Available online at www.sciencedirect.com
ScienceDirect
International Journal of Food Microbiology 121 (2008) 229–233
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Inhibition of *Listeria monocytogenes* in dairy products using the bacteriocin-like peptide cerein 8A

Delmar Bizani [a,b], Juliana A.C. Morrissy [a], Ana P.M. Dominguez [a], Adriano Brandelli [a,*]

[a] *Laboratório de Bioquímica e Microbiologia Aplicada, Instituto de Ciência e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, 91501-970 Porto Alegre, Brazil*
[b] *Centro Universitario La Salle, Av. Victor Barreto 2288, 92010-000 Canoas, Brazil*

Received 15 March 2007; received in revised form 15 August 2007; accepted 6 November 2007

Abstract

The efficacy of the antimicrobial peptide cerein 8A to control the development of *Listeria monocytogenes* in milk and soft cheese was investigated. The addition of 160 AU ml^{-1} cerein 8A to UHT milk resulted in a decrease of 3 log cycles in viable cells within the 14-day period at 4 °C. The viable counts of *L. monocytogenes* in pasteurized milk samples containing cerein 8A was lower than those observed in controls without bacteriocin. Addition of cerein 8A to Minas-type soft cheese caused a delay in the start of exponential growth phase, although similar counts were observed after day 6. When cerein 8A was used to control cheese surface contamination by *L. monocytogenes*, a decrease of 2 log cycles in viable counts of cerein-treated samples was observed during 30 days at 4 °C. This antimicrobial peptide shows potential use as a biopreservative for application in dairy products.

Keywords: Antimicrobial; Bacteriocin; Cheese; Listeriosis; Milk

1. Introduction

Listeria monocytogenes is a psychrotrophic microorganism that can grow at temperatures ranging from 1 to 45 °C, is highly salt-tolerant, and can initiate growth at a relatively low pH (Sorrells et al., 1989; Farber and Peterkin, 1991). These characteristics make *L. monocytogenes* particularly difficult to control in food, and therefore contamination by this bacterium could lead to a high risk factor. Soft cheeses are particularly sensitive to colonization by *L. monocytogenes*, most likely because of the frequent handling of cheese processing that may allow post-process contamination. Consumption of contaminated cheese was directly linked to several cases of listeriosis, and listeriosis outbreaks related to this food are relatively frequent (Farber and Peterkin, 1991; Reij et al., 2004). In addition, recalls due to *L. monocytogenes* in cheeses are reported (Muriana, 1996). This bacterium was isolated in 26.7% of Minas-type cheeses, a soft white cheese largely consumed in Brazil (Silva et al., 2004).

Bacteriocins are antimicrobial peptides widespread produced by bacteria. Those produced by lactic acid bacteria (LAB) have been the subject of intensive investigation because of their potential use as biopreservatives in the food industry (O'Sullivan et al., 2002). Several bacteriocins from LAB have been effectively characterized and tested in food systems to combat pathogenic and spoilage microorganisms. Nisin is the most well characterized bacteriocin and its use in food is permitted in more than 40 countries (Cleveland et al., 2001). Pediocin and nisin can reduce or inhibit *L. monocytogenes* in dairy products, particularly in pasteurized cheeses (Muriana, 1996).

Despite the intensive work on LAB, *Bacillus* is another interesting genus to investigate for antimicrobial peptides since this genus includes a variety of industrially important species and has a history of safe use in food and industry (Pedersen et al., 2002). The production of bacteriocins or bacteriocin-like substances has been already described for many *Bacillus* species

* Corresponding author. Fax: +55 51 3316 7048.
E-mail address: abrand@ufrgs.br (A. Brandelli).

0168-1605/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2007.11.016

such as *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus cereus*, among other (von Döhren, 1995; Stein, 2005).

B. cereus 8A was isolated from soils of native woodlands of Southern Brazil. This strain produces an antimicrobial peptide, cerein 8A, which inhibits several pathogenic and food-spoilage microorganisms (Bizani and Brandelli, 2002). This substance has a bactericidal effect on *L. monocytogenes* and *B. cereus*, apparently by disturbing the membrane function of target organisms (Bizani et al., 2005). The aim of this study was to evaluate the effect of cerein 8A on survival of *L. monocytogenes* in milk and Minas-type cheese.

