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ORIGINAL ARTICLE

# Brazilian artisanal cheeses as a source of beneficial *Enterococcus faecium* strains: characterization of the bacteriocinogenic potential

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**Abstract** Two bacterial isolates from milk and cheeses were selected based on their inhibition of *Listeria monocytogenes*, and classified as *Enterococcus faecium* based on 16S rRNA analysis. In MRS broth at 37 °C, bacteriocin-like substances (BLS) produced by *E. faecium* EM485 and EM925 were detected at 3,200 arbitrary units/mL. These peptides were inactivated by proteolytic enzymes, but not when treated with α-amylase, catalase and lipase. The two BLS remained stable at pH values ranging from 2.0 to 10.0, after exposure to 100 °C for 120 min and in the presence of surfactants and salts. DNA from both strains generated positive PCR results for enterocin A and B genes.

**Keywords** *Enterococcus faecium* · Bacteriocin · Bacteriocin-like substance · Anti-*Listeria monocytogenes* activity

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## Introduction

The physiological characteristics of *Enterococcus* spp. include tolerance to salts and acids, giving them a high survival rate in several food systems. Cheese and other fermented food products produced in the Mediterranean area are often rich sources of *Enterococcus* spp. and it is believed that the latter contribute to ripening and to the development of aroma (Giraffa 2003; Foulquié-Moreno et al. 2006; Franz et al. 2011) due to proteolysis, lipolysis and the production of diacetyl (Giraffa 2002). Furthermore, some studies have shown that bacteriocinogenic lactic acid bacteria (LAB), including *Enterococcus* spp., are common in Brazilian dairy products as well (Gomes et al. 2008; Frazzon et al. 2010; Moraes et al. 2010; Ortolani et al. 2010).

LAB are known for their production of antimicrobial compounds, including bacteriocins or bacteriocin-like peptides (De Vuyst and Vandamme 1994; Todorov 2009). Bacteriocins / bacteriocin-like substances (BLS) of LAB are defined as ribosomally synthesized proteins or protein complexes usually antagonistic to genetically closely related organisms (Nes and Johnsborg 2004). They are generally low molecular weight proteins that gain entry into target cells by binding to cell

surface receptors. In general their bactericidal mechanisms vary and may include pore formation and inhibition of peptidoglycan synthesis (James et al. 1991).

BLS peptides are classified into four classes, with most bacteriocins grouped in classes I and II. Class I peptides, known as lantibiotics (< 5 kDa), are post-translationally modified and contain lanthionine and β-methyl-lanthionine, while class II bacteriocins are small (< 10 kDa), heat-stable, cationic, hydrophobic and membrane-active peptides (de Vuyst and Vandamme 1994). Bacteriocins with the highly conserved N-terminal amino acid sequence YGNGVXaaC (Tyr-Gly-Asn-Gly-Val- Xaa-Cys), non-polar amino acids, one or more disulfide bridges and activity against *Listeria* spp. are grouped in class IIa (Eijsink et al. 1998; Ennahar et al. 2001). Bacteriocins that function in pairs, usually as two distinct peptides, are grouped in class IIb (Ennahar et al. 2001). Thiol-activated bacteriocins that rely on a *sec*-dependent secretion mechanism are grouped in class IIc (Hechard and Sahl 2002).

*Enterococcus* spp. species have been reported to produce bacteriocins belonging to classes I, IIa, IIb, IIc and III. Most of these bacteriocins are produced by *Enterococcus faecium* and *Enterococcus faecalis* (Foulquié-Moreno et al. 2006). Enterocin A (Aymerich et al. 1996), bacteriocin N15 (Losteinkit et al. 2001) and bacteriocin RC714 (Del Campo et al. 2001) produced by *E. faecium*, as well as enterocin SE-K4 (Eguchi et al. 2001), enterocin ST5HA (Todorov et al. 2010b) and bacteriocin 31 (Tomita et al. 1996) produced by *E. faecalis* are grouped in class IIa. Enterocins 1071A and 1071B produced by *E. faecalis* (Balla and Dicks 2005), and enterocins L50A and L50B produced by *E. faecium* (Cintas et al. 1998) belong to class IIb. Meanwhile, enterocin RJ-11 (Yamamoto et al. 2003) produced by *E. faecalis*, and enterocin P (Cintas et al. 1997), enterocin B (Casaus et al. 1997) and enterocin Q (Cintas et al. 2000) produced by *E. faecium* are grouped in class IIc.

