Food Microbiology 32 (2012) 38–47

Contents lists available at SciVerse ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Application of bacteriocinogenic *Enterococcus mundtii* CRL35 and *Enterococcus faecium* ST88Ch in the control of *Listeria monocytogenes* in fresh Minas cheese

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ARTICLE INFO

Article history:
Received 29 October 2011
Received in revised form
8 March 2012
Accepted 4 April 2012
Available online 14 April 2012

Keywords:
Bacteriocins
Minas cheese
Enterococcus mundtii
Enterococcus faecium
Listeria monocytogenes

ABSTRACT

Several strains of *Enterococcus* spp. are capable of producing bacteriocins with antimicrobial activity against important bacterial pathogens in dairy products. In this study, the bacteriocins produced by two *Enterococcus* strains (*Enterococcus mundtii* CRL35 and *Enterococcus faecium* ST88Ch), isolated from cheeses, were characterized and tested for their capability to control growth of *Listeria monocytogenes* 426 in experimentally contaminated fresh Minas cheese during refrigerated storage. Both strains were active against a variety of pathogenic and non-pathogenic microorganisms and bacteriocin absorption to various *L. monocytogenes*, *Enterococcus faecalis* ATCC 19443 and *Lactobacillus sakei* ATCC 15521 varied according to the strain and the testing conditions (pH, temperature, presence of salts and surfactants). Growth of *L. monocytogenes* 426 was inhibited in cheeses containing *E. mundtii* CRL35 up to 12 days at 8 °C, evidencing a bacteriostatic effect. *E. faecium* ST88Ch was less effective, as the bacteriostatic affect occurred only after 6 days at 8 °C. In cheeses containing nisin (12.5 mg/kg), less than one log reduction was observed. This research underlines the potential application of *E. mundtii* CRL35 in the control of *L. monocytogenes* in Minas cheese.

1. Introduction

Fresh Minas cheese (*Queijo Minas Frescal*) is a popular dairy product in Brazil, consumed all over the country. It is a fresh (non-ripened) cheese obtained by enzymatic coagulation of pasteurized bovine milk with rennet or other coagulating enzymes, and addition of lactic acid bacteria (LAB) is optional. Minas cheese has high water activity, pH above 5.0, low salt content and is not added of preservatives, resulting in an excellent substrate for growth of microorganisms and, consequently, the shelf-life scarcely exceeds 20 days under refrigeration (Souza and Saad, 2009).

Minas cheese is easy to manufacture and is produced by large dairy industries and also by small manufacturing plants, where *Listeria monocytogenes* as an environmental contaminant is common (Barancelli et al., 2011). The occurrence of the pathogen in Minas cheese has been frequently reported (Silva et al., 2001, 2004; Brito et al., 2008; Zocche et al., 2010). *L. monocytogenes* is particularly important in fresh cheeses like Minas cheese, because the growth is difficult to control due to the psychrotrophic characteristics and high salt tolerance of most strains (Swaminathan et al., 2007). Several studies have shown that *Listeria* strains are capable to survive for long periods in Minas cheese (Naldini et al., 2009; Pinto et al., 2009). *L. monocytogenes* causes listeriosis, a disease that affects pregnant women, the elderly, newborn and those who are immunocompromised. The pathogen has been detected in a wide range of foods, including dairy products and several large listeriosis outbreaks were linked to cheeses in the recent years (Koch et al., 2010; Fretz et al., 2010).

One technological alternative to conventional cheese preservation methods (chemical additives, salt, etc) is the use of indigenous LAB strains capable of producing bacteriocins with antilisterial activity. Bacteriocins are ribosomally synthesized peptides produced by several LAB and other bacteria as a defense mechanism against closely related bacteria (Gálvez et al., 2009).

