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

Esteban Vera Pingitore [a,b,*], Svetoslav Dimitrov Todorov [a], Fernando Sesma [b], Bernadette Dora Gombossy de Melo Franco [a]

[a] Universidade de São Paulo, Faculdade de Ciências Farmacêuticas, Departamento de Alimentos e Nutrição Experimental, Laboratório de Microbiologia de Alimentos, Av. Prof. Lineu Prestes 580 Bl 14, 05508-000 São Paulo, SP, Brazil
[b] Centro de Referencia para Lactobacilos, Chacabuco 145, 4000 San Miguel de Tucumán, Tucumán, Argentina

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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

* Corresponding author. Centro de Referencia para Lactobacilos, Chacabuco 145, 4000 San Miguel de Tucumán, Tucumán, Argentina. Tel.: +54 381 4311720; fax: +54 381 4005600.
E-mail addresses: estebanverapingitore@gmail.com, estebanvera@cerela.org.ar (E. Vera Pingitore).

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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

Table 1
Spectrum of activity of bacteriocins produced by *E. mundtii* CRL35 and *E. faecium* ST88Ch.

Test microorganisms	Medium and growth temperature	Inhibition zone (mm)*	
		CRL35	ST88Ch
L. monocytogenes Scott A	BHI, 37 °C	22	25
L. monocytogenes serological group 4b			
Strain 101	BHI, 37 °C	23	18
Strain 211	BHI, 37 °C	22	14
Strain 302	BHI, 37 °C	20	–
Strain 620	BHI, 37 °C	19	20
Strain 703	BHI, 37 °C	23	20
Strain 724	BHI, 37 °C	22	17
L. monocytogenes serological group 1/2a			
Strain 103	BHI, 37 °C	16	12
Strain 104	BHI, 37 °C	15	15
Strain 106	BHI, 37 °C	16	16
Strain 409	BHI, 37 °C	15	15
Strain 506	BHI, 37 °C	15	17
Strain 709	BHI, 37 °C	15	20
L. monocytogenes serological group 1/2b			
Strain 426	BHI, 37 °C	24	12
Strain 603	BHI, 37 °C	23	18
Strain 607	BHI, 37 °C	–	17
L. monocytogenes serological group 1/2c			
Strain 408	BHI, 37 °C	15	21
Strain 422	BHI, 37 °C	13	14
Strain 637	BHI, 37 °C	15	14
Strain 711	BHI, 37 °C	–	19
Strain 712	BHI, 37 °C	16	18
Bacillus cereus ATCC 11778	BHI, 30 °C	–	–
Bacillus mycoides	BHI, 30 °C	–	–
Cronobacter sakazakii CDC996-77	BHI, 37 °C	–	–
Cronobacter sakazakii CFS10	BHI, 37 °C	–	–
Cronobacter sakazakii CFS101	BHI, 37 °C	–	–
Cronobacter sakazakii E922	BHI, 37 °C	–	–
Cronobacter sakazakii SK90	BHI, 37 °C	–	–
Enterobacter aeroginosa ATCC 13048	BHI, 37 °C	–	–
Enterococcus faecalis ATCC 19443	MRS, 30 °C	14	–
Enterococcus faecium ET05	MRS, 30 °C	–	–
Enterococcus faecium ET12	MRS, 30 °C	–	–
Enterococcus faecium ET88	MRS, 30 °C	–	–
Enterococcus faecium SD100	MRS, 30 °C	13	–
Enterococcus faecium SD154	MRS, 30 °C	15	–
Enterococcus faecium ST211Ch	MRS, 30 °C	–	–
Enterococcus faecium ST62BZ	MRS, 30 °C	–	–
Enterococcus faecium ST88Ch	MRS, 30 °C	12	–
Enterococcus hirae DF105	MRS, 30 °C	11	14
Escherichia coli ATCC 8739	Luria, 37 °C	–	–
Escherichia coli O157:H7	Luria, 37 °C	–	–
Lactobacillus acidophilus LA14	MRS, 30 °C	–	12
Lactobacillus acidophilus La5	MRS, 30 °C	–	–
Lactobacillus acidophilus LAC4	MRS, 30 °C	–	–
Lactobacillus curvatus ET06	MRS, 30 °C	10	–
Lactobacillus curvatus ET30	MRS, 30 °C	11	–
Lactobacillus curvatus ET31	MRS, 30 °C	–	–
Lactobacillus delbrueckii ET32	MRS, 30 °C	–	–
Lactobacillus delbrueckii subsp. *bulgaricus* B1	MRS, 30 °C	–	–
Lactobacillus delbrueckii subsp. *bulgaricus* B15	MRS, 30 °C	–	–
Lactobacillus delbrueckii subsp. *bulgaricus* B16	MRS, 30 °C	–	–
Lactobacillus delbrueckii subsp. *bulgaricus* B2	MRS, 30 °C	12	–
Lactobacillus delbrueckii subsp. *bulgaricus* B5	MRS, 30 °C	–	–
Lactobacillus fermentum ET35	MRS, 30 °C	–	–
Lactobacillus paracasei subsp. *paracasei* DF60Mi	MRS, 30 °C	–	–
Lactobacillus plantarum ST16Pa	MRS, 30 °C	–	–
Lactobacillus sakei subsp *sakei* 2a	MRS, 30 °C	13	–
Lactobacillus sakei ATCC 15521	MRS, 30 °C	10	–
Lactococcus lactis subsp. *lactis* B17	MRS, 30 °C	–	–
Lactococcus lactis subsp. *lactis* DF02Mi	MRS, 30 °C	11	–
Lactococcus lactis subsp. *lactis* DF03Mi	MRS, 30 °C	13	–
Lactococcus lactis subsp. *lactis* DF04Mi	MRS, 30 °C	–	15
Lactococcus lactis subsp. *lactis* MK02R	MRS, 30 °C	–	16
Lactococcus lactis subsp. *lactis* R704	MRS, 30 °C	–	–
Lactococcus lactis subsp. *lactis* V69	MRS, 30 °C	–	–
Leuconostoc lactis DF05Mi	MRS, 30 °C	12	–
Listeria innocua ATCC 33090	BHI, 37 °C	22	–
Listeria seeligeri L21	BHI, 37 °C	–	–
Listeria welshimery L1	BHI, 37 °C	–	–

Table 1 (*continued*)

Test microorganisms	Medium and growth temperature	Inhibition zone (mm)*	
		CRL35	ST88Ch
Pediococcus pentosaceus ET34	MRS, 30 °C	–	–
Salmonella braenderup H9812	BHI, 37 °C	–	–
Salmonella enteritidis ATCC 13076	BHI, 37 °C	–	–
Salmonella typhimurium ATCC 14028	BHI, 37 °C	–	–
Staphylococcus aureus ATCC 25923	BHI, 37 °C	–	–
Staphylococcus aureus ATCC 29213	BHI, 37 °C	–	–
Staphylococcus aureus ATCC 6538	BHI, 37 °C	–	–

*Bacteriocin activity presented as diameter of inhibition zones (mm). – = no inhibition observed or inhibition zone less than 2 mm diameter.

saline to $OD_{660\ nm} = 0.3$. One milliliter of the cell suspension was transferred to a 2 mL sterile plastic cuvette and the $OD_{660\ nm}$ recorded over 60 min, using a spectrophotometer (Ultraspec 2000, Pharmacia Biotech). Auto-aggregation was determined using the following equation (Todorov and Dicks, 2008):

$$\%\ \text{Auto-aggregation} = [(OD_0 - OD_{60})/OD_0] \times 100$$

OD_0 refers to the initial OD and OD_{60} refers to the OD determined after 60 min. For determination of OD_{60} the cultures were centrifuged at 300 × *g* for 2 min at 20 °C.