In this study, strains isolated from artisanal Coalho cheese produced in Ceará state, Brazil were evaluated for their bacteriocinogenic potential. The strains were identified as *E. faecium*, their BLS partially characterized and their mode of action studied. To our knowledge, this is the first report on the production of BLS by LAB isolated from Coalho cheese. The objective of this work was to perform a partial characterization of the produced BLS, with the future aim of using the strains as a co-starter or adjusting culture in cheese or milk fermentations.

## Materials and methods

### Isolation of LAB and screening for BLS activity

LAB were isolated from artisanal Coalho cheese. Twelve samples of Coalho cheese made from raw milk, from distinct brands, were collected from different markets in the city of Fortaleza, Ceará, Brazil. Cheeses were originally produced in Vale do Jaguaribe and Sertões Cearenses, traditional Coalho cheese manufacturing regions in Ceará, Brazil.

Twenty-five grams of each cheese was homogenized in 225 mL 2 % (w/v) sodium citrate (Vetec) preheated to 45 °C in a Stomacher (Seward, 400). Serial dilutions were made in 0.1 % (w/v) peptone water (Merck, Darmstadt, Germany) and plated in duplicate on M17 agar (Oxoid, http://www.oxoid.com) and cultivated for 48 h at 30 °C and 42 °C. Total microbial populations were enumerated and 15–20 colonies were picked randomly from M17 and Rogosa agar count plates for each cheese at both temperatures. Subsequently, isolates were purified in MRS (Difco, http://catalog.bd.com) and were examined for cell morphology, Gram stain, catalase activity and acid production. Acid production was evaluated by coagulation and reduction of Litmus Milk medium (BBL) at 35 °C for 7 days. Microorganisms that were acid producers, Gram-positive, catalase-negative, with cocci shape were pre-selected for future study.

The cocci were tested for their ability to grow in skim milk at 10 °C and 45 °C, in APT broth (Himedia, http://www.himedialabs.com) at pH 4.4 and 9.6 and in the presence of 6.5 % NaCl (Harrigan 1998). Presumptive enterococci were pre-identified by API50CHL and API20Strep systems (BioMéureux®, Marcy-l'Etoile, France). The following microorganisms were used as control for identification: *E. faecalis* ATCC 19433, *Lactococcus lactis* subsp. *lactis* NCDO 2003 and *Streptococcus thermophilus* NCDO 1968.

All previously isolated microorganisms have been screened for the production of antimicrobial substances against three strains of *Listeria monocytogenes* and three strains of *E. faecalis* and, based on preliminary tests, isolates EM485 and EM925 were selected for future study based on their inhibitory spectrum and were screened for BLS production by the agar-spot-test method (Todorov 2008) against a panel of test microorganisms, including various strains of *Listeria* spp., *Enterococcus* spp., and *Lactobacillus* spp. (Table 1). Activity was expressed as arbitrary units (AU) per milliliter, with 1 AU defined as the reciprocal of the highest dilution showing a clear zone of inhibition (Mayr-Harting et al. 1972). *Listeria monocytogenes* 211 (collection of the Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil) was used as a sensitive test strain.

### Identification of isolates EM485 and EM925

Based on preliminary tests and largest inhibition zones, isolates EM485 and EM925 were selected for future studies. In addition to physiological and biochemical tests, and carbohydrate fermentation reactions (API50CHL and API20 Strep test strips) performed in preliminary screening, identification was confirmed by PCR using genus-specific primers Ent1 and

**Table 1** Antimicrobial spectrum activity of bacteriocin-like substances (BLS) produced by *Enterococcus faecium* EM485 and *Enterococcus faecium* EM925 determined by the spot-on-lawn method

