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# Antibacterial potential of *Enterococcus faecium* strains isolated from ewes' milk and cheese

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ABSTRACT

The study was conducted to evaluate the antibacterial activity of three bacteriocin-producing *Enterococcus faecium* strains (TW15, TW20 and TW22) isolated from ewes' milk and cheese sampled in the Patagonian region of Argentina. The strains were tested against spoilage and pathogens microorganisms showing antimicrobial activity towards 4 strains of *Listeria monocytogenes*, one strain of *Listeria innocua* and 2 strains of *Staphylococcus aureus*. *E. faecium* TW15, *E. faecium* TW20 and *E. faecium* TW22 were sensitive to vancomycin. Furthermore, investigation of virulence factors revealed the absence of the genes encoding them. The bacteriocin-like substances (BLISs) produced by the 3 strains were thermostable, pH resistant and can be expressed even in the presence of NaCl (3.0 g/100 g). Moreover, they prove to have a bactericidal mode of action. Results from physicochemical and biochemical characteristics of BLIS produced by these *E. faecium* strains make them potential candidates to aid in preservation of foods.
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## 1. Introduction

Lactic acid bacteria (LAB) are considered as food grade organisms that are safe to consume, having a long history of use in food. Due to their ability to produce several antimicrobial substances, including bacteriocins, they have the potential to be used in biopreservation. A number of bacteriocins with a spectrum of activity covering several target bacteria have been cited (Cocolin, Foschino, Comi, & Fortina, 2007; Todorov, Ho, Vaz-Velho, & Dicks, 2010), many of them being effective against *Listeria monocytogenes*. Investigation of bacteriocins which inhibit pathogens is becoming attractive for the food industry to be used as food additives.

Among LAB, the members of the genus *Enterococcus* are found in many food products, especially those from animal origin, such as dairy products (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). They are most frequently present in many traditional European cheeses prepared in the Mediterranean countries, mostly from raw ewes' or goats' milk, playing an important role in the ripening of these cheeses (Manolopoulou et al., 2003). Enterococci have the ability to produce bacteriocins, the so-called enterocins. Moreover, they are used in some countries as probiotics (Franz, Stiles, Schleifer, & Holzapfel, 2003). Although enterococci have a long history of use as artisanal cultures for preparation of various types of cheeses (Izquierdo, Marchioni, Aoude-Werner, Hasselmann, & Ennahar, 2009; Manolopoulou et al., 2003), sausages (Giraffa, 2002; Hugas, Garriga, & Aymerich, 2003) and olives (Ben Omar et al., 2004), they are sometimes associated with pathogenicity (Khan, Flint, & Yu, 2010). They have been reported to be the cause of endocarditis, bacteraemia, and several infections, as well as of multiple antibiotic resistances (Franz, Holzapfel, & Stiles, 1999; Kayser, 2003). Several virulence factors have been described (Jett, Huycke, & Gilmore, 1994) and the number of antibiotic-resistant enterococci (ARE), especially vancomycin-resistant enterococci (VRE), is increasing. However, when enterococcal isolates from various sources are tested for virulence factors, it has been found that incidence of virulence and pathogenicity is highest amongst clinical isolates (Mannu et al., 2003). Consequently, De Vuyst, Foulquié Moreno, and Revets (2003) concluded that enterococcal strains lacking haemolytic activity and not carrying cytolysin nor vancomycin resistance genes may be regarded as safe and can be used as starter cultures, co-cultures, or probiotics.

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On the assumption that a specific bacteriocin will have its own unique properties and usefulness in targeting microbial pathogens, isolation and purification of new bacteriocins will always prove beneficial (Osmanağaoğlu, 2007). This study was conducted to isolate and identify bacteriocin-producing enterococci from ewes' milk and cheese, as well as to characterize bacteriocin-like substances produced by them. The bacteria or their purified bacteriocin would ultimately be used to improve the hygiene and safety of food products.

## 2. Materials and methods

### 2.1. Sampling

Ewes' milk samples were obtained by mechanical milking of animals belonging to dairy farms located in Chubut (Argentine Patagonia). Samples were carried to the laboratory at 4 °C and then they were kept frozen at −30 °C until processed. Ewes' milk cheese is made in an artisanal fashion without using commercial starter cultures. Cheese samples were taken with a sterile cork borer (diameter: 8 mm) and were processed within 4 h of sampling.