2. Materials and methods

2.1. Bacterial strains

The producer strain was *B. cereus* 8A, isolated and characterized as described by Bizani and Brandelli (2002). Indicator strain was *L. monocytogenes* ATCC 7644. Strains were kept frozen in brain hearth infusion broth (BHI; Difco, Detroit, MI, USA) containing 20% glycerol at −21 °C.

2.2. Antimicrobial activity assay

The antimicrobial activity was detected by agar disk diffusion assay (Motta and Brandelli, 2002). Aliquots (20 μl) of bacteriocin were applied in paper disks on agar plates previously inoculated with a swab submerged in indicator strain suspension which corresponded to a 0.5 McFarland turbidity standard solution. Plates were incubated at 37 °C for 24 h. The bacteriocin titre was determined by serial two-fold dilution method (Kimura et al., 1998). Activity was defined as the reciprocal of the dilution after the last serial dilution giving an inhibition zone and was expressed as activity units (AU) per milliliter.

2.3. Production of cerein 8A

B. cereus 8A was grown in 100 ml of BHI broth in an orbital shaker at 125 rpm for 48 h at 30 °C. The culture was centrifuged at 10,000 *g* for 10 min at 4 °C, and the supernatant was sterilized with a 0.22 μm filter membrane (Millipore, Bedford, MA, USA) and stored at 4 °C until utilization. The bacteriocin was further purified by ammonium sulfate precipitation and 1-butanol extraction, as previously described (Bizani et al., 2005).

2.4. Addition of cerein 8A to milk samples

Fresh samples of UHT and pasteurized milk were obtained at a local market. Samples were manipulated under sterile conditions. An aliquot of cerein 8A (final concentration 160 AU ml^{-1}) was added to each 10 ml milk samples. Then, *L. monocytogenes* cells were inoculated to give 10^4 CFU ml^{-1}. Heat inactivated bacteriocin (30 min at 100 °C) was added to control tubes. Milk samples were stored at 4 °C and individual tubes were removed at 2-day intervals for evaluation of *L. monocytogenes* growth using Oxford Listeria selective agar (Acumedia, Lansing, MI, USA). All determinations were done using three independent samples. Total plate counts of original pasteurized milk were determined in Plate Count Agar (Acumedia, Lansing, MI, USA) according to standard procedures (Peeler and Maturin, 1992).

2.5. Addition of cerein 8A to Minas-type cheese

Cheeses were manufactured following the traditional procedure employed by Brazilian dairies (Buriti et al., 2005). Calcium chloride was initially added to the milk at a level of 0.25 g l^{-1}. Cerein 8A was added at same time of commercial rennet (Christian Hansen, Valinhos, Brazil) to a final concentration of 400 AU ml^{-1}. Then, *L. monocytogenes* cells were inoculated to give 10^2 CFU g^{-1}. Cheese samples were stored at 4 °C in sterile containers. Samples were removed at indicated intervals for evaluation of *L. monocytogenes*. Cheese samples (10 g) were homogenized with 90 ml of Listeria Enrichment Broth (Acumedia, Baltimore, MA, USA) in a blender for 60 s following by decimal serial dilutions before enumerating by plating in Oxford Listeria selective agar. Alternatively application of cerein 8A (400 AU ml^{-1}) was done on surface of manufactured cheese and then *L. monocytogenes* was inoculated by submerging the product in a suspension of 10^2 CFU ml^{-1} before incubation at 4 °C. All determinations were done using three independent samples.