| Test microorganism | Medium | Incubation temperature (°C) | BLS EM485 | BLS EM925 |
|---|---|---|---|---|
| *Enterococcus faecalis* | MRS | 30 | 2 / 2 | 2 / 2 |
| *Enterococcus faecium* | MRS | 30 | 2 / 9 | 2 / 9 |
| *Enterococcus mundtii* | MRS | 30 | 1 / 1 | 1 / 1 |
| *Enterococcus* spp. | MRS | 30 | 13 / 18 | 13 / 18 |
| *Lactobacillus acidophylus* | MRS | 30 | 0 / 2 | 0 / 2 |
| *Lactobacillus curvatus* | MRS | 30 | 0 / 3 | 0 / 3 |
| *Lactobacillus delbrueckii* | MRS | 30 | 0 / 2 | 0 / 2 |
| *Lactobacillus fermentum* | MRS | 30 | 0 / 3 | 0 / 3 |
| *Lactobacillus paracasei* | MRS | 30 | 4 / 8 | 5 / 8 |
| *Lactobacillus plantarum* | MRS | 30 | 0 / 4 | 0 / 4 |
| *Lactobacillus sakei* | MRS | 30 | 0 / 6 | 0 / 6 |
| *Lactococcus lactis* | MRS | 30 | 0 / 3 | 1 / 3 |
| *Leuconostoc mesenteroides* subsp. *mesenteroides* | MRS | 30 | 0 / 4 | 0 / 4 |
| *Listeria innocua* | BHI | 30 | 0 / 3 | 0 / 3 |
| *Listeria monocytogenes* | BHI | 30 | 27 / 29 | 29 / 29 |
| *Pediococcus acidilactici* | MRS | 30 | 0 / 4 | 0 / 4 |
| *Shigella flexneri* | BHI | 37 | 0 / 1 | 0 / 1 |
| *Salmonella* spp. | BHI | 37 | 0 / 7 | 0 / 7 |
| *Clostridium* spp. | BHI | 37 | 0 / 4 | 0 / 4 |
| *Escherichia coli* | BHI | 37 | 0 / 6 | 0 / 6 |

Ent2 (Ke et al. 1999). Additional confirmation of identity was obtained by amplifying genomic DNA with universal primers F8 and R1512 (Felske et al. 1997), and primers 16-1A and 23-1B (Tannock et al. 1999). The fragments amplified using F8 and R1512 and 16-1A and 23-1B primers were purified using Exonuclease 1 (Exo1; USB, http://www.affymetrix.com/estore/browse/brand/usb) and Shrimp Alkaline Phosphatase (SAP; USB). The smaller 16S–23S rRNA gene intergenic spacer region amplified using 16-1A and 23-1B primers was purified by 1.5 % low melting agarose preparative gel followed by QIAquick Gel extraction kit (Qiagen, Valencia, CA). Both purified fragments were sequenced and compared to sequences in GenBank using BLAST, Basic Local Alignment Search Tool.

Differentiation of the isolates (EM485 and EM925) was performed by random amplification of polymorphic DNA (RAPD) PCR. DNA was isolated according to the manufacturer's protocol using a ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA) using primers OPL-14 (5′-GTG ACA GGC T-3′) and OPL-20 (5′-TGG TGG ACC A-3′). Amplification reactions were performed according to Todorov et al. (2010a). The amplified products were separated by electrophoresis in 1.4 % (w/v) agarose gels in 0.5x TAE buffer at 100 V for 2 h. Gels were stained in TAE buffer containing 0.5 μg/mL ethidium bromide (Sigma Diagnostics, St. Louis, MO). Banding patterns were analyzed using Gel Compare, Version 4.1 (Applied Maths, Kortrijk, Belgium).

## Characterization of BLS EM485 and BLS EM925

### *Isolation of BLS EM485 and BLS EM925*

Strains EM485 and EM925 were cultured in MRS broth for 24 h at 30 °C. The cells were harvested (8,000 *g*, 10 min, 4 °C), the cell-free supernatant was adjusted to pH 5.0 with 1 M NaOH, heat-treated (80 °C for 10 min) and the level of antimicrobial activity determined by testing against *Listeria monocytogenes* 211.

### *Effects of enzymes, pH, detergents and temperature on BLS EM485 and BLS EM925*

One milliliter of a cell-free supernatant obtained from a 24-h culture of *E. faecium* EM485 and *E. faecium* EM925, prepared as described before, was added to 1 mg/mL α-amylase, catalase, Proteinase K, proteinase type XIV, pronase, trypsin, α-chymotrypsin, pepsin and papain (all from Sigma Diagnostics), respectively. Samples were incubated at 30 °C for 30 min and then heated at 95–97 °C for 5 min. In a separate experiment, the pH of 10 mL of cell-free supernatants was adjusted to 2.0, 4.0, 6.0, 8.0, 10.0 or 12.0 with 1 M HCl or 1 M NaOH and incubated at 30 °C for 1 h. Another batch of cell-free supernatants was treated with 10 mg/mL Triton X-100 (Sigma), Triton X-114 (Sigma), Tween 20 (Merck), Tween 80 (Merck), NaCl (Sigma), SDS (Sigma), urea (Merck), glycerol (Merck), milk (Difco) or EDTA (Merck), respectively, and incubated for 30 min at 30 °C. The effect of temperature on BLS EM485 and BLS EM925 was determined by incubating cell-free supernatants at 30, 37, 45, 60, 80 and 100 °C for 30 min and 2 h, respectively, and at 121 °C for 20 min. The pH of all samples was adjusted to 6.0 and BLS EM485 and BLS EM925 activity determined with *Listeria monocytogenes* 211 as the sensitive strain, as described above.