For evaluation of co-aggregation, strains *E. mundtii* CRL35 and *E. faecium* ST88Ch were grown in 10 mL MRS and *L. monocytogenes* 101, 211, 426, 603 and 703, *E. faecalis* ATCC 19443 and *Lb. sakei* ATCC 15521 in BHI or MRS, at 37 °C. Cells were harvested after 24 h (7000 × *g*, 10 min, 20 °C), washed, resuspended and diluted in 0.85% sterile saline to $OD_{660\ nm} = 0.3$. Then 500 µl of each suspension were mixed in a 2 mL sterile plastic cuvette and the $OD_{660\ nm}$ recorded over 60 min using a spectrophotometer (Ultraspec 2000, Pharmacia Biotech). The degree of co-aggregation was determined by OD readings of mixed cultures. Co-aggregation was calculated using the following equation (Todorov and Dicks, 2008):

$$\%\ \text{Co-aggregation} = [(OD_{tot} - OD_s)/OD_{tot}] \times 100$$

OD_{tot} refers to the initial OD, taken immediately after the tested strains were mixed and OD_S refers to the OD of the supernatant after 60 min. Experiments were conducted in triplicates on two separate occasions.

2.4. Control of *L. monocytogenes* in experimentally contaminated Minas cheese

2.4.1. Preparation of inoculum for experimental contamination of the cheeses

L. monocytogenes 426 was used for experimental contamination of Minas cheese. Cultures of *L. monocytogenes* 426, grown in TSB-YE broth for 24 h at 37 °C, and *E. mundtii* CRL35, *E. faecium* ST88Ch and *E. faecalis* ATCC 19443, grown in MRS broth for 24 h at 30 °C, were centrifuged at 6000 × *g* for 10 min at 10 °C, and washed three times with 0.85% sterile saline. The final suspensions were submitted to decimal serial dilutions and plated in TSA-YE or MRS agar for determination of the number of viable cells.

For preparation of cheeses containing *E. mundtii* CRL35, *E. faecium* ST88Ch or *E. faecalis* ATCC 19443, the cultures were added to the milk after the pasteurization step, to reach a level of 10^6 CFU/mL. For contamination with *L. monocytogenes* 426, the culture was added to the salted curd at the agitation step, as described below, to reach a level of 10^3 CFU/g.

2.4.2. Fresh Minas cheese manufacturing

Minas cheese was manufactured according to Scholz (1995), following the diagram presented in Fig. 1. For each batch of cheese, ten liters of pasteurized whole milk, purchased in a local supermarket, were re-pasteurized by heating at 63 °C for 30 min, cooled to 35 °C, and added of 2.5 mL of saturated $CaCl_2$ solution, 2.5 mL of 85% lactic acid (Chemco Indústria e Comércio de Produtos Químicos Ltda, Brazil) and 9.0 mL of commercial rennet (Fábrica de Coalhos e Coagulantes Bela Vista Produtos Enzimáticos Indústria e Comércio Ltda., Brazil). After 50 min, the curd was cut both vertically and horizontally into cubes of approximately 1 cm^3 using a plastic spatula. Cooking grade NaCl (2%) was added and the salted curd was agitated slowly for 30 min at 21 °C. The curd was transferred to perforated sterile plastic circular cheese containers (appr. 15 cm diameter) and maintained at 21 °C for 1 h for dripping. The cheeses were unmolded, packed in plastic bags, and stored under

Table 2
Adsorption of bacteriocins produced by *E. mundtii* CRL35 and *E. faecium* ST88Ch to *L. monocytogenes*, *E. faecalis* ATCC 19443 and *Lb. sakei* ATCC 15521, at 20 °C, 30 °C and 37 °C, at pH = 6.0.

	Adsorption (%) of bacteriocins produced by					
	E. mundtii CRL35			*E. faecium* ST88Ch		
	20 °C	30 °C	37 °C	20 °C	30 °C	37 °C
L. monocytogenes 426	43	72	72	66	100	100
L. monocytogenes 603	43	43	43	66	66	66
L. monocytogenes 101	72	84	84	100	100	100
L. monocytogenes 211	84	84	84	66	100	100
L. monocytogenes 703	68	72	72	100	100	100
E. faecalis ATCC 19443	43	43	43	66	66	66
Lb. sakei ATCC 15521	43	43	43	66	66	66

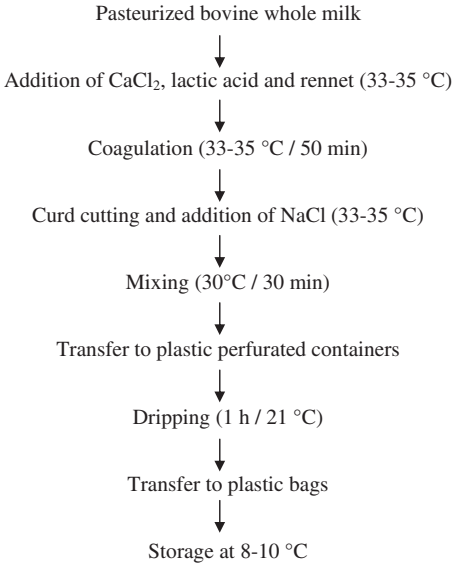

Fig. 1. Steps in Minas cheese production.

refrigeration (8–10 °C). Each package contained approximately 170 g of cheese.

The following batches of Minas cheese were manufactured:

A. Cheeses prepared with pasteurized milk containing *E. mundtii* CRL35, *E. faecium* ST88Ch or *E. faecalis* ATCC 19443, and experimentally contaminated with *L. monocytogenes* 426, added to the salted curd at the agitation step;
B. Cheeses prepared with pasteurized milk containing 12.5 mg/kg pure nisin (Sigma–Aldrich) and experimentally contaminated with *L. monocytogenes* 426, added to the salted curd at the agitation step;
C. Control cheeses, containing *L. monocytogenes* 426 only, or *E. mundtii* CRL35 only, or *E. faecium* ST88Ch only or *E. faecalis* ATCC 19443 only.
D. Control cheeses, containing no added bacteria or nisin.

2.4.3. Microbial examination of the cheeses

Experimentally contaminated cheeses were submitted to counts of *L. monocytogenes* and enterococci on time zero and every two days, up to twelve days of storage. This study was limited to twelve days due to the growth of molds and also because no significant growth of *L. monocytogenes* was observed in the following days. Twenty five grams of each sample were stomached with 225 mL of sterile 0.1% peptone water, submitted to decimal serial dilutions in the same diluent and pour-plated (1 mL) with TSA-YE. After solidification, plates were overlaid with 10 mL Listeria Selective Agar Base (Oxford Formulation, Oxoid) supplemented with Listeria Selective Supplement (Oxoid) and incubated at 37 °C for 24 h. For enumeration of enterococci, the decimal serial dilutions were plated on MRS agar, and incubated at 30 °C for 24 h. Non-contaminated control cheeses were also tested for *L. monocytogenes* and enterococci, using the described procedures. Growing colonies were counted and results expressed as CFU/g. The experiments were repeated three times.