3. Results and discussion

3.1. Inhibition of L. monocytogenes in milk

Samples of UHT milk were first tested to evaluate the effect of cerein 8A. The results can be visualized in Fig. 1. Growth of *L. monocytogenes* increased from day 5 in control samples, reaching about 8 $\log_{10}$ CFU ml^{-1} at the end of incubation. In

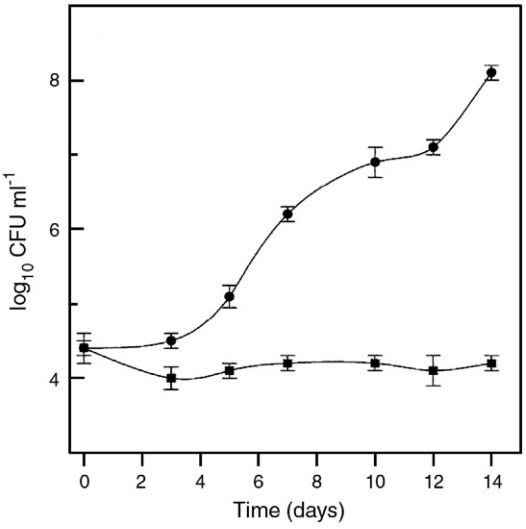

Fig. 1. Effect of cerein 8A to control *Listeria monocytogenes* in UHT milk at 4 °C. Heat inactivated (●) or 160 AU ml^{-1} cerein 8A (■) were added to milk samples before inoculation with *L. monocytogenes*. Each point is the mean ± S.E.M. of three independent experiments.

contrast, the viable counts of *L. monocytogenes* remained almost constant in the milk samples containing cerein 8A (Fig. 1).

Although the addition of bacteriocins to UHT milk may lack practical application, this product serves as an important system to evaluate the influence of milk components on the bacteriocin activity. Milk has been used as a food model to evaluate the efficacy of some bacteriocins against *L. monocytogenes*. Maisnier-Patin et al. (1995) used commercially available nisin (Nisaplin®) to investigate the additional lethality of bacteriocins on *L. monocytogenes* in skim milk during heating. They found that 25 or 50 IU of nisin per ml resulted in a significant reduction in the time required to achieve an equivalent reduction by heat alone. Addition of partially purified carnocin CP5 to skim milk (2000 AU ml^{-1}) inoculated with *L. monocytogenes* showed an immediate reduction in *L. monocytogenes* that recovered after either 2 days at 15 °C or 5 days at 7 °C (Mathieu et al., 1994). Pediocin 5 also reduced viable counts of *L. monocytogenes* in partially-skimmed milk (Huang et al., 1994).

Samples of pasteurized milk were also tested. Total plate counts of original milk were 4.1 log_{10} CFU ml^{-1}. Similar counts of *L. monocytogenes* were observed in controls and cerein-treated samples during the initial incubation time (Fig. 2). However, the addition of cerein 8A caused an increased reduction in the number of viable cells of *L. monocytogenes* during incubation for up to 5 days. The reduction of viable cells in pasteurized milk may suggest that cerein 8A has a synergism with some endogenous components of milk, such as lysozyme and lactoferrin (Nattress et al., 2001; Branen and Davidson, 2004), or also with metabolites produced by milk microflora. Indeed, bacteriocins and other inhibitory metabolites are widespread produced among LAB present in milk and dairy products (Rodríguez et al., 2000; Ayad et al., 2004).

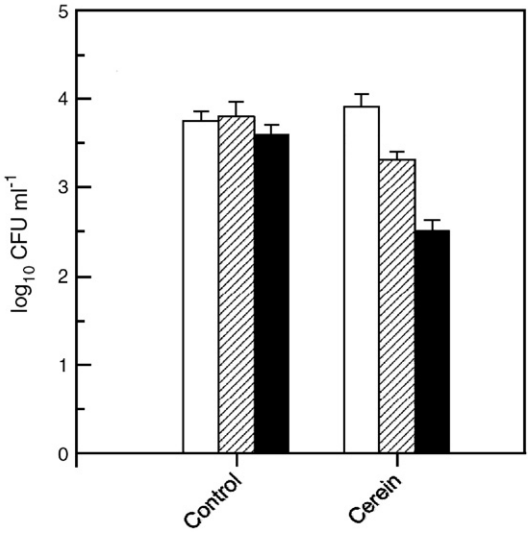

Fig. 2. Effect of cerein 8A to control *Listeria monocytogenes* in pasteurized milk at 4 °C. Heat inactivated (Control) or 160 AU ml^{-1} cerein 8A (Cerein) were added to milk samples before inoculation with *L. monocytogenes*. Samples were monitored at day 0 (white bars), 3 (dashed bars) and 5 (black bars). Bars are the means ± S.E.M. of three independent experiments.

