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Effect of Enterocin CCM 4231 on *Listeria monocytogenes* in Saint-Paulin Cheese

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ABSTRACT. The bacteriocin production by *Enterococcus faecium* strain in cheese milk and cheese was demonstrated. Purified enterocin CCM 4231 exhibited an anti-listerial effect during Saint-Paulin cheese manufacture. During cheese production the strain grew to a final concentration of 10.1 ± 0.01 log CFU per mL per g in cheese. Then only a slight decrease of the cell concentration was noticed during ripening and was almost stable for 8 weeks. No significant differences in pH were observed between the experimental and reference cheeses. Bacteriocin production during cheese manufacture was detected only in milk samples and curd, reaching a level of 100 AU/mL. After addition of purified enterocin CCM 4231 (concentration 3200 AU/mL) into the experimental cheese, the initial concentration of 6.7 ± 0.06 log CFU per mL of *Listeria monocytogenes* Ohio was reduced up to 1.9 ± 0.01 log CFU per mL per g. After 6 weeks and at the end of the experiment the difference of surviving cells of *L. monocytogenes* Ohio in ECH was only one or 0.7 log cycle compared to the control cheese. Although enterocin CCM 4231 partially inhibited *L. monocytogenes* in Saint-Paulin cheese manufacture, an inhibitory effect of enterocin added was shown in 1-week cheese; however, it was not possible to detect bacteriocin activity by the agar spot test. The traditional fermentation and ripening process was not disturbed, resulting in acceptable end-products, including sensory aspects.

Listeria monocytogenes is a psychotrophic Gram-positive food-borne pathogen which can be found in a large range of foods, including cheeses (Davies *et al.* 1997; Farias *et al.* 1999). It can also grow at refrigeration temperatures. *Listeria* spp. are able to grow in environments such as feces, soil and water. In humans or animals, the majority of infections probably result from ingestion of food or feed containing large numbers of *L. monocytogenes* (McLaugchlin 1997). Therefore, the developed control systems must exclude this pathogen from the food chain and provide conditions to avoid its multiplication. One of the possibilities involved in the control system to prevent contamination is the application of bacteriocins with predominant anti-listerial effect into the sources and/or directly into the food (feed) during their manufacture.

Bacteriocins are protein compounds with an antimicrobial effect against closely related bacteria (Klaenhammer 1993). This definition has been broadened somewhat in recent years, in that it has become evident that some bacteriocins may show inhibitory spectra that include unrelated genera (El-Ziney and Debevere 1998, Lauková *et al.* 2000). Generally, bacteriocins are low-molar-mass, cationic, amphiphilic peptides which tend to aggregate and are benign to the producing organism (Scanell *et al.* 2000). They have been examined both at applied and basic levels. At present the following classes of bacteriocins can be distinguished: lantibiotics, small, heat-stable peptides with four subgroups and large, heat-labile proteins (Nes *et al.* 1996). Bacteriocin-producing strains of lactic-acid bacteria should ensure biological preservation without changing sensory characteristics of products. In the reviews of Hanlin and Evancho (1994) and Holzapfel *et al.* (1995) many potential applications of protective cultures are presented, involving bacteriocin-producing strains with protective influence in the food systems. The potential use of bacteriocin (lantibiotic) nisin to control *L. monocytogenes* in cheese manufacture or in poultry was reported by Ferreira and Lund (1996) and by Mahadeo and Tatini (1994). The products themselves, cheeses including, are the source of bacteriocin-producing isolates – hence, their protective character could be used directly.

Interest in enterocins (bacteriocins produced by some enterococci; Lauková and Mareková 2001; Morovský *et al.* 2001) increased recently as it was recognized that production of bacteriocins active against *L. monocytogenes* is an extensively prevalent phenotype in enterococci (Giraffa 1995; Ennahar *et al.* 2000). The possibility of general use of enterocin(s) in food systems is limited only by legislative problems. Enterocin CCM 4231 was defined as a small, heat-stable substance with a broad antimicrobial spectrum, which is produced by *Enterococcus faecium* strain CCM 4231 (Lauková *et al.* 1993).