Bacteriocin-producing enterococci have been extensively studied and were already isolated from a variety of sources, with the proposed application as food preservative culture, for pathogen

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doi:10.1016/j.fm.2012.04.005

control (Giraffa, 2003; Cocolin et al., 2007; Gálvez et al., 2009; Bayoub et al., 2011; Hadji-Sfaxi et al., 2011). Research in the field is progressing exponentially, and a recent study reported antiviral activity by the bacteriocin produced by *Enterococcus* spp. strains isolated from cheese (Wachsman et al., 2003), soy beans (Todorov et al., 2005) and smoked salmon (Todorov et al., 2010).

Several enterococcal strains produce bacteriocins (enterocins), and most of them are class II bacteriocins, which are heat stable, cationic, hydrophobic and low molecular weight peptides with antilisterial activity. This group of bacteriocins presents a cationic region with the conserved YGNGVXC 'pediocin box' motif and two residues of cysteine joined by an S–S bridge stabilizing the formed ß-sheet structure (Franz et al., 2007; Heng et al., 2007).

Only few enterocins were tested *in situ* (Giraffa et al., 1995; Laucova et al., 1999; Izquierdo et al., 2009; Settanni et al., 2011), thus little is known about their action as biopreservatives in foods. In this study, we report results on some characteristics of two bacteriocinogenic enterococci strains (*Enterococcus mundtii* CRL35 and *Enterococcus faecium* ST88Ch) isolated from cheeses and their effectiveness in the control of *L. monocytogenes* in experimentally contaminated Minas cheese. Results were compared to those obtained in cheeses prepared with a non-bacteriocinogenic *Enterococcus faecalis* strain and with commercial nisin.

2. Materials and methods

2.1. Strains

Two bacteriocinogenic enterococci were used in this study: *E. mundtii* CRL35, isolated from yellow cheese in Tucumán, Argentina (Farías et al., 1996) and *E. faecium* ST88Ch, isolated from yellow cheese in Sao Paulo, SP, Brazil (Furtado et al., 2009). The strains used in the determination of the antimicrobial spectrum of the bacteriocinogenic enterococci are described in Table 1. Pure cultures were obtained in MRS broth (Difco) or BHI medium (Oxoid) at 37 °C, and stored at −80 °C in growth medium supplemented with glycerol (15%, v/v, final concentration).

2.2. Characterization of the bacteriocins CRL35 and ST88Ch

2.2.1. Spectrum of activity

E. mundtii CRL35 and *E. faecium* ST88Ch were inoculated (2%, v/v) into 100 mL MRS broth and incubated at 30 °C for 24 h. Cells were harvested (8000 × *g*, 15 min, 4 °C) and the pH of the cell-free supernatants adjusted to 6.0 with sterile 1 M NaOH, heated for 10 min at 80 °C, and then filter-sterilized (0.20 μm, Millipore). Bacteriocin activity was tested against target organisms shown in Table 1, using the agar-spot test method (Todorov, 2008). Results were expressed as diameter of the inhibition zone (mm).

2.2.2. Dynamics of growth and bacteriocin production

MRS broth was inoculated with an 18-h-old culture (2%, v/v) of *E. mundtii* CRL35 or *E. faecium* ST88Ch and incubated at 30 °C, without agitation. Optical density ($OD_{600\ nm}$), pH and antimicrobial activity in the broth were measured at regular intervals for 25 h, using *L. monocytogenes* 426 as sensitive strain. In a separate experiment, *E. mundtii* CRL35 or *E. faecium* ST88Ch were cultivated in 10% and 5% skim milk (Molico, Nestlé) at 30 °C, without agitation, for 24 h, and antimicrobial activity was measured as described before. Antimicrobial activity was expressed as arbitrary units AU/mL, calculated as follows: $a^b \times 100$, where "*a*" represents the dilution factor and "*b*" the highest dilution that produces an inhibition zone of at least 2 mm in diameter.