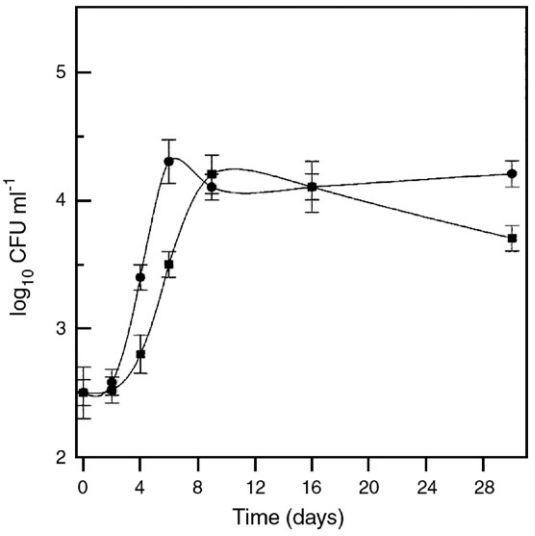

Fig. 3. Effect of cerein 8A to control *Listeria monocytogenes* in Minas-type cheese at 4 °C. (●) control, (■) 400 AU ml^{-1} cerein 8A. Each point is the mean ± S.E.M. of three independent experiments.

3.2. Inhibition of *L. monocytogenes* in Minas cheese

The addition of 400 AU ml^{-1} cerein 8A during the manufacture of Minas-type cheese caused a delay in the development of *L. monocytogenes* when compared with controls without bacteriocin (Fig. 3). Cell counts reached 4 log_{10} CFU ml^{-1} within 6 days in untreated cheese samples, while these values were reached from day 9 in bacteriocin-treated samples. In addition, a decrease in the viable cells of *L. monocytogenes* was observed after day 16 (Fig. 3).

Cerein 8A was applied in the cheese surface and the development of *L. monocytogenes* was monitored. The samples flooded in cerein 8A suspension have significant lower viable

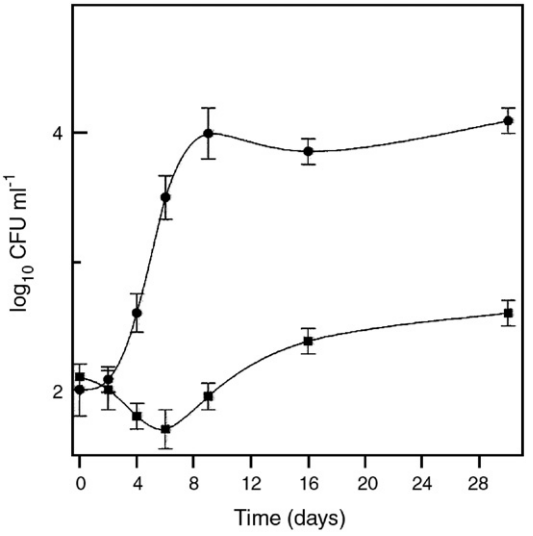

Fig. 4. Effect of cerein 8A to control *Listeria monocytogenes* on surface of Minas-type cheese at 4 °C. (●) control, (■) 400 AU ml^{-1} cerein 8A. Each point is the mean ± S.E.M. of three independent experiments.

counts when compared with the controls without bacteriocin addition (Fig. 4). The *L. monocytogenes* counts of the cerein-treated samples were below 2 $\log_{10}$ CFU ml^{-1} until day 10.