Here we describe growth and bacteriocin production by the CCM 4231 strain in cheese milk and evaluation of anti-listerial effect of enterocin CCM 4231 during the manufacture of Saint-Paulin cheese.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions. The bacteriocin-producing strain *Enterococcus faecium* CCM 4231 was cultivated in MRS broth and/or on MRS agar (*Oxoid*). An overnight culture of *L. monocytogenes* Ohio strain (an 0.1 % inoculum, concentration 10 CFU/nL, *i.e.* 10^7 CFU/mL; *INRA*, Jouy-en-Josas, France) propagated twice at 37 °C in Tryptone Soy Broth with 0.6 % yeast extract (TSBYE, *Oxoid*) was used for contamination of cheese milk. Commercial starter culture (CSC; composed from *Lactococcus lactis*, *L. lactis* subsp. *diacetilactis*, *L. cremoris* and one strain of *Leuconostoc* sp.) used in cheese-making was Flora Danica. It was propagated twice in sterile reconstituted skimmed milk at 30 °C before use in cheese-making.

Growth and bacteriocin production in cheese milk and cheese. 10-L vat of cheese milk pasteurized at 70 °C (experimental cheese milk, ECH) was inoculated with 1 % of an overnight culture of the CCM 4231 strain in milk (propagated twice at 37 °C in sterile reconstituted skimmed milk), resulting in an initial concentration of 100 CFU/nL (*i.e.* 10^8 CFU/mL). Control cheese (CCH) was made from the other milk vat, *i.e.* without inoculum. Growth of the CCM 4231 strain in cheese milk and cheese was checked during all cheese-making procedures by plating of appropriate dilutions of samples on M-*Enterococcus* agar (*Oxoid*). Plates were incubated at 37 °C for 2 d.

Cheese-manufacturing process. Saint-Paulin cheese was manufactured in 10-L vats from pasteurized milk, using standard technology for this type of cheese. Milk vats were divided into the experimental cheese vat (ECH), the control cheese vat (CCH) and the reference cheese vat (RCH). ECH and CCH vats were inoculated with 0.1 % (diluted suspension) of an overnight culture of *L. monocytogenes* Ohio strain in TSYBE. Then 50 mL of CSC was added to all milk vats. Purified enterocin CCM 4231 (3200 AU/mL; Lauková *et al.* 1999) was added to the experimental cheese fabricate. Rennet (2 mL per 10 L) and $CaCl_2$ (3 g per 10 L) were added. The curds were cut after 30 min into 4–6 mm cubes and scalded at 31 °C for 10 min. Whey was separated (50 %), followed by scalding at 28 °C for 50 min. Cheese was pre-pressed for 10 min and then was pressed for 2¼ h and salted for 14 h at 14 °C. Finally, it was kept at 14 °C for 8 weeks (ripening). Milk, curd, whey and cheese were aseptically sampled and analyzed immediately after sampling. Cheese homogenates in sterile trisodium citrate solution, and decimal dilutions in Ringer solution (*Oxoid*), were prepared and plated in triplicate on Oxford agar (*Oxoid*). Plates were incubated at 30 °C for 2 d.

Detection of L. monocytogenes *during the cheese production procedure.* For detection of *L. monocytogenes* in samples taken throughout cheese-ripening, an enrichment procedure was used. Cheese samples (10 g) were homogenized with 90 mL of a 0.1 % of sterile trisodium citrate solution in a Stomacher 80 (*Seward Laboratory*, UK). The appropriate dilutions in Ringer solution (1 mL) were then transferred to a tube containing *Listeria* enrichment broth (*Oxoid*; each dilution into 3 tubes). Tubes were cultivated at 29–30 °C for 2 d. The survival counts of *Listeria* were then taken on Oxford agar plates (30 °C, 2 d) and enumerated by the most probable number technique. pH in cheese homogenate was measured (pH Meter 3310; *Jenway*, UK). Sensory analysis was not done in our experiment.

