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ORIGINAL RESEARCH

Purification of two bacteriocins produced by *Enterococcus faecalis* DBFIQ E24 strain isolated from raw bovine milk

RICARDO M. MANZO,[1,2] MARÍA DE LAS MERCEDES CARDOSO,[1,2] GEORGINA G. TONARELLI[2] and ARTURO C. SIMONETTA[1]*

[1]*Cátedras de Microbiología y Biotecnología, Departamento de Ingeniería en Alimentos, Facultad de Ingeniería Química (F.I.Q.), Universidad Nacional del Litoral (U.N.L.), and* [2]*Departamento de Química Orgánica, Universidad Nacional del Litoral (U.N.L.), Ciudad Universitaria, Paraje "El Pozo", Santa Fe, Argentina*

Enterococcus faecalis *DBFIQ E24 was formerly characterised as a producer of antibacterial and antifungal substances. This strain is vancomycin sensitive, nonhaemolytic and gelatinase negative. SDS-PAGE revealed the presence of an active band located between 1060 and 3500 Da. After the application of a three-step purification procedure, two antimicrobial peptides were isolated. One of them is mostly active against* Bacillus cereus *with a molecular mass of 1364 Da, and the other one with a molecular mass of 1686 Da is mainly active against* Escherichia coli. *These results confirm the technological potential of* E. faecalis *DBFIQ E24 strain as a GRAS food biopreservative.*

Keywords *Enterococcus faecalis* strain, Enterocin, Purification, Food biopreservative, Foodborne bacteria.

INTRODUCTION

Bacteriocins are ribosomally synthesised peptides or proteins that exert, in an extracellular way, antimicrobial action against taxonomically related bacteria. Bacteriocins from lactic acid bacteria were isolated and characterised principally from *Lactococcus, Lactobacillus, Leuconostoc, Carnobacterium, Enterococcus* and *Pediococcus* genera. However, an important number of studies have shown that several bacteriocins can antagonise not only taxonomically related Gram (+) bacteria but also Gram (−) bacteria (Cardoso *et al.* 2012; Ramakrishnan *et al.* 2012). As a consequence of their antagonistic activity, bacteriocins have been used as food preservatives, as antibiotic complement in mastitis treatment (Balciunas *et al.* 2013) and as antiviral agents (Wachsman *et al.* 1999).

Enterococcus genus is an important part of the constitutive microbiota of fermented vegetables, meat products and most cheeses, not only artisanal but also industrially produced with pasteurised milk. In nature, they can be found ubiquitously because they are very resistant to temperature, pH, different antimicrobial substances, high salt concentration, reducing conditions and different substrates (food commodities; intestinal tract of mammals, poultry, fish and insects; food industries environments; among others). These bacteria play a key role in food maturation through different mechanisms such as proteolysis, lipolysis and citrate metabolism exerting, in this way, a deep influence on its flavour and taste (Foulquié Moreno *et al.* 2006). However, several virulence factors have been described in the genus and a number of virulent and pathogenic enterococci, especially vancomycin-resistant enterococci (VRE) strains, which is higher among clinical isolates (Mannu *et al.* 2003). Therefore, as shown by several scientific reports, enterococci strains lacking haemolytic activity (Belguesmia *et al.* 2011) and carrying neither cytolysin (haemolysin) nor vancomycin resistance genes may be regarded as safe and could be used as starter cultures, cocultures or probiotics. Thus, it is essential to make an adequate selection of the *Enterococcus* strain to be studied or used as a starter culture (De Vuyst *et al.* 2003; Ahmadova *et al.* 2013).

*Author for correspondence. E-mail: asimonet@fiq.unl.edu.ar

Enterocins are bacteriocins produced by enterococci species that have gained special attention in the last decade, owing to the fact that enterococci are present in a wide variety of raw and fermented foods in which most of them are usually active against many foodborne and pathogenic bacterial genera such as *Listeria*, *Clostridium*, *Vibrio* and *Escherichia* (Simonetta *et al.* 1997). Also, it has been proved that either enterocins or their corresponding cell-free supernatants inhibit the growth of several moulds (Cardoso *et al.* 2012), yeasts (Magnusson *et al.* 2003) and viruses, such as *Herpes simplex* type I and II (Wachsman *et al.* 2003).

Purification of bacteriocins involves many sequential and time-consuming chromatographic steps. The applied protocols reported consisted of four main basic steps: (i) ammonium sulphate protein precipitation, (ii) cation-exchange chromatography, (iii) hydrophobic interaction chromatography, and (iv) reversed-phase chromatography (RPC) using high-performance liquid chromatography (HPLC) or fast-protein liquid chromatography equipment. This purification protocol has successfully been applied in homogeneity purification of several bacteriocins such as lactococcin G (Nissen-Meyer *et al.* 1992), pediocin PA-1 (Nieto Lozano *et al.* 1992) and enterocin A (Aymerich *et al.* 1996). Slight variations of the general purification protocol described above have been introduced previously for enterocins purification with success.

Therefore, the objective of this work was the complementary characterisation and purification of two bacteriocins from *Enterococcus faecalis* DBFIQ E24 strain, a wild strain isolated from bovine raw milk. These peptide molecules are present in the cell-free supernatant, previously characterised as an antimicrobial substance with broad antagonistic activity against bacteria, yeast and moulds (Cardoso *et al.* 2012), which have increased their promising technological application in food industry namely as an antimicrobial biopreservative.

MATERIALS AND METHODS

Strains and cultures preservation

The bacteriocinogenic strain *E. faecalis* DBFIQ E24, isolated from raw cow milk produced in Santa Fe region (Argentina), was chosen from previous studies (Cardoso *et al.* 2012). *Escherichia coli* DBFIQ Ec9 and *Bacillus cereus* DBFIQ B28, belonging to the Departamento de Ingeniería en Alimentos (F.I.Q. – U.N.L.) collection, were employed as target strains. All bacterial strains were lyophilised to maintain a reference stock and frozen at –80 °C in M17 broth (Difco Laboratories, Detroit, MI, USA) for *Enterococcus* strain, or nutrient broth (Merck KGaA, Darmstadt, Germany) for nonlactic acid bacteria, with the addition of 15% (v/v) glycerol.