2.4.4. pH monitoring

At each sampling for bacterial counts, the pH was measured in the cheese homogenates in 0.1% peptone water, using a DMPH2 potentiometer (Digimed, Brazil).

2.4.5. Statistical analyses

Results of microbial counts in the cheeses were submitted to variance analyses (ANOVA). The growth of *L. monocytogenes* during storage was evaluated using regression analyses. Statistical differences were detected by analyses of contrast ($p < 0.05$). The statistical analyses were performed using the Assistat (Assistat – Statistical Assistance, Version 7.5 beta, 2008) software.

3. Results and discussion

3.1. Bacteriocin bioassay and spectrum of activity

Neutralized cell-free supernatant from 24-h-old cultures of *E. mundtii* CRL35 and *E. faecium* ST88Ch inhibited the growth of most *L. monocytogenes* strains (Table 1). The other tested microorganisms were inhibited in a strain-specific manner, but none of the Gram negative microorganisms was affected by the two bacteriocinogenic strains. Identical results were recorded with the agar-spot and well diffusion methods.

The assessment of the inhibitory spectrum is an important characteristic when evaluating possible applications of bacteriocin-producing strains as potential starter cultures, as their inhibitory activity plays a relevant role in the competition with other microorganisms in the food matrix, protecting it from the colonization by foodborne pathogens. The activity of the cell-free supernatants of the two bacteriocinogenic strains against most of the tested *L. monocytogenes* strains (Table 1) is quite interesting, particularly because many reports have shown that *L. monocytogenes* is an important pathogen in fresh and maturated cheeses (Silva et al., 2001, 2004; Brito et al., 2008; Zocche et al., 2010; Koch et al., 2010; Fretz et al., 2010).

3.2. Dynamics of growth and bacteriocin production

The production of bacteriocin increased with the growth of both bacteriocinogenic strains (Fig. 2). For *E. mundtii* CRL35 the maximum bacteriocin production (25 600 AU/mL) was recorded at 15 h of growth in MRS broth, and this maximum remained stable up to 21 h and decreased at 24 h. *E. faecium* ST88Ch produced lower levels of bacteriocin and the maximum production (6400 AU/mL) occurred after 18 h of growth in MRS broth, followed by decrease in activity to 3200 AU/mL in next 6 h.

Several studies have shown that bacteriocin production is dependent on the biomass. Todorov and Dicks (2009) reported that optimal levels of mundticin ST4SA, produced by *E. mundtii* ST4SA, were obtained in growth media that supported high biomass production, such as MRS. Similar bacteriocin production profile was reported for bacteriocin ST311LD, produced by *E. faecium* ST311LD isolated from fermented olives, in which maximal bacteriocin production was reported at 20 h in MRS broth, followed by a decrease in activity in the next 5 h (Todorov and Dicks, 2005). The decrease in activity of bacteriocins at the end of the monitored period might be explained by the degradation of the bacteriocin by extracellular proteolytic enzymes. Similar decrease has been observed for bacteriocins produced by *E. faecium* ST311LD (Todorov and Dicks, 2005), *E. mundtii* ST4SA (Todorov and Dicks, 2009) and *Pediococcus acidilactici* NRRL B5627 (Anastasiadou et al., 2008). From a metabolic point of view, this trend is characteristic of a primary metabolite production. When grown in reconstituted milk (5% or 10%) for 24 h at 30 °C (data not shown), both strains produced less bacteriocin than in MRS medium (6400 AU/mL for *E. mundtii* CRL35 and 800 AU/mL for *E. faecium* ST88Ch).

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

Addition of 1600 AU/mL of bacteriocins CRL35 or ST88Ch to 3 h-old cultures of *L. monocytogenes* strains 101, 211, 426, 603 and 703 affected their growth over 12 h in a similar pattern (Fig. 3), i.e. the strains presented an extended lag phase. The most sensitive strain to both bacteriocins was *L. monocytogenes* 426, as no viable cells could be detected up to 12 h. For this particular strain, both bacteriocins expressed a bactericidal mode of action. *L. monocytogenes* 603 was more sensitive to bacteriocin CRL35 than to bacteriocin ST88Ch. A resulting extended lag phase was also observed when the bacteriocins were added to 3 h-old cultures of *E. faecalis* ATCC 19443 and *Lb. sakei* ATCC 15521. Other bacteriocins, such as those produced by *Ped. acidilactici* HA-6111-2 and *E. faecium* ST5Ha, presented a similar behavior (Albano et al., 2007; Todorov et al., 2010).

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

As shown in Table 2, the absorption of the bacteriocin produced by *E. faecium* ST88Ch to *L. monocytogenes* 101, 211, 426, 603 and 703, *E. faecalis* ATCC 19443 and *Lb. sakei* ATCC 15521 was higher than bacteriocin produced by *E. mundtii* CRL35. For both bacteriocins, the

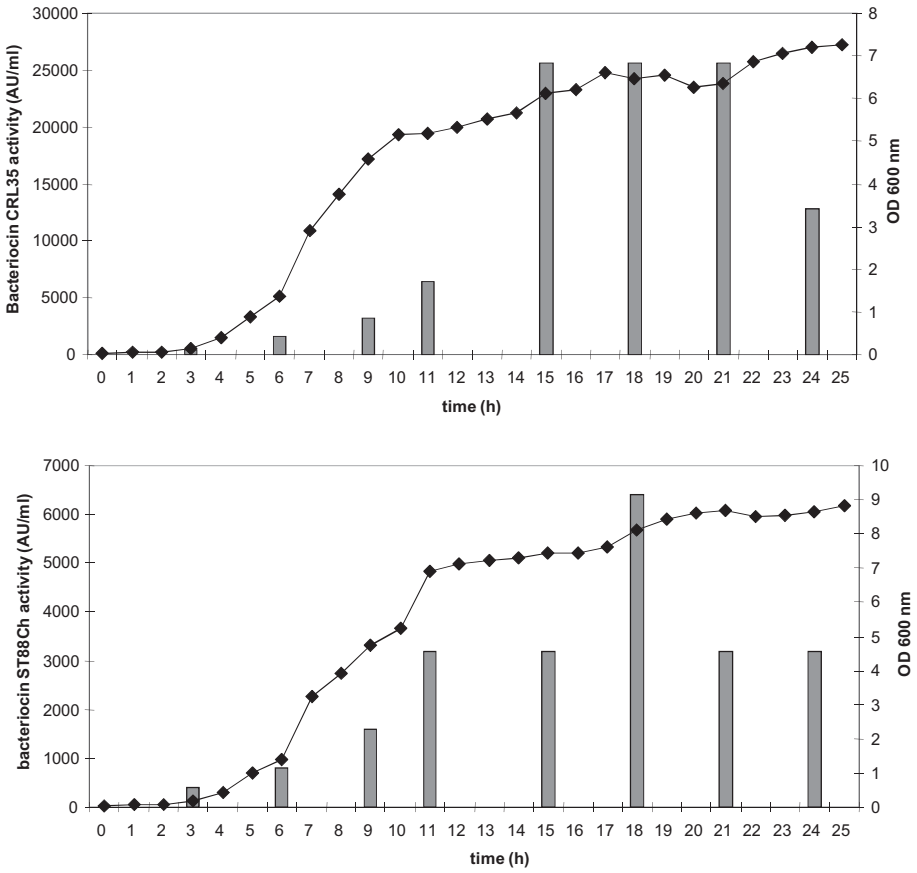

Fig. 2. Growth and bacteriocins production by *E. mundtii* CRL35 and *E. faecium* ST88Ch in MRS broth at 30 °C (sensitive strain: *L. monocytogenes* 426). Bars indicate the bacteriocin activity (AU/ml) and diamonds indicate the absorbance ($OD_{600\ nm}$).