Bacteriocin assay. Homogenized samples (in sterile 0.1 % of trisodium citrate solution) were heated at 80 °C for 10 min and centrifuged (10 000 ***g***) at 4 °C for 10 min. Then bacteriocin activity was tested by the agar spot technique (De Vuyst *et al.* 1996). Serial two-fold dilutions (in Ringer solution) of treated samples were spotted (10 µL) onto a bottom layer of 1.5 % (*W/V*) of TSY agar (*Oxoid*) inoculated with 500 µL of the indicator strain *L. monocytogenes* Ohio (A_{600} = 0.4). After pre-diffusion at 4 °C for 30 min, the plates were incubated at 37 °C for 18 h. The highest dilution showing a definite zone of growth inhibition was recorded. The titer of bacteriocin activity was quantified and expressed in arbitrary units (AU/mL), the reciprocal of the highest sample dilution showing inhibition.

RESULTS AND DISCUSSION

An initial level of 8.3 ± 0.03 log CFU/mL of *E. faecium* strain CCM 4231 was obtained in cheese milk. During cheese manufacture, the strain grew to reach 10.1 ± 0.1 log CFU per mL per g (Table I). During the ripening period (up to 8 weeks), only a slight decrease up to an initial cell concentration was found. No differences in pH were noted between ECH and CCH. At the end of the ripening period in both ECH and CCH even the same pH was measured. Bacteriocin activity was detected only in ECH milk samples and curd reaching a level of 100 AU/mL.

Purified enterocin CCM 4231 added to ECH reduced the initial cell concentration of *L. monocytogenes* (6.7 ± 0.06 log CFU per mL per g) to 2.4 ± 0.04 log CFU per mL per g (Table II) through one week of ripening, in comparison with CCH (6.0 ± 0.01). Although after 6 weeks of ripening as well as at the

end of the experiment, the difference in surviving of *Listeria* cells in ECH was only one log cycle (from 2.6 ± 0.01 to 1.6 ± 0.01) compared to CCH, an inhibitory effect of added enterocin was very clearly seen in one-week cheese. It was not possible to detect any activity by analytical agar spot test.

Table I. Growth and bacteriocin production of *E. faecium* in cheese milk[a]

Sample	Reference cheese pH	Experimental cheese	
		pH	log CFU/mL[b]
Cheese milk[c]	6.70 ± 0.03	6.62 ± 0.02	8.3 ± 0.03
Cheese milk with CSC[c,d]	6.63 ± 0.02	6.57 ± 0.02	9.9 ± 0.05
Curd[c]	6.62 ± 0.01	6.55 ± 0.01	9.3 ± 0.02
Cheese	6.77 ± 0.03	6.56 ± 0.04	10.1 ± 0.01
1 week of ripening	5.18 ± 0.04	4.97 ± 0.04	8.5 ± 0.04
6 weeks of ripening	4.97 ± 0.04	4.97 ± 0.04	8.6 ± 0.06
8 weeks of ripening	4.97 ± 0.04	4.97 ± 0.04	8.6 ± 0.06

[a]Averaged from triplicate plating or measuring.
[b]Cell concentration of *E. faecium* strain CCM 4231.
[c]Bacteriocin activity (100 AU/mL) detected by agar spot test.
[d]CSC – commercial starter culture.

Table II. Cheese manufacture with addition of enterocin CCM 4231 in cheese milk[a]

Sample	Reference cheese pH	Cheese with *Listeria*		Cheese with *Listeria* and enterocin	
		pH	*L. monocytogenes*[b]	pH	*L. monocytogenes*[b]
Milk	6.78 ± 0.06	6.78 ± 0.06	6.1 ± 0.04	6.94 ± 0.06	6.7 ± 0.06
Milk + enterocin[c]	–	–	–	6.85 ± 0.06	4.1 ± 0.04
Milk + enterocin[c] + CSC[d]	6.80 ± 0.06	6.68 ± 0.06	–	6.79 ± 0.06	1.6 ± 0.01
Curd	6.80 ± 0.05	6.67 ± 0.05	–	6.79 ± 0.05	1.6 ± 0.01
Cheese	6.71 ± 0.04	6.71 ± 0.04	6.1 ± 0.04	6.73 ± 0.03	2.1 ± 0.04
1 week of ripening	5.15 ± 0.06	5.42 ± 0.06	6.0 ± 0.01	5.27 ± 0.04	2.4 ± 0.04
6 weeks of ripening	5.12 ± 0.07	5.51 ± 0.05	2.6 ± 0.01	5.46 ± 0.05	1.6 ± 0.01
8 weeks of ripening	5.23 ± 0.05	5.42 ± 0.03	2.6 ± 0.01	5.49 ± 0.03	1.9 ± 0.01