DNA isolation and genetic identification of bacteriocin-producing strain

Enterococcus faecalis DBFIQ E24 was PCR-identified employing the 16S rDNA methodology. For this purpose, total DNA from *E. faecalis* was obtained from a pure 10 mL culture in Elliker broth (Difco Laboratories) grown at 30 °C for 16 h using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA). The amplification reaction of the 1500-bp fragment belonging to 16S rDNA was made using two universal primers, pA and pH, and employing 1 μL of total DNA (dilution 1:50) as template. The PCR mixture included 2.5 U of *Taq* polymerase (GE Healthcare, Uppsala, Sweden), 200 nM of dNTPs and 400 nM of each primer (Sigma-Genosys, The Woodlands, TX, USA) in a 50 μL final volume. A negative reaction control without DNA was performed. Amplification was performed in a GeneAmp PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 3 min at 94 °C, 36 cycles of 1 min at 94 °C, 2 min at 51 °C and 2 min at 72 °C, and a final step of 7 min at 72 °C. PCR products were visualised on 1.5% agarose gels in TBE buffer (pH 8.3), with added GelRed (Biotium, Hayward, CA, USA), and visualised by UV light. The PCR amplification product was purified using the GenElute™ PCR clean-up kit (Sigma-Aldrich), and the nucleotide sequences were determined by primer extension at the DNA Sequencing Service of Macrogen Inc. (Seoul, South Korea). Sequence data were assembled and compared using a sequence analysis software package available from the EMBL Spanish node (CNB, CSIC, Spain). The strain identity was evaluated by nucleotide–nucleotide BLAST sequence alignment and comparison using the NCBI database (http://www.ncbi.nlm.nih.gov/blast).

Cell-free supernatant production

From a stock culture, *E. faecalis* DBFIQ E24 was activated by two successive culturing procedures in M17 broth (Difco) at 37 °C for 24 h. The final culture was obtained by adding 30 mL of the last propagation culture to 1000 mL of M17 broth (Difco) and incubating at 37 °C for 24 h. The cell concentrations achieved were established between 9 $\log_{10}$ and 9.9 $\log_{10}$ CFU/mL. Then, cells were removed by centrifugation at 5200 g for 15 min at 4 °C. Afterwards, the Cell-free supernatant (CFS) obtained was 10-fold-concentrated at 70 °C in a Büchi RE 111 Rotavapor (Büchi Labortechnik AG, Postfach, Switzerland) followed by sterilisation by filtration using 0.22-μm-pore-diameter membranes (Sartorius-Stedim, Göttingen, Germany) and immediately used or stored at −20 °C until use within 1 month after their production. Anytime CFS was produced, a concentrated M17 broth (Difco) containing the same concentration of lactic acid as CFS was also prepared for utilisation as negative control in the subsequent assays. pH range of concentrated CFS was maintained at approximately 6.5–7.0.

Antimicrobial activity assay

Antagonistic activity, employing *Escherichia coli* DBFIQ Ec9 and *Bacillus cereus* DBFIQ B28 as sensitive strains, was assayed by the agar-well diffusion test. Each assay was performed in triplicate employing 15 mL of molten Nutrient Agar (Merck) and 60 μL of assayed samples, as described by Cardoso *et al.* (2012).

Purification of active peptides from E24 CFS

A total of 100 mL of 10-fold-concentrated E24 CFS was divided into 25-mL aliquots and first processed by adding solid ammonium acetate (pH 4.5) up to a 50 mM concentration and each one was applied to a SP-Sepharose Fast Flow (GE Healthcare) column (2.5 cm diameter by 35 cm height) previously equilibrated with 50 mM, pH 4.5 ammonium acetate ($AcNH_4$) buffer. The sample was recirculated for 1 h or until absorbance at 230 nm reached equilibrium and washed with equilibration buffer to remove nonbound proteins and other substances. Elution was made through a stepped gradient elution mode employing increasing concentrations from 0.05 to 0.4 M, pH 4.5 ammonium acetate buffer solutions (Guyonnet *et al.* 2000) using an Econo Gradient peristaltic pump (Bio-Rad Laboratories, Hercules, CA, USA). Absorbance at 230 and 280 nm was measured in 5 mL fractions, recovered at 2.5 mL/min flow rate using a fraction collector (model 2110; Bio-Rad). Fractions were gathered together, concentrated by a rotary evaporator (Büchi) and freeze-dried by lyophilisation for 24 h employing a vacuum freeze-dryer (Rificor, model L-M10-A-E50-CRT, Buenos Aires, Argentina). Samples were resuspended in water, equilibrated at pH 7.0 and assayed for antibacterial activity employing the agar-well diffusion assay against *B. cereus* and *E. coli* as target strains as mentioned above.

In addition, a protein precipitation assay employing different ammonium sulphate concentrations (20, 30, 40, 60, 80, 85, 90 and 100% saturation) was performed for recovering the maximum amount of the antibacterial substances. Salt was added progressively, and the suspension was maintained at 4 °C overnight without stirring to maximise the creation of a protein clot.

Furthermore, Q-Sepharose Fast flow resin (GE Healthcare) was also in the testing of the bacteriocin's purification because the active peak had appeared rapidly when eluted with ammonium acetate in a SP-Sepharose ion-exchange resin. The employed methodological conditions for anion-exchange chromatography were the same as in SP-Sepharose cation-exchange chromatography. Subsequently, the active fractions obtained by strong cation-exchange chromatography were pooled and trifluoroacetic acid (Merck) (TFA) was added up to a 0.1% (v/v) final concentration (Herraiz and Casal 1995). Next, the sample was applied to a 35-cm^3 prepacked C_{18} Sep-Pak Plus cartridge (Waters Corporation, Milford, MA, USA) for solid-phase extraction equilibrated with 0.1% (v/v) TFA (Merck) Milli-Q water (Waters Corp.) and allowed to interact with the solid phase through recirculation of the sample for 1 h. Afterwards, the column was washed with the same equilibration solution, and elution was made by a stepped increase of acetonitrile (JT Baker, Center Valley, PA, USA) (ACN) concentration from 20% (v/v) to 100% in 0.1% (v/v) TFA Milli-Q water solutions, using a 2.0 mL/min constant flow rate. Immediately, samples were concentrated to eliminate organic modifier, lyophilised, reconstituted with Milli-Q water and equilibrated at pH 7.0. Antibacterial activity was determined as described in the ion-exchange step.