### 2.2. Enterococci isolation and identification

Samples from milk and cheese were grown in azide-purple bromocresol broth with NaCl (6.5 g/100 ml). After incubation at 35 °C for 48 h, cell cultures were seeded in de Man Rogosa Sharpe (MRS) agar (Biokar Diagnostics, France) supplemented with NaCl (6.5 g/100 ml) (Anedra, Argentina), 40 μg/ml nalidixic acid (Sigma Aldrich, USA) and 10 μg/ml cycloheximide (Sigma Aldrich, USA). Predominant colony types were picked off the modified MRS agar and purified by streaking out on MRS. Isolates were maintained as frozen stocks in skimmed milk (20 g/100 ml) and glycerol (10 g/100 ml) at −30 °C.

The following tests were carried out for presumptive identification of the isolates: observation of colony characteristics and cell morphology, Gram staining, catalase and oxidase production, growth at 10 °C and 45 °C, growth in the presence of NaCl (6.5 g/100 ml) and at pH 9.6, as well as growth and esculin hydrolysis on bile-esculin agar (BEA) (Schleifer & Kilpper-Bälz, 1984). The pyrrolidonyl aminopeptidase activity was also tested with a commercial kit (Pyrrolidonyl peptidase strips; BioChemika, Sigma Aldrich, USA) following the manufacturer specifications.

Those colonies belonging to the *Enterococcus* genus were identified to species level by means of sugar fermentation according to the Manero and Blanch (1999) scheme.

### 2.3. Screening for antagonistic activity

#### 2.3.1. Bacterial strains and growth conditions

The bacterial strains used as indicator microorganisms as well as the conditions for growth are listed in Table 1. In all the cases, the indicator strains and the isolated *Enterococcus* strains, kept as frozen stocks in the suitable medium, were propagated twice in the appropriate culture media before use.

#### 2.3.2. Detection of inhibitory activity

Cell-free culture supernatants (CFSs) obtained by centrifugation of overnight cultures at 8000 *g* at 4 °C for 10 min were adjusted to pH 7.0 with 0.5 mol/l NaOH and heated at 100 °C for 5 min in order to inactivate endogenous proteases. Afterwards, they were filtered through 0.22 μm pore size cellulose acetate filter (Sartorius, Goettingen, Germany) and stored at −20 °C until use. The antimicrobial activity of the cell-free neutralized culture supernatants was determined by the agar well diffusion assay (AWDA) (Schillinger & Lücke, 1987). The supernatant (50 μl) was placed in wells (6 mm in diameter) cut in MRS, BHI, or Blood agar plates (15 ml) seeded with a stationary phase cell suspension of the indicator microorganisms (1 ml/100 ml) listed in Table 1. The plates were incubated at 30 or 35 °C for 24 h, with the exception of *Brochothrix thermosphacta* strains which were incubated at 25 °C for 48 h. The diameters of the zones of growth inhibition were then measured with a micrometre (Starrett, USA).

### 2.4. Determination of virulence factors

#### 2.4.1. Assay of haemolytic activity

Production of haemolysis was determined by streaking the *Enterococcus* cultures (grown in MRS broth for 18 h) in Brain Heart Infusion (BHI) agar plates supplemented with 50 g/100 ml human blood. After incubation at 37 °C for 24 h under aerobic conditions, plates were examined for haemolysis. Presence of zone of clearing around the colonies was interpreted as β-haemolysis.

#### 2.4.2. Assay of gelatinase activity

Production of gelatinase was tested on BHI agar plates containing 10 g/l peptone and 30 g/l gelatine. After overnight incubation at 37 °C, the plates were placed at 4 °C for 5 h before examination for zone of turbidity around the colonies indicating hydrolysis of gelatine.

#### 2.4.3. Vancomycin resistance

Minimum inhibitory concentration (MIC) was determined by the dilution method described by Sahm and Washington II (1992).

#### 2.4.4. PCR for the detection of virulence factors

Primers used for the amplification of genes *agg* (aggregation substance gene), *gelE* (coding for gelatinase), *cylB* (transport of cytolysin gene) and *cpd* (sex pheromones gene) as well as the general PCR conditions were those described by Eaton and Gasson (2001) and are reported in Table 2.