influence of temperature in the levels of absorption was strain dependent, but in general, the absorption increased with the temperature.

The levels of adsorption of the two bacteriocins to *L. monocytogenes* 426 according to the experimental conditions are shown in Table 3. The effect of each factor in the level of absorption varied according to the factor under evaluation and the bacteriocin. Presence of NaCl (1%) and milk (1%) and pH 4.0 and 8.0 resulted in higher absorption of bacteriocin CRL35 than bacteriocin ST88Ch. In counterpart, Tween 20 (1%), Tween 80 (1%) and pH 6.0 caused a higher absorption of bacteriocin ST88Ch than bacteriocin CRL35.

A previous study with bacteriocin AMA-K has also shown that the temperature influenced its adsorption to *Listeria innocua* LMG13568, *L. monocytogenes* Scott A and *Listeria ivanovii* susbp. *ivanovii* ATCC 19119 (Todorov, 2008). The absorption was higher at 30 °C and 37 °C and 45 °C than at 4 °C and 15 °C. However, other study indicated that temperature had no effect on the adsorption of plantaricin 423 to *E. faecium* HKLHS (Todorov and Dicks, 2006). Obviously temperature affects differently the absorption of different bacteriocins, and the effect depends on the sensitive strain used in this evaluation.

Differences in adsorption according to the pH were also observed for other bacteriocins. Optimal adsorption of buchnericin LB to *Lactobacillus plantarum* was recorded at pH 5.0–8.0 (Yildirim et al., 2002). For plantaricin 423, adsorption to *E. faecium* HKLHS was maximal at pH 8.0–10.0, and for *Lb. sakei* DSM20017, the maximal adsorption occurred between pH 2.0 and 6.0 (Todorov and Dicks, 2006). This pH dependence may be due to specific interaction between bacteriocins and the target cells or structural pH dependent modifications of the bacteriocins receptors on the target cell surface.

The effect of Tween 20, Tween 80 and different concentrations of NaCl in reducing the adsorption of bacteriocins to *Listeria* was also observed for bacteriocin AMA-K. In another study with bacteriocin HV219, increased adsorption to *E. faecium* HKLHS and *E. faecalis* E88 was detected in the presence of several chemicals (Triton X-100, β-mercapto-ethanol, ethanol, methanol, chloroform, NaCl, KCl, KH_2PO_4, K_2HPO_4, $MgCl_2$, Na-acetate, Na_2CO_3, Tris and NH_4-citrate) (von Mollendorff et al., 2007).

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

Auto-aggregation showed to be strain-specific, and values ranged from 27.7% for *L. monocytogenes* 603–86.5% for *E. faecium* ST88Ch (Fig. 4). The low levels of co-aggregation with *L. monocytogenes* may play an important role in preventing the formation of biofilms, and in this way eliminating the pathogen from the food matrix. In counterpart, the levels of auto-aggregation of *E. mundtii* CRL35 and *E. faecium* ST88Ch were high (71% and 86%, respectively), which may facilitate the action of their bacteriocins against the target pathogens.

3.6. Control of *L. monocytogenes* 426 in experimentally contaminated Minas cheese

As shown in Fig. 5, *L. monocytogenes* 426 grew fast in fresh Minas cheese during storage under refrigeration (8–10 °C). From an initial