Nisin has been tested in dairy products, causing inhibition of *L. monocytogenes* in ricotta-type cheese (Davies et al., 1997). The use of 1.25 mg ml^{-1} nisin protected the cheeses for up to 11 days while 2.5 mg ml^{-1} increased the shelf-life to 55 days. The presence of the mould inhibitor potassium sorbate may have contributed to the antimicrobial effectiveness of nisin, since it has been shown that this preservative acts in combination with nisin enhancing the listericidal effect compared with nisin alone (Buncic et al., 1995). Although nisin has been used to control *L. monocytogenes* in cheese, strains presenting increased tolerance or resistance to nisin have been reported (Rasch and Knochel, 1998; Martinez et al., 2005). Therefore, research for new substances presenting antilisterial activity is a very important field.

The use of cerein 8A may increase the shelf-life of Minas cheese. The food-borne pathogen *L. monocytogenes* was effectively controlled, for a period of up to 4 weeks at 4 °C. The less effective activity of incorporated cerein 8A in comparison with its application in cheese surface may be associated with inactivation by endogenous food enzymes or binding to components of the food matrix, which has been reported for other bacteriocins (de Vuyst and Vandamme, 1994). The chemical composition and the physical conditions of food can have a significant influence on the activity of the bacteriocin (Cleveland et al., 2001). Indeed, the effective nisin concentration to control *L. monocytogenes* in cottage cheese (Ferreira and Lund, 1996) and ricotta cheese (Davies et al., 1997) have been reported as 2000 and 100 IU ml^{-1}, respectively.

Camembert cheese elaborated with a nisin-producing *L. lactis* has reduced added *L. monocytogenes* population by over 3 log cycles through the second week of ripening. Upon prolonged ripening, *L. monocytogenes* increased, especially on the surface (Maisnier-Patin et al., 1992). The use of LAB itself to control *L. monocytogenes* has also been described. The surface contamination of Munster cheese could be prevented by spraying a cell suspension of *L. plantarum* pediocin AcH producer (Ennahar et al., 1998). In this regard, cerein 8A was capable to inhibit surface contamination of *L. monocytogenes* in Minas cheese, suggesting its potential use as biopreservative in dairy products.

Acknowledgement

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil.

References

Ayad, E.H.E., Nashat, S., El-Sadek, N., Metwaly, H., El-Soda, M., 2004. Selection of wild lactic acid bacteria isolated from traditional Egyptian dairy products according to production and technological criteria. Food Microbiol. 21, 715–725.

Bizani, D., Brandelli, A., 2002. Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. strain 8A. J. Appl. Microbiol. 93, 512–519.

Bizani, D., Motta, A.S., Morrissy, J.A.C., Terra, R.M., Souto, A.A., Brandelli, A., 2005. Antibacterial activity of cerein 8A, a bacteriocin-like peptide produced by *Bacillus cereus*. Int. Microbiol. 8, 125–131.

Branen, J.K., Davidson, P.M., 2004. Enhancement of nisin, lysozyme, and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lactoferrin. Int. J. Food Microbiol. 90, 63–74.

Buncic, S., Fitzgerald, C.M., Bell, R.G., Hudson, J.A., 1995. Individual and combined listericidal effects of sodium lactate, potassium sorbate, nisin and curing salts at refrigeration temperature. J. Food. Saf. 15, 247–264.

Buriti, F.C.A., Rocha, J.S., Assis, E.G., Saad, S.M.I., 2005. Probiotic potential of Minas fresh cheese prepared with the addition of *Lactobacillus paracasei*. LWT-Food Sci. Technol. 38, 173–180.

Cleveland, J., Montville, T.J., Nes, I.F., Chikindas, M.L., 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Int. J. Food Microbiol. 71, 1–20.

Davies, E.A., Bevis, H.E., Delves-Broughton, J., 1997. The use of the bacteriocin, nisin, as a preservative in ricotta-type cheeses to control the food-borne pathogen *Listeria monocytogenes*. Lett. Appl. Microbiol. 24, 343–346.

de Vuyst, L., Vandamme, E., 1994. Bacteriocins of Lactic Acid Bacteria. Microbiology, Genetics and Applications. Blackie Academic and Professional, London.

Ennahar, S., Assobhei, O., Hasselmann, C., 1998. Inhibition of *Listeria monocytogenes* in a smear-surface soft cheese by *Lactobacillus plantarum* WHE 92, a pediocin AcH producer. J. Food Prot. 61, 186–191.