The active fraction was heated at 37 °C for 30 min and analysed in a RP-HPLC equipment (Gilson, model 811C, Middleton, WI, USA) using a C_{18} analytical column (pore diameter: 90 Å, size particle: 4 μm, size: 250 × 4.60 mm, Jupiter Proteo model) purchased from Phenomenex (Torrance, CA, USA). Elution was carried out by applying a continuous linear gradient from 5% to 80% (v/v) of solvent B [ACN in 0.08% (v/v) TFA] in solvent A [Milli-Q water in 0.1% (v/v) TFA]. Constant flow rate employed was 0.8 mL/min, and sample volume loaded was 10 μL. Sensitivity range was kept at 0.2 absorbance units full-scale (AUFS), and detection was performed at 220 and 280 nm.

Through analysis by RP-HPLC, the following chromatographic fractions were obtained: fraction I, from 0.0 to 10.60 min; fraction II, from 10.60 to 12.16 min; fraction III, from 12.16 to 15.60 min; and fraction IV, from 15.60 to 30.00 min. Finally, fractions were pooled, concentrated, lyophilised, resuspended with Milli-Q water, equilibrated at pH 7.0 and assayed for antimicrobial activity as previously mentioned.

Protein quantitation

Protein concentration of different samples was determined by the bicinchoninic acid method employing bovine serum albumin (BSA; Sigma-Aldrich) as protein standard (Walker 2002). Briefly, samples (100 μL) were appropriately diluted and incubated at 60 °C for 30 min. Then, absorbance was measured at 562 nm in a UV/VIS spectrophotometer (Metrolab, model 21700, Buenos Aires, Argentina).

Tricine–SDS-polyacrylamide gel electrophoresis

Cell-free supernatant and partially purified samples were separated on a one-dimensional tricine–SDS-polyacrylamide gel electrophoresis as described by Schägger and von Jagow (1987). Each tricine–SDS-PAGE gel (dimensions: 8 × 7.3 × 0.075 cm) was prepared with the following composition: the separating gel (16.5% T, 3% C) was overlaid by the spacer gel (10% T, 3% C) and the stacking gel (4% T, 3% C) was poured on top of the spacer gel. Peptide fractions were prepared by mixing in a 1:1 (v/v) dilution with sample buffer and boiled for 5 min in a water-bath. Ultralow-range molecular weight markers from 1 060 to 26 600 Da (Sigma-Aldrich) were used. Gels were run at room

temperature and constant voltage (80 V) for 210 min with an initial current of 55 mA and finishing with 22 mA or until 0.02% (w/v) Coomassie blue G-250 tracking dye has reached the bottom line of the corresponding gel. Electrophoresis was performed in a Bio-Rad equipment model Mini-PROTEAN 3 Cell with a Bio-Rad PowerPac Basic Power Supply (Bio-Rad Laboratories, Hercules, CA, USA). Gels were silver-stained according to the method described by Blum *et al.* (1987).

N,N′-tetramethylene-ethylenediamine (TEMED), ammonium persulphate, acrylamide, N,N′-methylene-bis-acrylamide, 2-mercaptoethanol, tricine, *tris* (*hydroxymethyl*)-*aminomethane (Trizma base),* sodium dodecyl sulphate (SDS), Coomassie blue G-250 and glycerol were acquired from Bio-Rad Laboratories.

Bacteriocinogenic activity direct detection in SDS-PAGE gels

Antimicrobial activity direct detection was performed in polyacrylamide gels according to Bhunia and Johnson (1992) with some modifications. SDS-PAGE gel was cut in two pieces. One piece was silver-stained employing Blum *et al.* (1987) methodology. The other slice, with the purpose of assaying antimicrobial activity, was extensively washed with deionised water and fixed with 2-propanol: acetic acid: water (20:10:70) (v/v/v) mixture for 2 h at room temperature. Then, the fixed SDS-PAGE gel was washed three times with Milli-Q water. Each wash consisted of a 10-min duration. Finally, gel was put on a sterile Petri dish with Nutrient Agar (Merck) and overlaid with 0.8% (w/v) soft Nutrient Agar (Merck) inoculated with 6.0 $\log_{10}$ CFU/mL of *B. cereus* DBFIQ B28 and *E. coli* DBFIQ Ec9 as target strains. The plate was incubated for 18 h at 37 °C, and clear zones due to inhibition of target strain were measured.

Determination of virulence factors

Haemolysis assay

Enterococcus faecalis DBFIQ E24 strain was activated by three successive culturing procedures in M17 broth at 37 °C for 24 h. Then, enterococci were inoculated by streaking the liquid culture onto Blood Agar Base (Difco) plates containing 7% (v/v) of factor 0 Rh (−) human blood and incubated overnight at 37 °C. Haemolytic reaction was recorded by the observation of a clear zone of hydrolysis around the colonies (β-haemolysis), a partial hydrolysis and greening zone (α-haemolysis) or no reaction (γ-haemolysis) (De Vuyst *et al.* 2003).

Vancomycin susceptibility test

To evaluate the glycopeptide antibiotic resistance of *E. faecalis* DBFIQ E24, Foulquié Moreno *et al.* (2003) protocol was followed.

Gelatinase assay

Assay for the presence of gelatinase activity in *E. faecalis* DBFIQ E24 strain was performed using Brain Heart Infusion Agar (BHI; Difco) plates with the addition of 10 g/L peptone and 30 g/L gelatin. After overnight incubation at 37 °C, grown enterococci culture plates were placed at 4 °C for 5 h. Examination for the presence or absence of a turbidity zone around the colonies was carried out as indicative of gelatin liquefaction (Rivas *et al.* 2012).

Mass spectrometry analysis

Fractions obtained by RP-HPLC were analysed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS). A total of 10 μL of each fraction, previously lyophilised and dissolved in Milli-Q water (Waters Corp.) containing 0.1% (v/v) TFA, was mixed with a matrix solution containing 10 μL of α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) in 30% (v/v) aqueous ACN and 0.1% (v/v) TFA. Then, a 0.5-μL aliquot of this mixture was deposited on a stainless steel probe tip and allowed to dry at room temperature for 5 min. Samples were measured on a MALDI-TOF/TOF Ultraflex II mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with an ion-trap source with visualisation optics and a low-energy N_2 UV laser (337 nm). Data is presented as the m/z ratio (mass / charge) and AUFS (absorbance units full-scale).

