pediocin activity decreased from 64,000 to 2,000 AU per g. Garde et al. (10) also described a decrease in the recovery of enterocin activity from a Hispanic type of cheese after 15 days of ripening at 14°C. In contrast, Ryan et al. (28) reported that the levels of lactacin 3147 remained relatively constant during 6 months of ripening at 8°C. The primary cause of the decline in the pediocin titers after 6 months of ripening was probably the sensitivity of pediocin to proteinases, in addition to autoysis of the starter and release of peptidases. However, other factors, including depletion of carbon and energy sources and binding of pediocin to cheese components, may also have contributed to the decrease in pediocin production and/or activity.
Although the level of pediocin activity recovered from the experimental cheese after 6 months of ripening at 8°C was lower than the levels recovered earlier, the number of pathogen cells was reduced to <10 CFU per g. As the residual L. monocytogenes population did not increase, the persistence of L. monocytogenes in an otherwise hostile environment could be attributed in part to individual cells that were entrapped in the casein matrix, which may have sequestered the pathogen somewhat from exposure to pediocin. The residual L. monocytogenes population may also have included a subpopulation of pediocin-resistant cells that over a longer time period could grow to levels sufficient to detect. However, it is also possible that the low pH, low water activity, and low ripening temperature provided additional hurdles which suppressed the growth and survival of residual L. monocytogenes cells in Cheddar cheese.

In conclusion, a genetically modified starter culture that produced pediocin in situ improved the microbiological safety of Cheddar cheese contaminated with L. monocytogenes. The acid-producing quality of the bacteriocinogenic starter culture and the composition of the Cheddar cheese produced with it were not altered by the acquisition of pMC117. In addition, the Standard of Identity of Cheddar Cheese was maintained, as pediocin was produced in situ and, thus, was not considered an additive. The use of genetically enhanced starter cultures that produce bacteriocins in situ may lessen microbiological safety problems with foods that do not undergo high-heat treatment and/or foods in which the use of chemical preservatives is not suitable.

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