[a]Averaged from triplicate plating or measuring.
[b]Cell concentration of *L. monocytogenes* strain Ohio after inoculation of cheese milk (log CFU/mL); bacteriocin activity could not be detected by agar spot test.
[c]Added to 3200 AU/mL.
[d]Commercial starter culture.

Growth of the CCM 4231 strain in cheese milk was comparable with that in skimmed milk (Laukoá *et al.* 1999). Growth started from an initial level of 100 CFU per µL per g and after 6 h and/or 8 h 100 and 1000 CFU per nL per g were found; bacteriocin activity was 400–6400 AU/mL. Nunez *et al.* (1997) described that *E. faecalis* strain INIA 4 was able to produced enterocin in competition with milk native microflora during the manufacture of Manchego cheese from raw ewe milk (high activity was found after 6–8 h of growth of the producing strain). The CCM 4231 strain produced bacteriocin in cheese milk also after 6 or 8 h, in exponential growth phase (*unpublished data*).

Inability to detect bacteriocin activity in cheese by the plating method (at laboratory level), in spite of the inhibitory effect was shown, could be probably explained by the metabolic activity of the bacteriocin-producing strain which could be restrained by the regular starter bacteria. Giraffa *et al.* (1995) reported an insignificant slight difference in the viable counts of thermophilic starter streptococci in Talleggio cheese manufacture. We have also this kind of experience during yoghurt production which was experimentally contaminated with *Staphylococcus aureus* and treated by enterocin CCM 4231 (Laukoá *et al.* 1999, 2001). The other explanation could be the concentration of CFU of the initial inoculum. The protocol used for bacteriocin titration from a cheese matrix permitted to overcome the intrinsic difficulties of a solid matrix, making it possible to use bacteriocin titration by the diffusion agar spot test (Giraffa *et al.* 1995). Because of relatively low pH (5.46 ± 0.05; *see* Table II) strain Ohio was probably able to initiate growth. On the other

hand, in CCH cheese a similar pH was measured (5.51 ± 0.05) and a decrease in Ohio cells was found. However, difference of one log cycle and/or 0.7 log cycle was maintained in Ohio cells counting between ECH and CCH, as well. Inhibition of *Listeria* cells by enterocin CRL35 during goat cheese manufacture was reported by Farias *et al.* (1999). Moreover, levels of *Listeria* sp. in raw milk are usually less than 100/mL which probably can be eliminated by the bacteriocin (Vlaemynck, *unpublished data*). Kelly *et al.* (1996) reported that one of possible way to limit the growth of undesired microorganisms in minimally processed products is the use of protective cultures. The traditional fermentation and ripening process was not disturbed, resulting in sensorially acceptable end-products.

This experiment was performed in the *Agricultural Research Center, Department of Animal Product Quality and Transformation Technology* (Melle, Belgium) in 1997. Research support provided by *EU–Copernicus Project* ERB CIPACT 940160 (a part of final tests was also supported by the project of *Slovak Scientific Agency* VEGA no. 2/6106/99) and the skilful technical assistance of A. van De Walle as well as the other technical assistants are gratefully appreciated. Thanks are due to Dr. G. Waes, director of *Agricultural Research Center* in Melle, for the acceptance of the experiment. In addition, the experiments with enterocin had pilot character at that time; that is, only preliminary results were presented in comparison with recently applied enterocins in dairy products.

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