### *Production of BLS EM485 and BLS EM925*

Two milliliters of a 24 h culture of strains EM485 and EM925, respectively, were inoculated into 100 mL MRS broth and incubated at 30 °C. Changes in optical density at 600 nm ($OD_{600}$) and pH were monitored hourly for 36 h. BLS EM485 and BLS EM925 activity was measured every 3 h, as described above.

### *Mode of BLS EM485 and BLS EM925 activity*

BHI broth (200 mL) was inoculated with 1 % (v/v) overnight culture of *Listeria monocytogenes* 211 and incubated for 3 h at

37 °C. Filter-sterilized cell-free supernatant (20 mL) was added to the culture and changes in $OD_{600}$ recorded every hour for 10 h. A cell count of *Listeria monocytogenes* 211 on BHI supplemented with 2 % agar plates was performed 10 h from the beginning of the experiment.

*Adsorption of BLS EM485 and BLS EM925 to producer cells*

Adsorption of BLS EM485 and BLS EM925 to producer cells was studied according to Yang et al. (1992). An 18-h-old culture was adjusted to pH 5.0 with 1 M NaOH, 10 mL of the cells harvested (8,000 *g*, 15 min, 4 °C) and washed with an equal volume of sterile 0.1 M phosphate buffer (pH 6.5). The pellet was re-suspended in 10 mL 100 mM NaCl, pre-adjusted to pH 2.0 with 1 M HCl, and stirred for 1 h at 4 °C. Cells were harvested (3,000 *g*, 30 min, 4 °C) and the cell-free supernatant adjusted to pH 7.0 with sterile 1 M NaOH. Activity of BLS EM485 and BLS EM925 was tested as described above.

*Screening for presence of bacteriocin genes*

Total DNA was isolated from *E. faecium* EM485 and *E. faecium* EM925 using a ZR Fungal/Bacterial DNA Kit (Zymo Research) according to the manufacturer's instructions. DNA was submitted to PCR reactions to detect genes responsible for codification of the following bacteriocins: enterocin A, enterocin P, enterocin B and enterocin L50B (Aymerich et al. 1996; Cintas et al. 1998; Du Toit et al. 2000) (Table 2). The PCR reaction was prepared using primers at 10 pM/μL and conditions as described previously (Aymerich et al. 1996; Cintas et al. 1998; Du Toit et al. 2000) adjusting the annealing temperature according to the specification of the primers used. The amplified products were separated by electrophoresis on agarose gels in 0.5× TAE buffer. Agarose gels were stained in TAE buffer containing 0.5 μg/mL ethidium bromide (Sigma Diagnostics).

## Results and discussion

Spectrum of antimicrobial activity

The selected isolates (20 in total) were subjected to pre-screening by testing for the production of antimicrobial substances based on the spot-on-lawn method against three strains of *Listeria monocytogenes* and three strains of *E. faecalis*. Two of the microbial isolates obtained from artisanal Coalho cheese inhibited the growth of *Listeria monocytogenes*. These strains, named EM485 and EM925, were selected for further study and screened against a panel of test organisms (Table 1). Cell-free supernatants obtained from *E. faecium* EM485 and *E. faecium* EM925, adjusted to pH 6.0, inhibited the growth of *Enterococcus* spp., *Lactobacillus paracasei* and *Listeria monocytogenes*. In addition cell-free supernatants obtained from *E. faecium* EM925 inhibit the growth of *Lactococcus lactis*. However, none of the other strains included in the panel test were inhibited by the cell-free supernatant from both *E. faecium* strains tested (Table 1). In general, this narrow spectrum of activity was revealed to be unique for a BLS produced by *E. faecium*. Most bacteriocins described for *E. faecium* were found to be active against a much broader range of microbial genera and species (de Vuyst and Vandamme 1994). However, the narrow spectrum of activity recorded for bacteriocin T8 has been described as typical for only class IIa bacteriocins (De Kwaadsteniet et al. 2006). It is important to underline the very strong activity of BLS produced by *E. faecium* EM485 and *E. faecium* EM925 against *Listeria monocytogenes* and *Enterococcus* spp., which may lead to important applications in the biopreservation of fermented food products.