### 2.5. Genetic identification of bacteriocin-producing strains

Bacterial isolates from which the CFSs maintained their antibacterial activity throughout the characterization of antibacterial compounds were selected for genetic identification. Genomic DNA from the strains was extracted from overnight Tripticase Soy Broth (TSB) (Difco, USA) cultures using the kit (Wizard Genomic, Promega, Wisconsin) following manufacturer's recommendations. The 16S rRNA gene sequence (corresponding to positions 27-1492 in the *Escherichia coli* gene) was PCR amplified as described by DeLong (1992), using a DNA thermal cycler Multigene Gradient (Labnet International Inc., Woodbridge, NJ). Sequencing on both strands of PCR-amplified fragments was performed using the dideoxy chain termination method by the commercial services of Macrogen Inc. (Seoul, Korea). The 16S rRNA homology searches against the NCBI database were carried out using BLAST program (Altschul, Gish, Miller, Myers, & Lipman, 1990).

### 2.6. PCR detection of enterocin structural genes

PCR amplification for structural enterocin A, B, P, LB50A and LB50B genes was performed with the specific primers listed in Table 3 using the conditions described by De Vuyst et al. (2003).

### 2.7. Characterization of the antimicrobial activity

#### 2.7.1. Thermal stability

In all the instances described along this point, the remaining activity of the CFSs after each treatment was determined by the

**Table 1**
Culture conditions and antimicrobial activities of cell-free supernatants from *Enterococcus faecium* TW 15, *E. faecium* TW 20 and *E. faecium* TW 22.

| Indicator strains | Growth media and temperature (°C) | Antimicrobial activity | | |
|---|---|---|---|---|
| | | *E. faecium* TW 15 | *E. faecium* TW 20 | *E. faecium* TW 22 |
| *Listeria innocua* ATCC 33090 | BHI, 30 | + | + | + |
| *L. monocytogenes* ATCC 7644 | BHI, 35 | + | + | + |
| *L. monocytogenes* TW 35 | BHI, 35 | + | + | + |
| *L. monocytogenes* TW 5 | BHI, 35 | + | + | + |
| *L. monocytogenes* TW 11 | BHI, 35 | + | + | + |
| *Bacillus subtilis* DBFIQ Bs | Blood agar, 35 | − | − | − |
| *B. megaterium* DBFIQ Bm | Blood agar, 35 | − | − | − |
| *B. cereus* DBFIQ Bc | Blood agar, 35 | − | − | − |
| *Lactococcus lactis* ssp. *cremoris* TW CM6 | MRS, 30 | − | − | − |
| *Lc. lactis* ssp. *lactis* TW CM7 | MRS, 30 | − | − | − |
| *Lactobacillus plantarum* CRL 691 | MRS, 30 | − | − | − |
| *Brochothrix thermosphacta* ACU 396 | BHI, 25 | − | − | − |
| *Br. thermosphacta* ACU 405 | BHI, 25 | − | − | − |
| *Staphylococcus aureus* ATCC 25923 | BHI, 35 | − | − | − |
| *S. aureus* TW 1 | BHI, 35 | − | − | − |
| *S. aureus* ATCC 6538 | BHI, 35 | + | + | + |
| *S. aureus* ATCC 29213 | BHI, 35 | − | − | − |
| *S. aureus* FBUNT | BHI, 35 | + | + | + |
| *Escherichia coli* ATCC 25922 | BHI, 35 | − | − | − |
| *E. coli* TW 1 | BHI, 35 | − | − | − |
| *E. coli* O157:H7 | BHI, 35 | − | − | − |
| *E. coli* FBUNT | BHI, 35 | − | − | − |
| *Salmonella* sp. TW 18 | BHI, 35 | − | − | − |
| *Shigella flexneri* TW 19 | BHI, 35 | − | − | − |
| *Pseudomonas aeruginosa* FBUNT | BHI, 30 | − | − | − |

Positive signs (+) indicate antimicrobial activity. Negative signs (−) indicate no antimicrobial activity.
TW: Universidad Nacional de la Patagonia San Juan Bosco, Trelew (Chubut, Argentina). DBFIQ: Food Engineering Department, Faculty of Chemical Engineering, Universidad Nacional del Litoral (Santa Fé, Argentina). CRL: Centro de Referencia de Lactobacilos, CERELA (CONICET) (Tucumán, Argentina). ACU: Austral Chaco University (Chaco, Argentina).

AWDA with *Listeria innocua* ATCC 33090 as indicator strain. A positive control, consisting of freshly prepared extracellular extract, was tested in parallel.

To test for heat sensitivity, neutralized CFSs were heated to 100 and 121 °C for 5, 15 and 30 min. Immediately after each treatment, samples were cooled under refrigeration and residual activity was determined.

To test the stability of CFSs during three freeze–thaw cycles, they were frozen at −30 °C during 24 h and thawed for 20 min at 4 °C. The remaining activity was then assayed.