2.2.3. Effect of bacteriocins on growth of L. monocytogenes, E. faecalis ATCC 19443 and Lactobacillus sakei ATCC 15521

E. mundtii CRL35 and *E. faecium* ST88Ch were cultivated in MRS broth for 18 h at 30 °C, without agitation. Cells were harvested (8000 × *g*, 15 min, 4 °C), the pH of the cell-free supernatants adjusted to 6.0 with sterile 1 M NaOH, heated for 10 min at 80 °C, and then filter-sterilized (0.20 μm, Millipore). A 20 mL aliquot of the filter-sterilized supernatant (pH was added to 100 mL culture of *L. monocytogenes* strains 101, 211, 426, 603 and 703, *E. faecalis* ATCC 19443 and *Lb. sakei* ATCC 15521) in early exponential phase and middle exponential phase and incubated for 16 h. Optical density readings at 600 nm were recorded at 1 h-interval.

In a separate set of experiments, cultures (100 mL) of *L. monocytogenes* strains 101, 211, 426, 603 and 703, *E. faecalis* ATCC 19443 and *Lb. sakei* ATCC 15521 in early exponential phase and middle exponential phase were harvested (5000 × *g*, 5 min, 4 °C), washed twice with 0.85% sterile saline and resuspended in 100 mL of 0.85% sterile saline. Equal volumes of the cell suspensions and the filter-sterilized (0.20 μm, Minisart®, Sartorius) bacteriocins CRL35 and ST88Ch were mixed. Viable cell numbers were determined before and after incubation for 1 h at 37 °C by plating onto BHI agar or MRS agar. Cell suspensions of *L. monocytogenes* 101, 211, 426, 603 and 703, *E. faecalis* ATCC 19443, *Lb. sakei* ATCC 15521 with no added bacteriocin served as controls.

2.2.4. Adsorption of bacteriocins to L. monocytogenes, E. faecalis ATCC 19443 and Lb. sakei ATCC 15521

Adsorption of bacteriocins produced by *E. mundtii* CRL35 and *E. faecium* ST88Ch to target strains described in Table 2 was tested according to Todorov (2008). The target strains were grown overnight in MRS or BHI broth at 30 °C and then centrifuged (8000 × *g*, 15 min, 4 °C). Cells were washed twice with sterile 5 mM phosphate buffer (pH 6.5) and resuspended to the original volume in the same buffer. Each cell suspension was mixed with an equal volume of filter-sterilized bacteriocins CRL35 and ST88Ch and incubated at 37 °C for 1 h. After removal of cells (8000 × *g*, 15 min, 25 °C), the activity of unbound bacteriocins in the adsorption supernatant was determined as described before. All experiments were done in duplicate. The percentage of adsorption to target the cells was calculated according to the following formula:

$$\%\ \text{adsorption} = 100 - \left(\frac{\text{bacteriocin activity after treatment}}{\text{original bacteriocin activity}} \times 100\right)$$

The effect of temperature in the adsorption of the bacteriocins produced by *E. mundtii* CRL35 and *E. faecium* ST88Ch to *L. monocytogenes* 101, 211, 426, 603 and 703, *E. faecalis* ATCC 19443 and *Lb. sakei* ATCC 15521 was also tested at 20 °C and 30 °C at pH 6.0. The effect in the adsorption of the bacteriocins to *L. monocytogenes* 426 was evaluated at different pH (4.0, 6.0 and 8.0) and with the addition of 1% NaCl, 1% Tween 20, 1% Tween 80 and 1% skim milk at 20 °C, 30 °C and 37 °C. Filter-sterilized bacteriocins CRL35 and ST88Ch were added to the treated cells, as described before, and incubated for 1 h at 37 °C. The cells were harvested and the antimicrobial activity in the cell-free supernatant determined as described before.

2.3. Aggregation of E. mundtii CRL35 and E. faecium ST88Ch with L. monocytogenes 426, E. faecalis ATCC 19443 and Lb. sakei ATCC 15521

For evaluation of auto-aggregation, strains *E. mundtii* CRL35 and *E. faecium* ST88Ch were grown in MRS broth (Difco) for 24 h at 37 °C. The cells were harvested by centrifugation at 7000 × *g* for 10 min at 20 °C, washed, resuspended and diluted in 0.85% sterile