Identification of isolates EM485 and EM925

According to the results of sugar fermentation reactions based on API50CHL, the isolates were classified as *Lactococcus lactis* (99.2 %); however, based on API20Strep test strips the strain was 99.7 % related to *E. faecium* (data not shown).

**Table 2** Primer sequences and results used in the investigation of presence of bacteriocin (enterocin A, enterocin P, enterocin B and enterocin L50B) genes in total DNA isolated from *Enterococcus faecium* EM485 and *Enterococcus faecium* EM925

| Bacteriocin genes | *Enterococcus faecium* EM485 | *Enterococcus faecium* EM925 | Primers (5′ – 3′) | Reference |
|---|---|---|---|---|
| Enterocin A | +[a] | + | F: GAG ATT TAT CTC CAT AAT CT<br>R: GTA CCA CTC ATA GTG GAA | Aymerich et al. 1996 |
| Enterocin P | - | - | F: ATG AGA AAA AAA TTA TTT AGT TT<br>R: TTA ATG TCC CAT ACC TGC CAA ACC | Gutierrez et al. 2005 |
| Enterocin B | + | + | F: GAA AAT GAT CAC AGA ATG CCT A<br>R: GTT GCA TTT AGA GTA TAC ATT TG | Du Toit et al. 2000 |
| Enterocin L50B | - | - | F: ATG GGA GCA ATC GCA AAA TTA<br>R: TAG CCA TTT TTC AAT TTG ATC | Cintas et al. 1998 |

[a] + indicates positive results for genes corresponding to known bacteriocins in *E. faecium* EM485 and *E. faecium* EM925

Identification based only on sugar fermentation profiles very often generates such conflicting results. In order to properly identify the isolates, we applied PCR with genus-specific primers. Amplification of genomic DNA from isolates EM485 and EM925 with genus-specific primers produced a 112 bp fragment, which corresponded in size to that of *E. mundtii* CRL35 (data not shown). The 16S rRNA amplified from isolates EM485 and EM925 revealed 99 % homology to the 16S rRNA sequence of *E. faecium*. As a result, the isolates EM485 and EM925 were regarded to be strains of *E. faecium*.

The isolates (EM485 and EM925) were differentiated by random amplification of polymorphic DNA (RAPD) PCR applying primers OPL-14 and OPL-20 and shown that these two isolates are not representative of the same strain (Fig. 1).

## Effect of enzymes, pH, detergents and temperature on BLS EM485 and BLS EM925

The activity of BLS EM485 and BLS EM925 was completely abolished by treatment with proteinase K, pronase, trypsin, pepsin and papain, but not by treatment with α-amylase or catalase (Table 3). This suggested that the activity of BLS EM485 and BLS EM925 is not dependent on glycosylation and not related to the effect of $H_2O_2$. Similar results have been reported for other bacteriocins of *Enterococcus* spp. (de Vuyst and Vandamme 1994; Todorov and Dicks 2005b; Todorov et al. 2005, 2010a, b). Leuconocin S, produced by *Leuconostoc paramesenteroides* (Lewus et al. 1992) and carnocin 54 from *Leuconostoc carnosum* (Keppler et al. 1994) are examples of amylase-sensitive bacteriocins.

BLS EM485 and BLS EM925 remained stable after incubation (30 °C) at pH 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 (Table 3). Additionally, treatment with Triton X-110, Triton X-114, Tween 20, Tween 80, SDS, NaCl, urea, milk, glycerol and EDTA had no effect on the activity of the bacteriocins EM485 and EM925 (Table 3). Similar behaviors were reported for the bacteriocin J46 produced by *Lactococcus lactis* subsp. *cremoris* (Hout et al. 1996). However, plantaricin C19 secreted by *Lactobacillus plantarum* C19 (Atrih et al. 2001) and bacteriocin HV219 from *Lactococcus lactis* susbp. *lactis* HV219 (Todorov et al. 2006) lost activity after treatment with SDS or Triton X-100. However, treating the enterocin EJ97 produced by *E. faecalis* EJ97 (Gálvez et al. 1998), bozacin B14 from *Lactococcus lactis* subsp. *lactis* B14 (Ivanova et al. 2000) and pediocin ST18 produced by *Pediococcus pentosaceus* ST18 (Todorov and Dicks 2005a) with SDS did not affect their antimicrobial activity.