Farber, J.M., Peterkin, P.I., 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. 55, 476–511.

Ferreira, M.A., Lund, B.M., 1996. The effect of nisin on *Listeria monocytogenes* in culture medium and long-life cottage cheese. Lett. Appl. Microbiol. 22, 433–438.

Huang, J., Lacroix, C., Daba, H., Simard, R.E., 1994. Growth of *Listeria monocytogenes* in milk and its control by pediocin 5 produced by *Pediococcus acidilactici* UL5. Int. Dairy J. 4, 429–443.

Kimura, H., Sashihara, T., Matsusaki, H., Sonomoto, K., Ishizaki, A., 1998. Novel bacteriocin of *Pediococcus* sp. ISK-1 isolated from well-aged bed of fermented rice bran. Ann. N.Y. Acad. Sci. 864, 345–348.

Maisnier-Patin, S., Deschmps, N., Tatini, S.R., Richards, J., 1992. Inhibition of *Listeria monocytogenes* in Camembert cheese made with a nisin-producing starter. Lait 72, 249–263.

Maisnier-Patin, S., Tatini, S.R., Richards, J., 1995. Combined effect of nisin and moderate heat on destruction of *Listeria monocytogenes* in milk. Lait 75, 81–91.

Martinez, B., Bravo, D., Rodriguez, A., 2005. Consequences of the development of nisin-resistant *Listeria monocytogenes* in fermented dairy products. J. Food Prot. 68, 2383–2388.

Mathieu, F.M., Michel, A., Lebrihi, A., Lefebvre, G., 1994. Effect of the bacteriocin carnocin CP5 on the viability of *Listeria monocytogenes* ATCC 15313 in salt solution, broth and skimmed milk, at various incubation temperatures. Int. J. Food Microbiol. 22, 155–172.

Motta, A.S., Brandelli, A., 2002. Characterization of an antibacterial peptide produced by *Brevibacterium linens*. J. Appl. Microbiol. 92, 63–71.

Muriana, P.M., 1996. Bacteriocins for control of *Listeria* spp. in food. J. Food Prot. 59, S54–S63.

Nattress, F.M., Yost, C.K., Baker, L.P., 2001. Evaluation of the ability of lysozyme and nisin to control meat spoilage bacteria. Int. J. Food Microbiol. 22, 111–119.

O'Sullivan, L., Ross, R.P., Hill, C., 2002. Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. Biochimie 84, 593–604.

Pedersen, P.B., Bjrnvad, M.E., Rasmussen, M.D., Petersen, J.N., 2002. Cytotoxic potential of industrial strains of *Bacillus* sp. Regul. Toxicol. Pharmacol. 36, 155–161.

Peeler, J.T., Maturin, L.J., 1992. Bacteriological Analytical Manual, 7th ed. AOAC International, Arlington.

Rasch, M., Knochel, S., 1998. Variations in tolerance of *Listeria monocytogenes* to nisin, pediocin PA-1 and bavaricin. Lett. Appl. Microbiol. 27, 275–278.

Reij, M.W., Den Aantrekker, E.D., ILSI European Risk Analysis in Microbiology Task Force, 2004. Recontamination as a source of pathogens in processed foods. Int. J. Food Microbiol. 91, 1–11.

Rodríguez, E., González, B., Gaya, P., Nuñez, M., Medina, M., 2000. Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk. Int. Dairy J. 10, 7–15.

Silva, W.P., Techera, S.C., Jantzen, M.M., von Laer, A.E., Lima, A.S., Mata, M.M., 2004. Presence of *Listeria monocytogenes* on handmade cheese "tipo Minas" commercialized in Pelotas city, Brazil. Alimentaria 357, 57–60.

Sorrells, K.M., Enigl, D.C., Herfield, J.R., 1989. Effect of pH, acidulant, time and temperature on the growth and survival of *Listeria monocytogenes*. J. Food Prot. 52, 571–573.

Stein, T., 2005. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol. Microbiol. 56, 845–857.

von Döhren, H., 1995. Peptides. Biotechnology 28, 129–171.