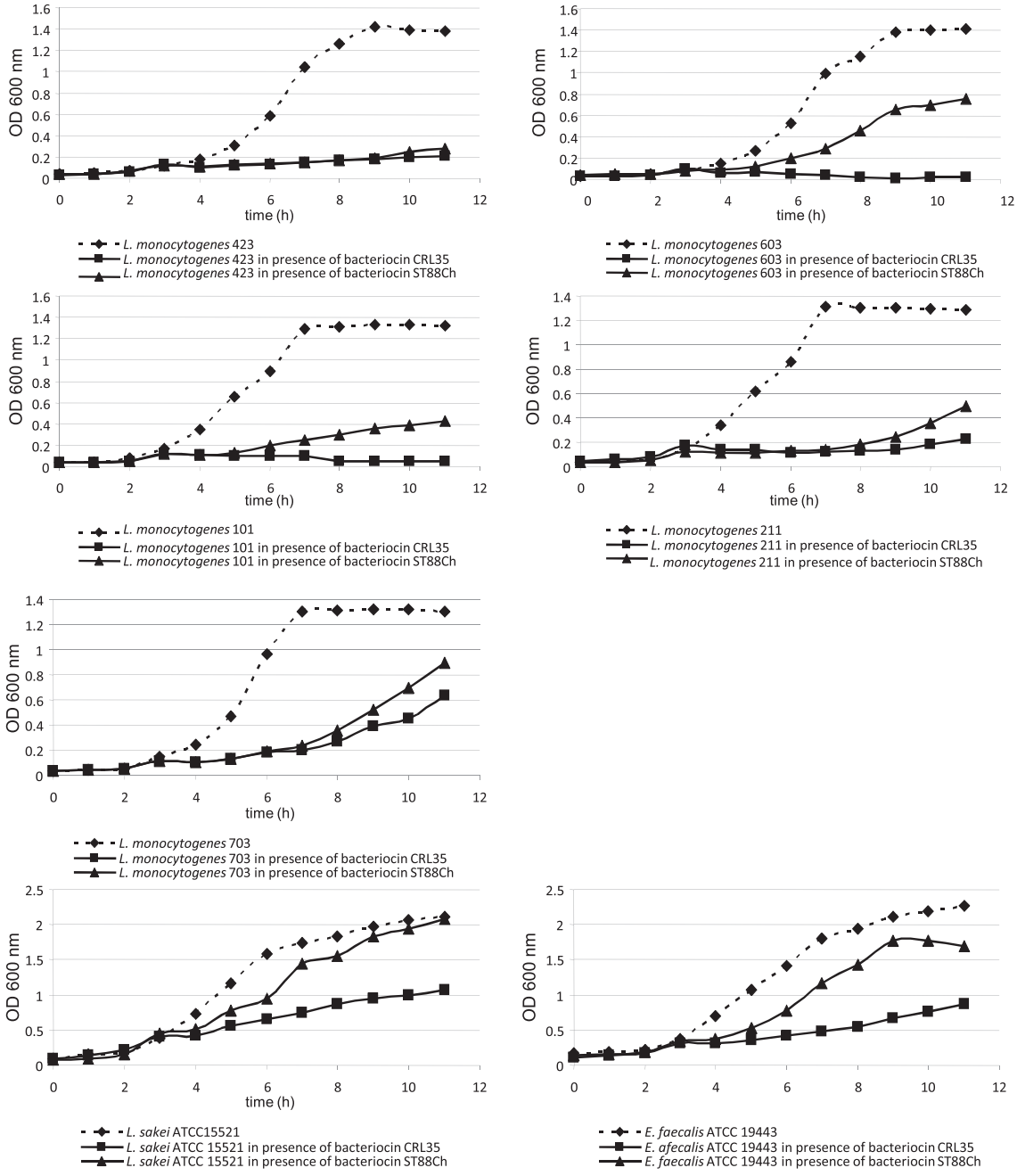

Fig. 3. Effect of bacteriocins produced by *E. mundtii* CRL35 and *E. faecium* ST88Ch on growth of *L. monocytogenes* strains 101, 211, 426, 603 and 703, *E. faecalis* ATCC 19443 and *Lb. sakei* ATCC 15521.

Table 3
Adsorption of bacteriocins produced by *E. mundtii* CRL35 and *E. faecium* ST88Ch to *L. monocytogenes* 426, under different experimental conditions.

	Adsorption (%) of bacteriocins produced by					
	E. mundtii CRL35			*E. faecium* ST88Ch		
	20 °C	30 °C	37 °C	20 °C	30 °C	37 °C
NaCl (1%)	84	84	84	66	66	66
Tween 20 (1%)	68	72	72	100	100	100
Tween 80 (1%)	68	72	72	66	100	66
Milk (1%)	84	84	84	66	66	66
at pH 4.0	84	84	84	66	66	100
at pH 6.0	43	72	72	66	100	100
at pH 8.0	68	68	68	33	33	33

count of 10^3 CFU/g, the level of contamination increased to 10^6 CFU/g in six days and to 10^8 CFU/g in 12 days (Q3 in Fig. 5). When the cheeses were added of the bacteriocinogenic *E. mundtii* CRL35 strain, the growth of *L. monocytogenes* 426 was completely inhibited (Q5 in Fig. 5). After 12 days under refrigeration, the counts of the pathogen remained the same as the initial inoculum, suggesting a bacteriostatic action of the bacteriocin. In cheeses containing bacteriocinogenic *E. faecium* ST88Ch (Q7 in Fig. 5), the inhibition of *L. monocytogenes* 426 was less marked and was similar to that achieved by the non-bacteriocinogenic *E. faecalis* ATCC 19443 (Q9 in Fig. 5). In the first six days, the growth of the pathogen was similar to that in cheeses without any bacteriocinogenic strain, but afterwards the bacteriostatic effect become evident. The counts of *L. monocytogenes* 426 after 12 days were 3 logs lower than that in

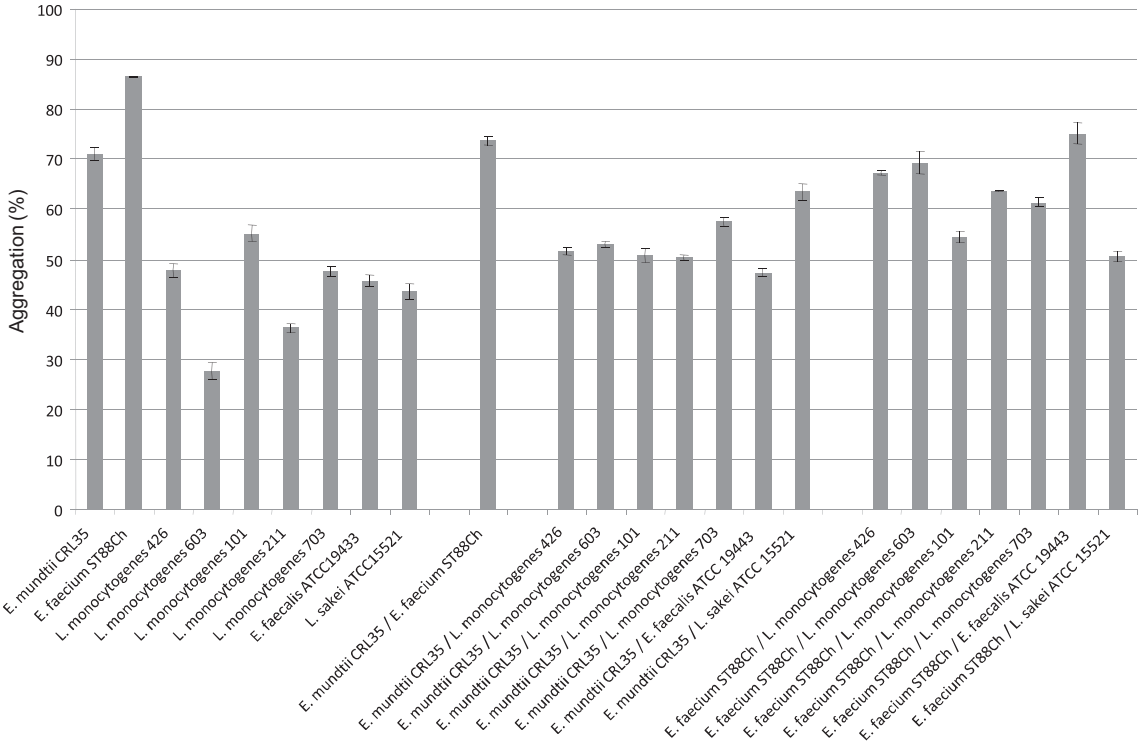

Fig. 4. Auto-aggregation and co-aggregation of *E. mundtii* CRL35, *E. faecium* ST88Ch, *L. monocytogenes* strains 101, 211, 426, 603 and 703, *E. faecalis* ATCC 19443 and *Lb. sakei* ATCC 15521. Each result represents an average of three independent experiments.

cheeses with no added bacteriocinogenic strain. Curiously, nisin had only a small effect in the inhibition of *L. monocytogenes* 426 in the Minas cheese (Q2 in Fig. 5), as counts after 12 days were half log lower than in cheeses without nisin. It should be noted that the prepared Minas cheeses not added of *L. monocytogenes* 426 did not contain *Listeria* (Q1, Q4, Q6 and Q8) and the counts of autochthonous lactic acid bacteria were low (Fig. 6). Fig. 6 also shows that all added lactic acid bacteria maintained the viability in the Minas cheese along the 12 days of storage under refrigeration, despite the presence of *L. monocytogenes* 426.