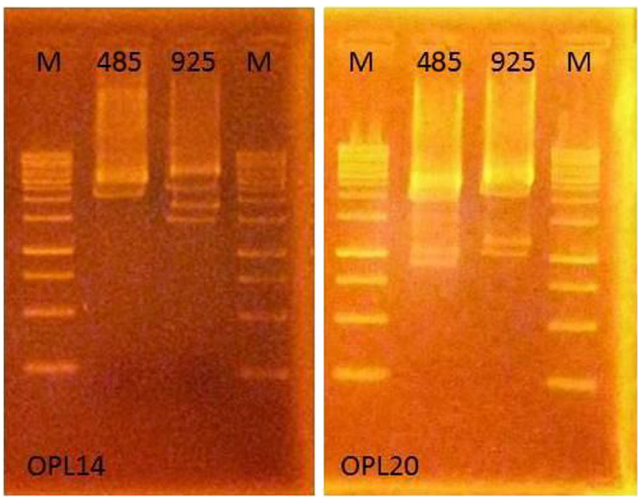


**Fig. 1** Agarose gels showing DNA fingerprint obtained after random amplification of polymorphic DNA (RAPD)-PCR with primers OPL14 (*left*) and OPL20 (*right*). Lanes: *M* O'GeneRuler$^{tm}$ 1 kb DNA ladder (Fermentas, http://ThermoScientificBio.com/Fermentas) *485 E. faecium* EM485, *925 E. faecium* EM925

**Table 3** Effects of enzymes, pH, detergents and temperature on stability of BLS EM485 and BLS EM925

| Treatment | EM485 BLS | EM925 BLS |
|---|---|---|
| Enzymes | | |
| α-amylase, lipase | + | + |
| proteinase K, proteinase type XIV, trypsin, pepsin, papain, pronase, α-chymotrypsin | - | - |
| Detergents, protease inhibitors, salts | | |
| Triton X-100, Triton X-114, Tween 20, Tween 80, SDS, NaCl, urea, EDTA, milk, glycerol | + | + |
| pH | | |
| 2.0 | + | + |
| 4.0 - 10.0 | + | + |
| 12.0 | + | + |
| Temperature | | |
| 121 °C (20 min) | -/+ | -/+ |
| 30, 37, 45, 60, 80, 100 °C (2 h) | + | + |

As reported in Table 3, BLS EM485 and BLS EM925 (pH 6.0) proved to be heat tolerant and remained stable after 2 h at 100 °C. However, a slight decrease in activity was observed upon heat treatment at 121 °C for 20 min (data not shown). This finding was consistent with a number of bacteriocins / BLS produced by *Lactobacillus* and *Enterococcus* spp. (Klaenhammer 1988; Van Reenen et al. 1998; Ko and Ahn 2000; Todorov et al. 2005; Todorov and Dicks 2005c). Moreover, lactocin NK24, produced by *Lactococcus lactis* NK24, lost 87.5 % of its activity after 30 min at 100 °C and was completely inactivated after 15 min at 121 °C (Lee and Paik 2001). In the case of lactocin MMFII secreted by *Lactococcus lactis* MMFII, only 8.3 % activity was recorded after 30 min at 110 °C and 25 % after 30 min at 80 °C and 90 °C (Ferchichi et al. 2001). Nisin from *Lactococcus lactis* subsp. *lactis* WNC20 was inactivated after 15 min at 121 °C when incubated at pH 7.0, but not when incubated at pH 3.0 (Noonpakdee et al. 2001).