RESULTS AND DISCUSSION

Genetic identification of bacteriocin-producing enterococci

Phenotypic identification of *E. faecalis* DBFIQ E24 strain was firstly performed by employing the biochemical keys and taxonomic data provided by the Bergey's Manual of Systematic Bacteriology, 2nd ed. (De Vos *et al.* 2009). In addition, their identity was confirmed by 16S rDNA partial sequencing, and the peptide profile was acquired and analysed by MALDI-TOF mass spectrometry analysis and compared with the existing database.

Purification of enterocin E24

After SP-Sepharose ion-exchange chromatography, five fractions were collected and assayed for antimicrobial activity against *E. coli* DBFIQ Ec9 and *B. cereus* DBFIQ B28. Figure 1 shows that washout and A (0.1 M $AcNH_4$) fractions were the only active samples.

Fraction A was the fraction with the highest activity (266 AU/mL) and was selected as the initial sample for solid-phase extraction (SPE) with a C_{18} reversed-phase chromatography cartridge. In that way, specific activity of active-eluted fraction obtained by C_{18} SPE was 30.6 (AU/mg), and a 10.2 times purification compared to CFS was achieved. However, recovery percentage was low (2%) because only partial column retention of total activity was observed (Table 1).

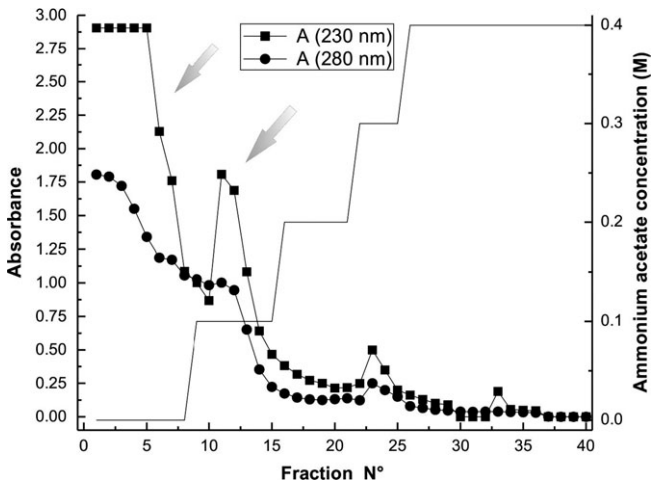

Figure 1 SP-Sepharose Fast Flow ion-exchange chromatography profile employing 0.1 M to 0.4 M $AcNH_4$, pH 4.5 buffer solutions. Concentrated CFS equilibrated with 0.05 M $AcNH_4$, pH 4.5 was used as starting sample. Five fractions were collected: washout fraction (1–9); fraction A (10–16); fraction B (17–21); fraction C (22–25); and fraction D (26–40). Arrows indicate active fractions. The straight line shows the ammonium acetate stepwise gradient concentration. Ion-exchange chromatography was performed to process a 100 mL volume of concentrated CFS. In all cases, the chromatographic profile was identical.

In addition, Q-Sepharose Fast Flow ion-exchange chromatography was employed, but no retention in the anionic resin was evidenced, confirming the cationic nature of the bacteriocinogenic peptides. Furthermore, attempts to recover the bacteriocins, precipitation with ammonium sulphate was carried out but results were unsatisfactory because antimicrobial activity was always present in the supernatant and no activity was recovered in the pellet, even at 100% ammonium sulphate saturation.

After C_{18} SPE purification step, it was found that only washout and 20% ACN elution fractions were active against both the bacterial strains assayed.

As it can be seen in Table 1, an active 20% ACN elution fraction presented a 33 AU/mL titre. Washout fraction was more active against *E. coli* DBFIQ Ec9 than against *B. cereus* DBFIQ B28, and the 20% ACN-eluted fraction has undergone an opposite behaviour in relation to antimicrobial activity. After this step, a pronounced drop in antimicrobial activity and recovery yield (0.3%) was evidenced (Table 1). In spite of these results, purification studies were continued with 20% ACN-eluted fraction.

Afterwards, RP-HPLC analysis of retained C_{18} SPE active fraction was performed to reveal the peptide profile, as shown in Figure 2. After the analysis of RP-HPLC chromatogram, four fractions were collected and evaluated for antibacterial activity against both bacterial strains. Fraction I was composed of several peaks of hydrophilic nature, slightly? retained by RP-HPLC column (below 20% ACN). This sample presented antibacterial activity, mainly against *E. coli* DBFIQ Ec9. Fraction II (30% ACN) showed one major peak, and fraction III did not show any peaks; however, both fractions did not show any antibacterial activity. Finally, in fraction IV, the most hydrophobic sample, only few peptides were observed and one major peak highlighted over the others (43% ACN). This fraction was mainly active against *B. cereus* DBFIQ B28, as shown in Figure 3. The same performance was observed in the C_{18} SPE purification step where washout fraction was more active against the Gram (−) than the Gram (+) strain, while 20% ACN-eluted fraction showed a reverse behaviour as mentioned before. In that sense, *E. coli* DBFIQ Ec9 revealed similar results than *B. cereus* DBFIQ B28 but was better antagonised by fraction I (data not shown).

After every purification step, an important decrease in activity and yield was detected. However, the active fractions obtained by RP-HPLC could be analysed by mass spectrometry.

Haemolysis assay

E. faecalis DBFIQ E24 did not show α- and β-haemolysis reactions when this strain was inoculated onto Blood Agar Base containing 7% (v/v) of factor 0 Rh (-) human blood.

Vancomycin susceptibility test

Enterococcus faecalis DBFIQ E24 is sensitive to the glycopeptide antibiotic vancomycin because the minimal inhibitory concentration (MIC) value was below 2 μg/mL. For considering a strain to be resistant to this antibiotic, MIC value has to be above 32 μg/mL, when the cited assay method is employed.

Gelatin hydrolysis

No gelatin-degradative activity was shown by *E. faecalis* DBFIQ E24 strain after the application of the agar plate methodology.