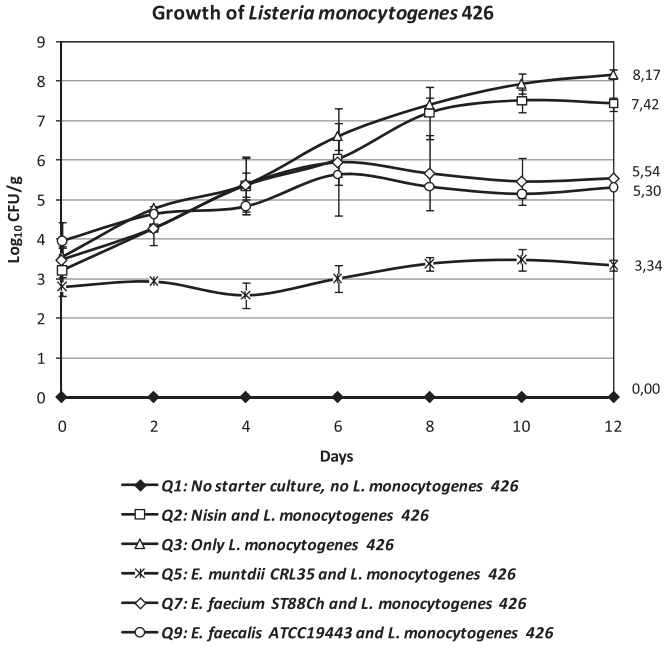

Fig. 5. Growth of *L. monocytogenes* 426 in experimentally contaminated Minas cheese stored under refrigeration (8–10 °C) up to 12 days, in the presence of bacteriocinogenic *E. mundtii* CRL35 and *E. faecium* ST88Ch, non-bacteriocinogenic *E. faecalis* ATCC 19443 and nisin (12.5 mg/kg). Results are average of three independent experiments.

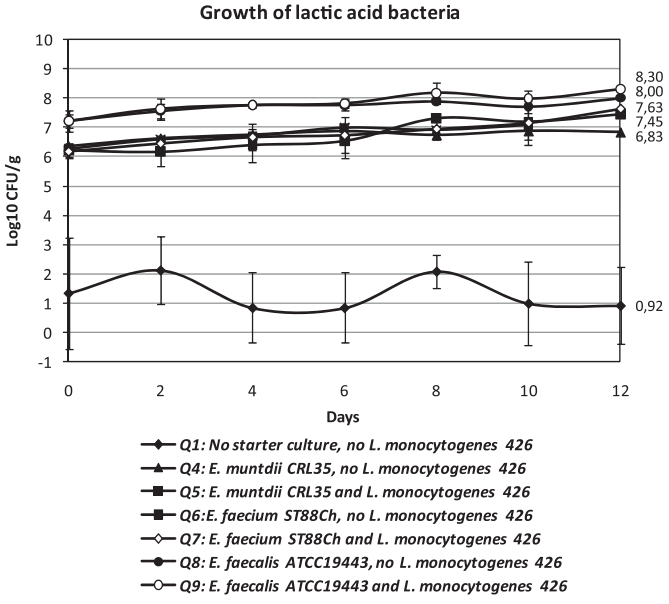

Fig. 6. Growth of bacteriocinogenic *E. mundtii* CRL35 and *E. faecium* ST88Ch and non-bacteriocinogenic *E. faecalis* ATCC 19443 in experimentally contaminated Minas cheese stored under refrigeration (8–10 °C) up to 12 days. Results are average of three independent experiments.

The pH of the cheeses containing the bacteriocinogenic and the non-bacteriocinogenic strains dropped from initial 5.8 to 5.2 after 12 days. The inhibition of *L. monocytogenes* 426 cannot be attributed to this decrease of pH, as this pathogen can grow well at pH 5.0, even under refrigeration. No visible changes in the physical characteristics (texture or color) of the cheeses containing *E. mundtii* CRL35 or *E. faecium* ST88Ch were observed.

These results confirm previous findings indicating that the inhibitory activity of bacteriocins in culture media is not always reproducible in food systems (*in situ*) (Schillinger et al., 1996; Collins et al., 2011). Several factors present in the food can influence the inhibitory effect, such as interaction with additives/ingredients, adsorption to food components, and inactivation by food enzymes and pH changes in the food. Low solubility and uneven distribution in the food matrix and limited stability of bacteriocin during food shelf-life are additional factors that influence the activity of bacteriocins in foods. The food microbiota has an important role, especially the microbial load and diversity, as sensitivity to the bacteriocins is variable among bacteria and even among strains belonging to the same species. Microbial interactions in the food system may be responsible for changes in the sensitivity to the bacteriocins. The target microorganisms play also an important role, depending on the physiological stage (growing, resting, starving or viable but non-cultivable cells, stressed or sub-lethally injured cells, endospores), the protection by physico-chemical barriers (microcolonies, biofilms, slime) and the development of resistance/adaptation (Gálvez et al., 2009).

Studies on the application of bacteriocins produced by bacteriocinogenic strains in cheeses indicate that results may vary according to the bacteriocinogenic strain and the type of cheese. Izquierdo et al. (2009) reported that *E. faecium* WHE 81, capable to produce at least two bacteriocins, was effective in the control of *L. monocytogenes* in Munster cheese, a red smear soft cheese. In counterpart, Nascimento et al. (2008) observed that in Brazilian Minas cheese, the counts of *L. monocytogenes* in samples containing bacteriocinogenic strains did not differ from those in samples containing non-bacteriocinogenic lactic acid bacteria. Settanni et al. (2011) have recently reported that several non-starter bacteriocinogenic lactic acid bacteria were capable to extend the shelf-life of a fresh cheese (Tosèla) similar to the Brazilian Minas cheese.

The low activity of nisin observed in the Minas cheese samples was surprising as this bacteriocin has been used for many years in cheeses to control of post-processing contaminant pathogens, mainly *L. monocytogenes* and *Staphylococcus aureus* (Gálvez et al., 2008). Nisin producing strains have been reported to inhibit *Listeria* in several types of cheeses (cottage, Camembert, Manchego) (Gálvez et al., 2008). Nevertheless, nisin producing strains may not offer the technological properties required for cheese making, such as fast acidification and proteolytic activity (O'Sullivan et al., 2002).

Among the tested enterococci, strains, *E. mundtii* CRL35 presented a good potential for application in Minas cheese for the control of *L. monocytogenes* in this product. Preliminary results (unpublished data) indicate that this strain is negative for gelatinase, aggregation substance, extracellular surface protein, sexual-pheromones and vancomycin resistance genes (vanA, vanB, vanC), indicating safety for application of this strain in cheese. Ongoing study with this and other enterococci strains isolated from Brazilian foods will bring more light to the controversial topic of their use for biopreservation of foods. In addition, several studies in the last years have demonstrated safe application of enterococci in foods (Ogier and Serror, 2008; Gálvez et al., 2009; Martin-Platero et al., 2009; Franz et al., 2011).

Several *E. mundtii* strains have been shown to produce bacteriocins, such as mundticin L (Feng et al., 2009), mundticin QU 2 (Zendo et al., 2005), mundticin ST15 (De Kwaadsteniet et al., 2005), and mundticin KS (Kawamoto et al., 2002), but none of them have been tested as biopreservatives in cheeses. Further studies with semi-purified or pure bacteriocin produced by *E. mundtii* CRL35, associated or not to other antimicrobial hurdles, are necessary to evaluate the application of this strain/bacteriocin for the improvement of safety and quality of this type of cheese.