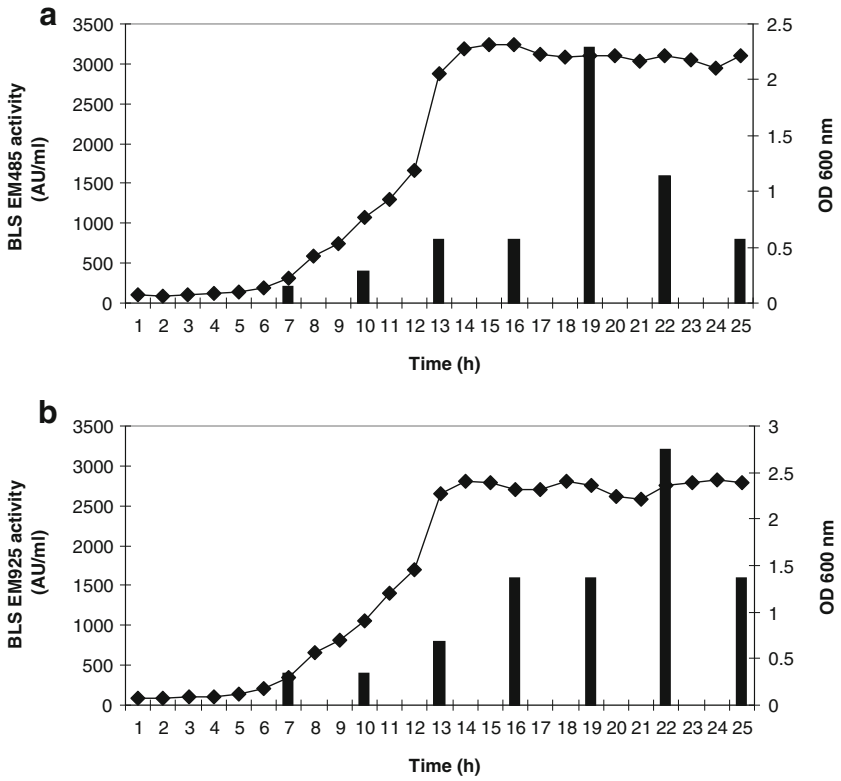


**Fig. 2** Growth rate of **a** *Enterococcus faecium* EM485 and **b** *Enterococcus faecium* EM925 (*filled diamonds*) and bacteriocin-like substance (BLS) production in arbitrary units (AU)/mL (*bars*) in MRS broth. BLS activity was determined against *Listeria monocytogenes* 211

Production of BLS EM485 and BLS EM925

The cell density of *E. faecium* EM485 increased from 0.07 to 2.21 ($OD_{600}$) during 24 h of growth at 30 °C (Fig. 2). Production of BLS EM485 increased from 200 AU/mL after 6 h of growth to 3,200 AU/mL during the following 12 h and decreased to 800 AU/mL during the next 6 h (Fig. 2). A similar profile of growth and BLS production was recorded for *E. faecium* EM925. The cell density of *E. faecium* EM925 increased from 0.08 to 2.39 ($OD_{600}$) during 24 h of growth at 30 °C while production of BLS EM925 increased from 400 AU/mL after 6 h of growth to 3,200 AU/mL during the following 15 h before decreasing to 1,600 AU/mL during the next 3 h (Fig. 2). Optimal production of BLS EM485 and BLS EM925 was recorded during stationary growth, which may suggest that the peptide is a secondary metabolite. Similar findings were reported for plantaricin ST31 (Todorov et al. 1999); bacteriocin ST311LD produced by *E. faecium* ST311LD isolated from spoiled black olives (Todorov and Dicks 2005b); bacteriocin ST4V produced by *E. mundtii* ST4V (Todorov et al. 2005); and bacteriocins produced by *E. faecium* ET05, *E. faecium* ET12 and *E. faecium* ET88, respectively, isolated from smoked salmon (Tomé et al. 2009). The pH of the cultures decreased from 6.50 to approximately 5.10 during the first 9 h of fermentation, then to approximately 4.20 during the following 15 h (data not

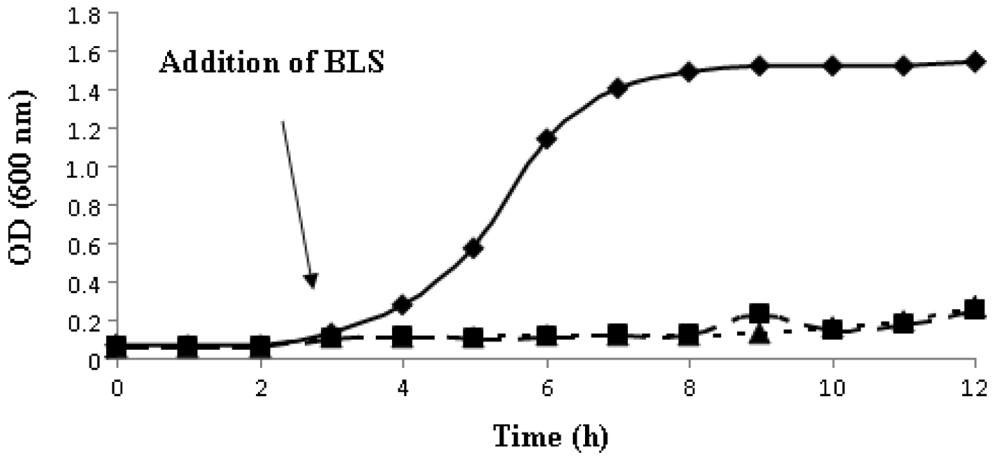


**Fig. 3** Effect of BLS produced by *Enterococcus faecium* EM485 (*squares*) and *Enterococcus faecium* EM925 (*triangles*) on the growth of *Listeria monocytogenes* 211 over a period of 12 h. The control (*diamonds*) is growth in the absence of bacteriocins