Direct detection of bacteriocinogenic activity

Figure 4 shows the results obtained after the activity assay was performed in a discontinuous gradient polyacrylamide gel employing a 33-fold-concentrated CFS against *B. cereus* DBFIQ B28 as target strain. In addition, the detection of antimicrobial activity was also performed with *E. coli* DBFIQ Ec9 as a sensitive strain, which showed similar results to *B. cereus* DBFIQ B28 (data not shown). The antagonist effect achieved was bacteriocin-specific, because other sections of polyacrylamide gel did not produce any inhibition effect in the target strains. Furthermore, before the application of the gel slab to the Petri dish, the gel was extensively washed during 30 min to assure an antibacterial activity-specific effect. Likewise, from Figure 4, it can be observed that the band with antibacterial activity against the target strains was between 1060-Da- and 3500-Da-molecular-weight markers, confirming the previous results obtained through

Table 1 Purification protocol for antibacterial peptides from CFS of *E. faecalis* DBFIQ E24 strain

Fraction	*Volume (mL)*	*Total Activity (AU)*[a]	*Total protein (mg)*[b]	*Specific Activity (AU/mg)*	*Yield (%)*	*Purification fold*[d]
10-fold-concentrated CFS	100	26 600	8900	3	100	1
0.1 M $AcNH_4$ SP-Sepharose	2	532	17.4	30.6	2	10.2
20% ACN C_{18} SPE	2	66	3	22	0.3	7.3
RP-HPLC fraction I[c]	0.5	33	0.5	66	0.12	22
RP-HPLC fraction IV[e]	0.5	33	0.1	330	0.12	110

[a]Total activity was expressed as the titre of the corresponding fraction multiplied per total volume. Titre, in AU/mL, was defined as the reciprocal of the maximum serial two fold dilution that generated an inhibition zone of 7.5 mm (employing the agar diffusion method), divided by the sample volume tested (in mL). The diameter of the wells (7 mm) was subtracted from the inhibited zone. The target strains employed for bacteriocin detection were *B. cereus* DBFIQ B28 and *E. coli* DBFIQ Ec9.

[b]Protein concentration was determined by the bicinchoninic acid method.

[c]Fraction active against *E. coli* DBFIQ Ec9.

[d]Purification fold is the number of times that target antibacterial peptide was purified in relation to the concentrated CFS.

[e]Fraction active against *B. cereus* DBFIQ B28.

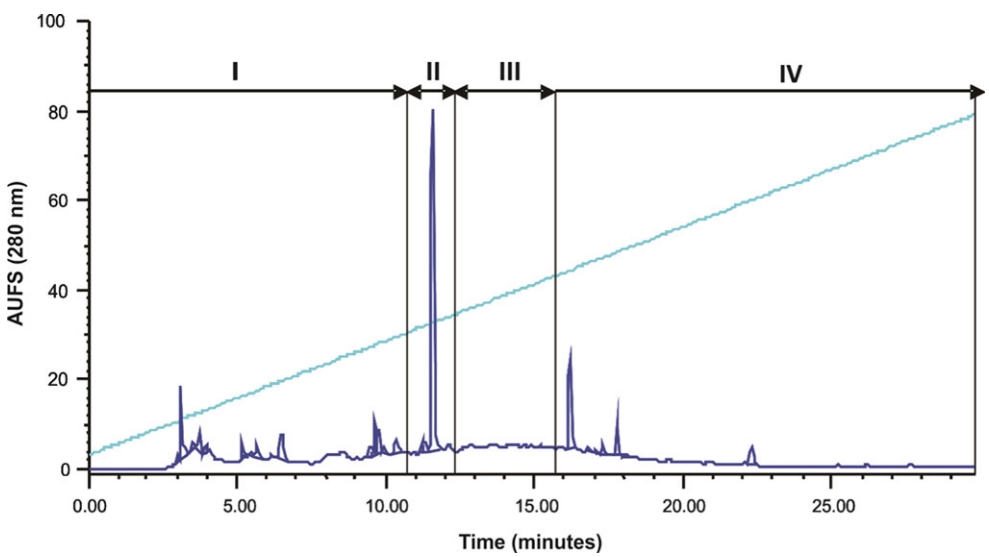

Figure 2 RP-HPLC chromatographic profile of 20% ACN-eluted fraction from C_{18} SPE. UV detection was performed at 280 nm, and four fractions were collected: I (from 0.00 to 10.60 min); II (from 10.60 to 12.16 min); III (from 12.16 to 15.60 min); and IV (from 15.60 to 30.00 min). The continuous straight line indicates acetonitrile gradient from 5% (v/v) at the beginning to 80% (v/v) at the end of the run.

apparent molecular weight determination employing ultrafiltration and dialysis techniques (Cardoso *et al.* 2012), which achieved a value <2000 Da.

Mass spectrometry

MALDI-TOF results of the four RP-HPLC fractions are shown in Figure 5. Analyses of mass spectra revealed the presence of a main peak in the active fraction I with m/z: 1686 Da. Fractions II and III, which turned out to be nonactive against target strains assayed, showed many peaks under and above 2000 and 1000 Da, respectively. Finally, fraction IV showed the presence of many peaks of interest with m/z: 1073 Da; 1187 Da; and 1364 Da. However, the first two peaks were not considered because both were also present in the nonactive fraction III. Mass spectra of fraction III and fraction IV, as can be seen in Figure 5, possess the same peptide profile, although different intensity in each peptide, with exception of 1364-Da-molecular-weight peptide. Moreover, fraction IV is an active fraction mainly against *B. cereus* DBFIQ B28, whereas fraction III was not active against target strain assayed. For this reason, activity was attributed to the presence of these distinctive peptides in fraction IV. In this way, two bacteriocins were detected: one with a

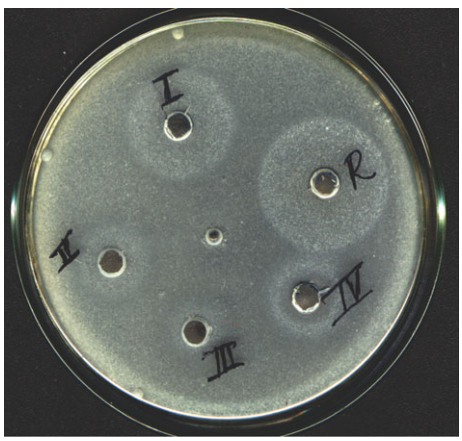

Figure 3 Antibacterial assay of RP-HPLC fractions obtained against *B. cereus* DBFIQ B28 employing the agar-well diffusion method. Diameter of wells: 7 mm. Fractions I and IV were the only active fractions against the two strains assayed. R (from residue) fraction is formed by nonretained samples during HPLC chromatographic process owing to the aggregation process that these bacteriocins underwent? The picture is only representative of the results achieved by the agar-well diffusion method. Each sample was assayed in triplicate.