4. Conclusions

The present study provides strong evidence that bacteriocin-producing enterococci can perform efficiently in the control of *L. monocytogenes* in fresh Minas cheese during refrigerated storage. Among the two cheese enterococci isolates studied, *E. mundtii* CRL35 presented a more evident inhibitory effect than *E. faecium* ST88Ch. Initial *L. monocytogenes* contamination levels of 10^3 CFU/g, obtained by experimental contamination during manufacture of Minas cheese in pilot scale, remained unchanged up to 12 days of storage under refrigeration when the cheeses were added of *E. mundtii* CRL35, while in cheeses not added of bacteriocinogenic strains the counts of *L. monocytogenes* after 12 days under refrigeration were as high as 10^8 CFU/g.

Based on the proven safety of *E. mundtii* CRL35 for hundreds of years due to their presence as natural starter in Tafí type artisanal cheeses (Farías et al., 1996), this strain presents a good potential for biopreservation of other fermented products like Minas cheese.

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References

Albano, H., Todorov, S.D., van Reenen, C.A., Hogg, T., Dicks, L.M.T., Teixeira, P., 2007. Characterization of a bacteriocin produced by *Pediococcus acidilactici* isolated from "Alheira", a fermented sausage traditionally produced in Portugal. International Journal of Food Microbiology 116, 239–247.

Anastasiadou, S., Papagianni, M., Filiousis, G., Ambrosiadis, I., Koidis, P., 2008. Pediocin SA-1, an antimicrobial peptide from *Pediococcus acidilactici* NRRL B5627: production conditions, purification and characterization. Bioresource Technology 99, 5384–5390.

Barancelli, G.V., Camargo, T.M., Reis, C.M.F., Porto, E., Hofer, E., Oliveira, C.A.F., 2011. Incidence of *Listeria monocytogenes* in cheese manufacturing plants from Northeast region of Sao Paulo, Brazil. Journal of Food Protection 74, 816–819.

Bayoub, K., Mardad, I., Ammar, E., Serrano, A., Soukri, A., 2011. Isolation and purification of two bacteriocins 3D produced by *Enterococcus faecium* with inhibitory activity against *Listeria monocytogenes*. Current Microbiology 62, 479–485.

Brito, J.R.F., Santos, E.M.P., Arcuri, E.F., Lange, C.C., Brito, M.A.V.P., Souza, G.N., Cerqueira, M.M.P.O., Beltran, J.M.S., Call, J.E., Liu, Y., Porto-Fett, A.C.S., Luchansky, J.B., 2008. Retail survey of Brazilian milk and Minas Frescal cheese and a contaminated dairy plant to establish prevalence, relatedness and sources of *Listeria monocytogenes* isolates. Applied and Environmental Microbiology 79, 4954–4961.

Cocolin, L., Foschino, R., Comi, G., Fortina, M.G., 2007. Description of the bacteriocins produced by two strains of *Enterococcus faecium* isolated from Italian goat milk. Food Microbiology 24, 752–758.

Collins, B., Cotter, P.D., Hill, C., Ross, R.P., 2011. The impact of nisin on sensitive and resistant mutants of *Listeria monocytogenes* in cottage cheese. Journal of Applied Microbiology 110, 1509–1514.

De Kwaadsteniet, M., Todorov, S.D., Knoetze, H., Dicks, L.M.T., 2005. Characterization of a 3,944 Dalton bacteriocin, produced by *Enterococcus mundtii* ST15, with activity against Gram-positive and Gram-negative bacteria. International Journal of Food Microbiology 105, 433–444.

Farías, M.E., Farías, R.N., De Ruiz Holgado, A.P., Sesma, F., 1996. Purification and N-terminal amino acid sequence of enterocin CRL 35, a 'pediocin-like' bacteriocin