shown). Considering that the BLS was reported to be active following exposure at pH 4.0 (Fig. 2), the decrease in antimicrobial activity (from 3,200 AU/mL to 800 AU/mL and 1,600 AU/mL, respectively) detected once the pH of the MRS medium was about 4.1 cannot be ascribed to the change in culture pH. Indeed, as reported by Yang et al. (1992) and Van Reenen et al. (1998), it is unlikely that such a small modification in pH could trigger a sudden interaction of BLS EM485 and BLS EM925 from the surface of the producer cell. On the contrary, the increase in BLS activity may be due to the metabolism of remaining nutrients or medium component(s) not required for cell growth or accumulation of the BLS in the cells' environment.

### Mode of activity

Growth of *Listeria monocytogenes* 211 treated with BLS EM485 or BLS EM925 (320 AU/mL final BLS activity / concentration) was completely inhibited over a period of 10 h (Fig. 3). The $OD_{600}$ of the control (not treated with BLS EM485 or BLS EM925) increased from 0.07 to 1.55 over the same period (Fig. 3) representing bacterial growth of $10^7$ CFU/mL. The cell count of *Listeria monocytogenes* 211 on BHI supplemented with 2 % agar plates in BLS-treated samples showed less than 100 CFU/mL. Similar findings were reported for bacteriocin ST13BR (Todorov et al. 2004) and bacteriocin ST311LD (Todorov and Dicks 2005b), whereas bacteriocins ST28MS and ST26MS repressed the growth of *Lactobacillus casei* LHS for only 2 h (Todorov and Dicks 2005c).

### Adsorption of BLS EM485 and BLS EM925 to producer cells

Low EM485 and EM925 BLS activity was detected after treatment of 18-h-old cells of *E. faecium* EM485 and EM925 with 100 mM NaCl. However, the activity was lower than that recorded in the cell-free supernatant, suggesting that BLS EM485 and BLS EM925 adsorb to the surface of the producer cells in very low concentrations. This is in accordance with results reported for plantaricin C19, whose maximal adsorption to producer cells was recorded between pH 5 and 7, with complete loss of adsorption at pH 1.5 and 2.0 (Atrih et al. 2001). In the case of plantaricin ST31 (Todorov et al. 1999), pediocin ST18 (Todorov and Dicks 2005a) and bozacin B14 (Ivanova et al. 2000), no bacteriocin activity was recorded on the cell surface of the producer strains.

### Screening for presence of bacteriocin genes

On the basis of the PCR reactions targeting enterocin P and enterocin L50B, no clear evidence for the presence of these genes in the total DNA of *E. faecium* EM485 and *E. faecium* EM925 was obtained. However, when we targeted enterocin A and enterocin B in PCR reactions with genomic DNA from *E. faecium* EM485 and *E. faecium* EM925, we had positive results. The sequence of the generated amplicons was 100 % identical to the targeted bacteriocin genes (enterocin A and enterocin B). Based on these results, we can say that both strains carry genes for production of enterocin A and enterocin B, but in order to conclude if one or both of these genes are expressed, appropriate biochemical protein purification and mass spectrometry needs to be performed. It is important to underline that some of the PCR reactions generated a product corresponding to the size of the target bacteriocin genes (enterocin P and enterocin L50B) and to a fragment generated by the positive controls. However, after the sequencing of PCR products, no homology to the targeted bacteriocin genes was observed. In contrast, sequence of the PCR products obtained by the positive controls generated 100 % homology. This is a significant remark indicating the importance of sequencing PCR products in order to confirm the identity of the products generated.

## Conclusions

This is the first study reporting the production of BLSs by LAB strains isolated from artisanal Coalho cheeses from Brazil with reference to bacteriocinogenic potential. The BLS secreted by *E. faecium* EM485 and EM925 are heat resistant and stable between pH 2.0 and 10.0, adsorb to the surface of the producer cell in low concentrations and are produced at higher levels during the stationary phase of fermentation. Considering the stability, efficiency and the narrow spectrum of antimicrobial activity exhibited by BLS EM485 and BLS EM925, *E. faecium* EM485 and EM925 could be used in a mixed starter culture for fermentation of milk products.

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