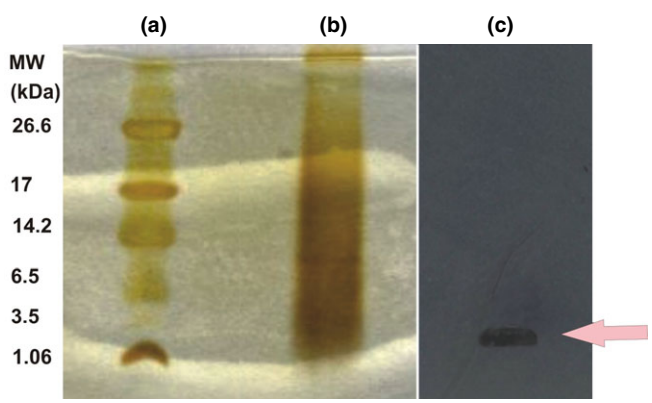

Figure 4 Direct detection of antimicrobial activity from concentrated CFS employing the methodology described by Bhunia and Johnson (1992). Bacteriocin's molecular weight was estimated using electrophoresis ultralow molecular weight marker (5 μL/well) from Sigma, which include the following proteins: triosephosphate isomerase from rabbit muscle: 26.6 kDa; myoglobin from horse heart: 17 kDa; α-lactalbumin from bovine milk: 14.2 kDa; aprotinin from bovine lung: 6.5 kDa; insulin chain B, oxidised, bovine: 3496 kDa; bradykinin: 1.06 kDa (a). For SDS-PAGE analysis, 5 μL of partially purified CFS sample per well were applied and gel was silver-stained according to Blum et al. (1987) (b). On the other hand, for direct detection of antimicrobial activity, 25 μL of 33-fold-concentrated cell-free supernatant were used where *B. cereus* DBFIQ B28 was employed as target strain; the arrow at the bottom right corner of the Figure indicates a band with antimicrobial activity (c).

molecular weight of 1 686 Da present in fraction I, and the other one with a molecular weight of 1 364 Da, detected in fraction IV.

The contributions of enterococci to either food organoleptic properties or the ability to produce bacteriocins are major characteristics for their application in food technologies, but it is essential to evaluate GRAS status of micro-organisms. Hence, the evaluation of haemolytic activity and antibiotic resistance, in particular vancomycin sensitivity, is essential to employ an enterococci strain either in food industry or as veterinary product manufacture, as a bacteriocinogenic GRAS bacterium.

First of all, γ-haemolysis reaction (no haemolysis) was detected when *E. faecalis* DBFIQ E24 was grown in Blood Agar Base containing 7% (v/v) factor 0 Rh(-) human blood. This result has reinforced the potential technological application either for this strain or its cell-free supernatant. Similar results were found by Sabia *et al.* (2008) and De Vuyst *et al.* (2003).

On the other hand, the emphasis has been focused on vancomycin resistance because it is considered the last treatment of both multiple-resistant Gram (+) and enterococcal infections usually causing nosocomial bacteremia, endocarditis and related illnesses (Houben 2003).

Minimal inhibitory concentration (MIC) achieved for this glycopeptide antibiotic was 2 μg/mL, which indicates that *E. faecalis* DBFIQ E24 strain is antibiotic sensitive. Cut-off MIC value must be placed above 32 μg/mL, to establish an enterococcus as a vancomycin-resistant strain (Sabia *et al.* 2008). Other scientific reports have also informed the detection of a very low percentage of vancomycin-resistant enterococci strains (De Vuyst *et al.* 2003; Sabia *et al.* 2008).

Furthermore, no gelatinase activity was measured in *E. faecalis* DBFIQ E24 strain. Gelatinase is an extracellular metalloendopeptidase that acts on gelatin, casein, haemoglobin, collagen and bioactive peptides. It has been shown that this enzyme was produced by an elevated percentage of pathogenic *E. faecalis* strains isolated from hospitalised patients and also demonstrated an increased pathogenicity trait in an animal model (Singh *et al.* 1998), suggesting that enterococci proteases also contribute to virulence. The best way to evaluate whether this enzyme activity is present in enterococci cells is through agar plate methodology because although *gelE* gene could be present in the genome, it is not always expressed (Eaton and Gasson 2001).

Due to the heterogeneity of bacteriocins, it is almost impossible to standardise a unified purification protocol, so it must be empirically designed according to the characteristics of the obtained cell-free supernatant. For instance, Guyonnet *et al.* (2000) purified the bacteriocins produced by *Lactobacillus sakei* 2675 and 2525 employing the same purification protocol, achieving a recovery percentage of 10% and 50% respectively. Besides, an active peptide produced by *E. faecalis* 336 strain was purified with a 66% recovery using the same purification protocol.

On the other hand, bacteriocins are present in extremely low quantities so that recovery percentages after purification