produced by *Enterococcus faecium* CRL 35. Letters in Applied Microbiology 22, 417–419.
Feng, G., Guron, G., Churey, J.J., Worobo, R., 2009. Characterization of mundticin L, a class IIa anti-listeria bacteriocin from *Enterococcus mundtii* CUGF08. Applied and Environmental Microbiology 75, 5708–5713.
Franz, C.M., van Belkum, M.J., Holzapfel, W.H., Abriouel, H., Gálvez, A., 2007. Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. FEMS Microbiology Reviews 3, 293–310.
Franz, C.M., Huch, M., Abriouel, H., Holzapfel, W., Gálvez, A., 2011. Enterococci as probiotics and their implications in food safety. International Journal of Food Microbiology 151, 125–140.
Fretz, R., Pichler, J., Sagel, U., Much, P., Ruppitsch, W., Pietzka, A.T., Stoger, A., Huhulescu, S., Heuberger, S., Appl, G., Werber, D., Stark, K., Prager, R., Flieger, A., Karpiskova, R., Pfaff, G., Allerberger, F., 2010. Multinational listeriosis outbreak due to quargel, a sour milk curd cheese, caused by two different *L. monocytogenes* serotype 1/2a strains, 2009–2010. Eurosurveillance 15, 2–3.
Furtado, D.N., Todorov, S.D., Chiarini, E., Destro, M.T., Landgraf, M., Franco, B.D.G.M., 2009. Goat milk and cheeses may be a good source for antilisterial bacteriocin-producing lactic acid bacteria. Biotechnology and Biotechnological Equipment 23, 775–779.
Gálvez, A., López, R.L., Abriouel, H., Valdivia, E., Omar, N.B., 2008. Application of bacteriocins in the control of foodborne pathogenic and spoilage bacteria. Critical Reviews in Biotechnology 28, 125–152.
Gálvez, A., Dubois-Dauphin, R.R., Ghalfi, H.H., Campos, D., Thonart, P., 2009. Description of two *Enterococcus* strains isolated from traditional Peruvian artisanal-produced cheeses with a bacteriocin-like inhibitory activity. Biotechnologie Agronomie Societé et Environement 13, 349–356.
Giraffa, G., 2003. Functionality of enterococci in dairy products. International Journal of Food Microbiology 88, 215–222.
Giraffa, G., Picchioni, N., Neviani, E., Carminati, 1995. Production and stability of an *Enterococcus faecium* bacteriocin during Taleggio cheesemaking and ripening. Food Microbiology 12, 301–307.
Hadji-Sfaxi, I., El-Ghaish, S., Ahmadova, A., Batdorj, B., Blay-Laliberté, G., Barbier, G., Haertlé, T., Chobert, J.M., 2011. Antimicrobial activity and safety of use of *Enterococcus faecium* PC4.1 isolated from Mongol yogurt. Food Control 22, 2020–2027.
Heng, N.C.K., Wescombe, P.A., Burton, J.P., Jack, R.W., Tagg, J.R., 2007. The diversity of bacteriocins in gram-positive bacteria. In: Riley, M.A., Chavan, M.A. (Eds.), Bacteriocins: Ecology and Evolution. Springer-Verlag, Berlín, pp. 45–92.
Izquierdo, E., Marchioni, E., Aoude-Werner, D., Hasselmann, C., Ennahar, S., 2009. Smearing of soft cheese with *Enterococcus faecium* WHE 81, a multi-bacteriocin producer, against *Listeria monocytogenes*. Food Microbiology 26, 16–20.
Kawamoto, S., Shima, J., Sato, R., Eguchi, T., Ohmomo, S., Shibato, J., Horikoshi, N., Takeshita, K., Sameshima, T., 2002. Biochemical and genetic characterization of mundticin KS, an antilisterial peptide produced by *Enterococcus mundtii* NFRI 7393. Applied and Environmental Microbiology 68, 3830–3840.
Koch, J., Dworak, R., Prager, R., Becker, B., Brockmann, S., Wicke, A., Wichmann-Schauer, H., Hof, H., Werber, D., Stark, K., 2010. Large listeriosis outbreak linked to cheese made from pasteurized milk, Germany, 2006–2007. Foodborne Pathogens and Disease 7, 1581–1584.
Laucova, A., Czikkova, S., Dobransky, T., Burdova, O., 1999. Inhibition of *Listeria monocytogenes* and *Staphylococcus aureus* by enterocin CCM 4231 in milk products. Food Microbiology 16, 93–99.
Martin-Platero, A.M., Valdivia, E., Maqueda, M., Martínez-Bueno, M., 2009. Characterization and safety evaluation of enterococci isolated from Spanish goats' milk cheeses. International Journal of Food Microbiology 132, 24–32.
Naldini, M.C.M., Viotto, W., Kuaye, A., 2009. Behaviour of *Listeria monocytogenes* inoculated into Minas frescal cheese made by direct acidification or lactic culture during refrigerated storage. International Journal of Dairy Technology 62, 361–365.
Nascimento, M.S., Moreno, I., Kuaye, A.Y., 2008. Applicability of bacteriocin-producing *Lactobacillus plantarum, Enterococcus faecium* and *Lactococcus lactis* ssp. *lactis* as adjunct starter in Minas Frescal cheesemaking. International Journal of Dairy Technology 61, 352–357.
Ogier, J.C., Serror, P., 2008. Safety assessment of dairy microorganisms: the *Enterococcus* genus. International Journal of Food Microbiology 126, 291–301.
O'Sullivan, L., Ross, R.P., Hill, C., 2002. Potential of bacteriocin-producing lactic acid bacteria for improvements of food safety and quality. Biochimie 84, 593–604.
Pinto, M.S., Carvalho, A.F., Pires, A.C.S., Paula, J.C.J., Sobral, D., Magalhaes, F.A.R., 2009. Survival of *Listeria innocua* in Minas traditional Serro cheese during ripening. Food Control 20, 1167–1170.
Schillinger, U., Geisen, R., Holzapfel, W.H., 1996. Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. Trends in Food Science & Technology 71, 58–64.
Scholz, W., 1995. Elaboración de quesos de oveja y de cabra. Acribia S.A, Zaragoza.
Settanni, L., Franciosi, E., Cavazza, A., Cocconcelli, P.S., Poznanski, E., 2011. Extension of Tosèla cheese shelf-life using non-starter lactic acid bacteria. Food Microbiology 26, 16–20.
Silva, M.C.D., Destro, M.T., Hofer, E., Tibana, A., 2001. Characterization and evaluation of some virulence markers of *Listeria monocytogenes* strains isolated from Brazilian cheeses using molecular, biochemical and serotyping techniques. International Journal of Food Microbiology 63, 275–280.
Silva, W.P., Techera, S.B.C., Jantzen, M.M., Von Laer, A.E., Lima, A.S., Mata, M.M., 2004. *Listeria monocytogenes* en quesos tipo Minas producidos artesanalmente y comercializados en Pelotas, RS, Brasil. Alimentaria (Madrid) 359, 57–60.
Souza, C.H.B., Saad, S.M.I., 2009. Viability of *Lactobacillus acidophilus* La-5 added solely or in co-culture with a yoghurt starter culture and implications on physico-chemical and related properties of Minas fresh cheese during storage. LWT – Food Science and Technology 42, 633–640.
Swaminathan, B., Cabanes, D., Zhang, W., Cossart, P., 2007. Listeria monocytogenes. In: Doyle, M.P., Beuchat, L.R. (Eds.), Food Microbiology: Fundamentals and Frontiers. ASM Press, Washington, pp. 322–341.
Todorov, S.D., 2008. Bacteriocin production by *Lactobacillus plantarum* AMA-K isolated from Amasi, a Zimbabwean fermented milk product and study of adsorption of bacteriocin AMA-K to *Listeria* spp. Brazilian Journal of Microbiology 38, 178–187.
Todorov, S.D., Dicks, L.M.T., 2005. Optimization of bacteriocin ST311LD production by *Enterococcus faecium* ST311LD, isolated from spoiled black olives. Journal of Microbiology 43, 370–374.
Todorov, S.D., Dicks, L.M.T., 2006. Parameters affecting the adsorption of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum* 423 isolated from sorghum beer. Biotechnology Journal 1, 405–409.
Todorov, S.D., Dicks, L.M.T., 2008. Evaluation of lactic acid bacteria from kefir, molasses and olive brine as possible probiotics based on physiological properties. Annals of Microbiology 58, 661–670.
Todorov, S.D., Dicks, L.M.T., 2009. Effect of modified MRS medium on production and purification of antimicrobial peptide ST4SA produced by *Enterococcus mundtii*. Anaerobe 15, 65–73.
Todorov, S.D., Wachsman, M.B., Knoetze, H., Meincken, M., Dicks, L.M.T., 2005. An antibacterial and antiviral peptide produced by *Enterococcus mundtii* ST4V isolated from soy beans. International Journal of Antimicrobial Agents 25, 508–513.
Todorov, S.D., Wachsman, M., Tomé, E., Dousset, X., Destro, M.T., Dicks, L.M.T., Franco, B.D.G.M., Vaz-Velho, M., Drider, D., 2010. Characterisation of an antiviral pediocin-like bacteriocin produced by *Enterococcus faecium*. Food Microbiology 27, 869–879.
von Mollendorff, J.W., Todorov, S.D., Dicks, L.M.T., 2007. Factors affecting the adsorption of bacteriocins to *Lactobacillus sakei* and *Enterococcus* sp. Applied Biochemistry and Biotechnology 142, 209–220.
Wachsman, M.B., Castilla, V., de Ruiz Holgado, A.P., de Torres, R.A., Sesma, F., Coto, C.E., 2003. Enterocin CRL35 inhibits late stages of HSV-1 and HSV-2 replication in vitro. Antiviral Research 58, 17–24.
Yildirim, Z., Avşar, Y.K., Yildirim, M., 2002. Factors affecting the adsorption of buchnericin LB, a bacteriocin produced by *Lactobacillus buchneri*. Microbiology Research 157, 103–107.
Zendo, T., Eungruttanagorn, N., Fujioka, S., Tashiro, Y., Nomura, K., Sera, Y., Kobayashi, G., Nakayama, J., Ishizaki, A., Sonomoto, K., 2005. Identification and production of a bacteriocin from *Enterococcus mundtii* QU 2 isolated from soybean. Journal of Applied Microbiology 99, 1181–1190.
Zocche, F., Bastos, C.P., França, R.C., Silva, W.P., 2010. Comparación entre dos métodos para extracción de DNA de *Staphylococcus aureus* en queso tipo minas frescal. Alimentaria 43, 110–116.