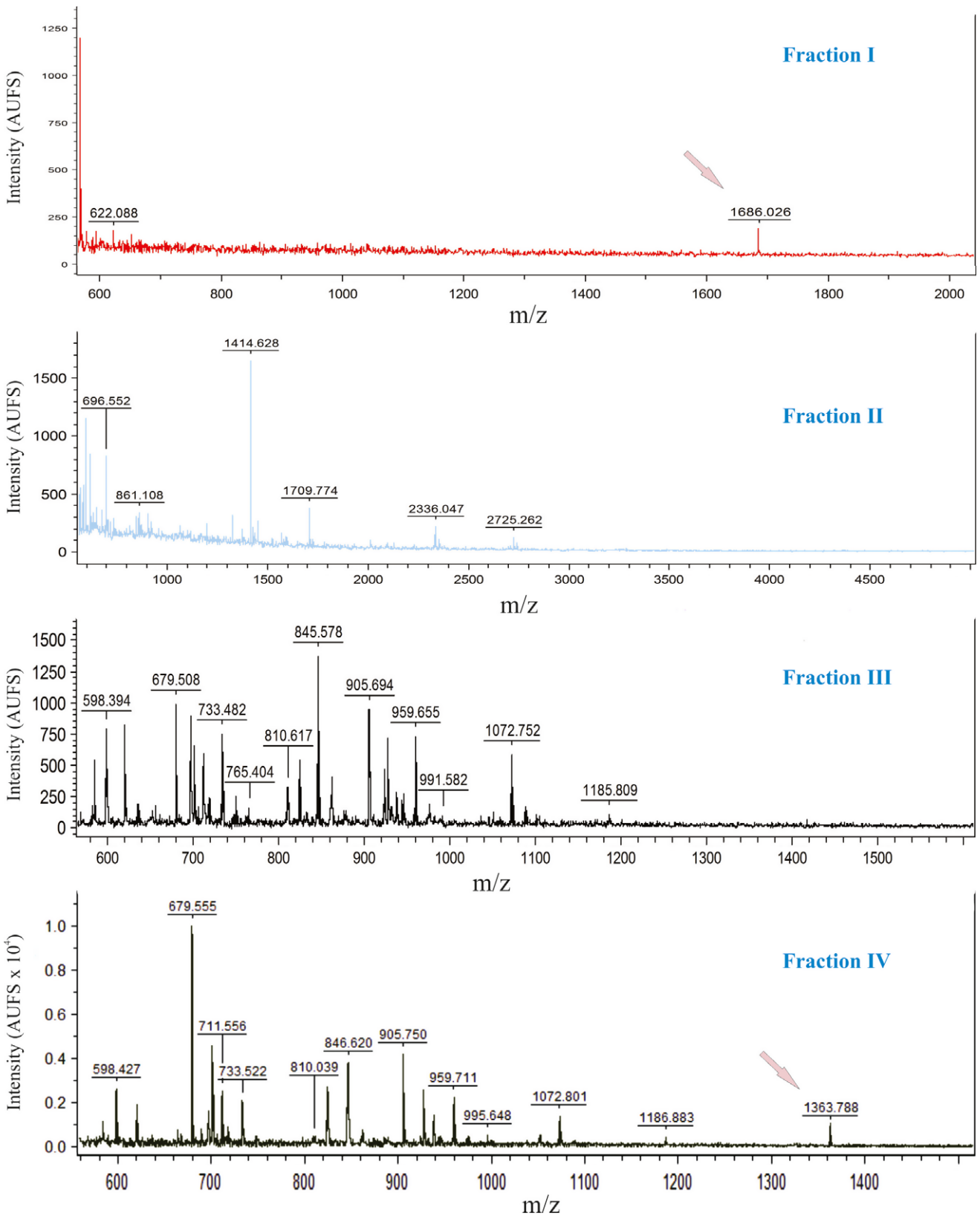

Figure 5 MALDI-TOF mass spectrometry analyses of the four fractions obtained from RP-HPLC. Arrows indicate the peptides with antibacterial activity.

must be high and purification protocols must be efficient (Guyonnet *et al.* 2000).

In this research, after SP-Sepharose chromatography, a small increase in specific activity and the level of purification was seen but recovery was low (2%); these results are in agreement with those reported by Herranz *et al.* (1999), who obtained 1.6% and 3.8% yield for *E. faecium* AA13 and *E. faecium* G16 bacteriocins purification, respectively. Also, an 86 and 194 AU/mg specific activity values were obtained, being much higher than those found in the present report. Furthermore, Aktypis *et al.* (1998) obtained a 6% yield and a specific activity value of 32 AU/mg after DEAE–cellulose chromatography of thermophilin T from *Streptococcus thermophilus* ACA-DC 0040 after their purification process.

After the analysis of the applied purification protocols reported here, a constant loss of activity was observed being probably attributed to the formation of bacteriocin complexes with low retention by the different chromatographic columns and thus was observed in the washout fractions (Aktypis *et al.* 1998; Motta *et al.* 2007). Besides, the detected peptides may not be the only antimicrobial compounds present in CFS, as reported by Cardoso *et al.* (2012), where hydrogen peroxide, short-chain fatty acids and low molecular weight modified peptides, mainly lipopeptides, were also present in crude CFS.

Acidocin CH5 purification from *Lactobacillus acidophilus* CH5 employing SP-Sepharose ion-exchange chromatography as a purification step revealed a 4% recovery and a purification fold of 66 times in comparison with initial specific activity (Chumchalová *et al.* 2004). Furthermore, utilisation of carboxymethyl-Sephadex CM-25 ion-exchange chromatography for antimicrobial substances purification from *Enterococcus faecalis* MRR 10-3 achieved a 56.05% recovery and a purification fold of 0.79 times (Martín-Platero *et al.* 2006). Moreover, after CFS application in a SP-Sepharose Fast Flow column, a recovery of 61% and a 106 times purification fold were obtained for enterocin P from *E. faecium* P13 purification protocol, as described by Cintas *et al.* (1997).

There are a myriad of bacteriocin purification protocols however, for a successfull purification, strategies must be selected and optimized for each antimicrobial peptide taking into account the physicochemical properties that each bacteriocin, in particular, possess. In the current study, our data showed that, after the C_{18} SPE purification step, two active fractions were obtained, one eluted in the washout fraction and the other one eluted with 20% ACN. This would suggest the presence of two antimicrobial substances with different hydrophobicity but with similar molecular weight, as indicated in the direct detection of antimicrobial activity assay and in the ultrafiltration results previously reported by Cardoso *et al.* (2012).

Martín-Platero *et al.* (2006) reported a 4.01% recovery yield and a 300 times purification fold after C_{18} SPE was used in the purification of the enterocins MR10A and MR10B. The authors suggested that the low recovery could be explained considering the conformational change in the bacteriocin structure, as a result of the action of organic solvents during the chromatographic process. These results are not in agreement with the present research, where a decrease in both parameters was observed. Additionally, an increase in biological activity after hydrophobic interaction chromatography was observed by many authors, suggesting that this purification process could lead to the elimination of inhibitors of bacteriocinogenic activity as reported for enterocin P (Cintas *et al.* 1997) and enterocin A (Casaus *et al.* 1997).

Other researchers have also obtained scarce yields after the application of enterocin's purification protocols. For instance, Herranz *et al.* (1999) found a low recovery yield after hydrophobic interaction chromatography (3.8% and 5.9%) was used for the purification of enterocins from *E. faecium* AA13 and *E. faecium* G16, respectively. The low retention of the antimicrobial peptides could be partially explained due to their natural hydrophobic characteristics which allow them to form macromolecular aggregates with a hydrophobic centre, thus exposing the hydrophilic zones towards elution solvent and, finally, not being retained in the chromatographic column (Aktypis *et al.* 1998; Motta *et al.* 2007). Aggregation is a common characteristic of bacteriocins and was proved for the bacteriocins produced by *E. faecalis* DBFIQ E24, because active fractions with a molecular weight larger than 3000 Da after the ultrafiltration analysis were obtained. Furthermore, the effect of certain surfactants promoted bacteriocin disaggregation, but their addition to CFS was not used in this work reported here because they tend to interfere in the antimicrobial activity assay (Cardoso *et al.* 2012).

Previous data reported in literature show that most of bacteriocins are hydrophobic peptides, preferentially active against Gram (+) bacteria (Pascual *et al.* 2008). However, after reversed-phase HPLC analysis, fractions I and IV turned out to be antimicrobial towards a Gram (-) and Gram (+) bacterium, respectively, revealing the existence of two different bacteriocins in the same CFS.

After the purification of active substances from *E. faecalis* DBFIQ E24 CFS, two bacteriocins were obtained. The more hydrophilic bacteriocin, with a molecular weight of 1 686 Da, was present in fraction I, which was preferentially active against *E. coli* DBFIQ Ec9 target strain, and the second one, a more hydrophobic peptide, with a molecular weight of 1 364 Da was present in fraction IV being mostly active against *B. cereus* DBFIQ B28 strain. In addition, an increase in antimicrobial activity was observed when the two separated fractions were combined, suggesting a synergistic action between these two antimicrobial peptides (data not shown). This difference in hydrophobicity has been explained by the disparity in elution profile between these two bacteriocins, where the fraction I was eluted between 5

and 25% of ACN as mobile phase and the fraction IV was recovered in the 40-70% ACN range. This behaviour partially explains their dissimilar antimicrobial spectra in direct relation to the functional unstabilisation of cytoplasmatic membranes of target cells owing to the hydrophobic interactions between the antimicrobial peptide and the membrane.

On the other hand, most of enterocins described in literature up to date have low molecular weights, between 2 and 8 kDa, in agreement with enterocins purified in this research.

Bacteriocin action is preferentially performed in outer cell membrane structures through proton-motive force dissipation, the depletion of essential molecules for target bacteria and the inhibition of the peptidoglycan synthesis, finally leading to cell death (Hasper *et al.* 2006). Because this force can be divided in two components, the hydrophilic peptide could exert its action against Gram (-) bacteria eliminating the membrane potential through a feasible electrostatic interaction with proteins similar to porines (e.g. the iron-chelator transporters) which enter into the periplasm and act on the inner membrane (Pagès *et al.* 2008) but not altering the internal pH gradient. This contributes to a diminishing protection of the lipopolysaccharide (LPS) membrane and increasing cell membrane permeability, finally causing cell death (Javed *et al.* 2011).

However, a more hydrophobic peptide could achieve both effects and consequently cell death, owing to a significant reduction of intracellular ATP and the inability to maintain intracellular K^+ and Mg^{2+} concentration, being more effective against Gram (+) bacteria, which possess a developed peptidoglycan cell wall.

Previous results performed with *E. faecalis* DBFIQ E24 CFS have demonstrated a bactericidal and a lytic mode of action against *B. cereus* DBFIQ B28 and *E. coli* DBFIQ Ec9 strains (Cardoso *et al.* 2012). This could be due to a combined action between the two active peptides, which synergistically act towards both bacterial strains additionally to the probable presence of certain chelating substances. However, these conclusions need to be verified.

Although activity of bacteriocins against Gram (−) bacteria is well known, their mode of action remains unidentified. However, the mode of action of sakacin C2, a novel broad-spectrum bacteriocin produced by *Lactobacillus sakei* C2 against *E. coli* ATCC 25922, was determined (Gao *et al.* 2011). The authors pointed that the mode of action of sakacin C2 is mainly bactericidal by causing the inhibition of the cell growth and depolarisation of the transmembrane electrical potential ($\Delta\Psi$). The dissipation of $\Delta\Psi$ may favour the insertion of sakacin C2 into the cell membrane, causing the pore formation, which finally leads to complete cell disintegration. This scientific report was the first that explained the mode of action of a bacteriocin against Gram (-) bacteria. In our case, the bacteriocin isolated from fraction I is also active against *E. coli*, despite the fact that its mode of action remains to be verified.

It was demonstrated that the addition of lipase to an *E. coli* MTCC118 suspension synergised enterocin lethal action by altering its mode of action. Although lipase by itself does not possess an antibacterial effect, it may improve enterocin action through the production of pores on the cell surface and subsequent leakage of intracellular components, finally causing cell death (Ramakrishnan *et al.* 2012). As a corollary to this, *E. faecalis* DBFIQ E24 has been previously characterised as a strongly lipolytic strain (Carrasco *et al.* 1992).

N-terminal amino acid sequences of active peptides from fractions I and IV were assayed by automated Edman degradation using a gas-phase model 470A sequencer/model 120A phenylthiohydantoin analyser (Applied Biosystems). However, sequencing was not possible, probably because the N-terminal end was blocked. This result may indicate the presence of modified amino acids, as a result of post-translational modifications (Blom *et al.* 1999). In this sense, according to the classification provided by Klaenhammer (1993), both bacteriocins would belong to class II due to their thermostability, activity at wide pH range and low molecular weight as previously reported by Cardoso *et al.* (2012). In addition, because both enterocins were characterised in the same cell-free supernatant, where a possible synergistic action could be present, bacteriocins would belong to class IIc or IId.

CONCLUSIONS

E. faecalis DBFIQ E24 proved to be an interesting *Enterococcus* strain with promising technological properties owing to its ability to produce many antimicrobial substances active against fungi, yeasts and bacteria, as previously reported by Cardoso *et al.* (2012). In this report, the enterococci strain was identified by genetic techniques, and the absence of an overt virulence activity was determined, allowing it to be considered as a GRAS strain, which would suggest that the analysed enterococcal strain may be regarded as safe and could be recommended for its utilisation in food biopreservation.

Moreover, the capacity to produce bacteriocins has been described by identifying two active peptides with similar molecular weight and physicochemical properties but with different antimicrobial spectra, where enterocin from RP-HPLC fraction I was mainly active against *E. coli*, being one of the few characterised that inhibited a Gram (−) bacterium.

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