The effect of extended storage at low temperature on bacteriocin-like substances (BLISs) stability was also evaluated by placing CFSs in an incubator device at 4 °C for 7, 14 and 21 days. CFSs were consequently tested for residual activity.

#### 2.7.2. Effect of pH on bacteriocin activity

To check the pH stability of BLIS, 1 ml portions of the CFSs of the selected strains, obtained as described above, were adjusted to pH values of 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5 by adding the appropriate volumes of 4 mol/l HCl or 4 mol/l NaOH. The pH values were measured by means of a glass electrode attached to a pH meter (Oakton®, Eutech Instruments, Singapore). Then, the samples were sterilized by filtration through 0.22 μm (Sartorius, Germany), incubated for 2 h at 30 °C and antimicrobial activity was determined by the AWDA described above. Negative controls, aimed at elucidating the possible role of acid pH values in the inhibition of *L. innocua* ATCC 33090 and *Staphylococcus aureus* FBUNT, were prepared by testing portions of non-inoculated MRS broth whose pH values were adjusted to 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5. CFSs from the bacteriocin-producer *Lactobacillus plantarum* ATCC 8014 was used as positive control (Lash, Mysliwiec, & Gourama, 2005).

#### 2.7.3. Enzyme sensibility

The inhibitory substances produced by the isolated strains were tested for susceptibility to trypsin, pepsin, bromeline and pronase (1 mg/ml). Overnight cultures were centrifuged at 8000 *g* at 4 °C for 10 min. While the rest of the supernatants were used as crude extracts, CFSs for trypsin and pronase were adjusted to pH 7.5 with 0.5 mol/l NaOH. The samples were incubated for an hour at each optimum enzyme temperature (25 °C: trypsin and bromeline;

**Table 2**
PCR amplification of potential enterococcal virulence factors.

| Gen | Primer | Sequence (5′–3′) | Size (bp) |
|---|---|---|---|
| *agg* | TE3 | AAGAAAAAGAAGTAGACCAAC | 1553 |
| | TE4 | AAACGGCAAGACAAGTAAATA | |
| *gelE* | TE9 | ACCCCGTATCATTGGTTT | 419 |
| | TE10 | ACGCATTGCTTTTCCATC | |
| *cylB* | TE15 | ATTCCTACCTATGTTCTGTTA | 843 |
| | TE16 | AATAAACTCTTCTTTTCCAAC | |
| *cpd* | TE51 | TGGTGGGTTATTTTTCAATTC | 782 |
| | TE52 | TACGGCTCTGGCTTACTA | |

**Table 3**
Specific terminal primers for the PCR detection of enterocin structural genes.

| Enterocin | Forward primer | Reverse primer |
|---|---|---|
| A | 5-GGT ACC ACT CAT AGT GGA AA-3 | 5-CCC TGG AAT TGC TCC ACC TAA-3 |
| B | 5-CAA AAT GTA AAA GAA TTA AGT ACG-3 | 5-AGA GTA TAC ATT TGC TAA CCC-3 |
| P | 5-GCT ACG CGT TCA TAT GGT AAT-3 | 5-TCC TGC AAT ATT CTC TTT AGC-3 |
| LB50A | 5-ATG GGA GCA ATC GCA AAA TTA-3 | 5-TTT GTT AAT TGC CCA TCC TTC-3 |
| LB50B | 5-ATG GGA GCA ATC GCA AAA TTA-3 | 5-CCT ACT CCT AAG CCT ATG GTA-3 |

37 °C: pronase and pepsine). The remaining activity after each treatment was determined with the AWDA, as described above.

The sensibility of the CFSs to the degradative enzymes lysozyme and lipase was also tested. In this case, the CFSs were neutralized to pH 7.2 with 0.5 mol/l NaOH, treated with each enzyme and incubated for an hour at 25 °C (lysozyme) and 37 °C (lipase). The remaining antimicrobial activity was tested as described previously.

### 2.8. Bacteriocin-like substances production

#### 2.8.1. Kinetics of growth and BLIS biosynthesis

The time of incubation at which the selected strains exhibited the maximum BLIS production was determined as follows: sterile flasks containing 50 ml of MRS broth were inoculated with 1 ml/100 ml BLIS-producing strain and incubated at 30 °C. Then, 1-ml aliquots were retrieved after 0, 1.5, 3, 4.5, 6, 9, 12, 15, 24 and 36 h and population growth was monitored by determining optical density (OD) at 600 nm. The production of bacteriocin along the bacterial growth was monitored at 0, 3, 6, 9, 12, 24 and 36 h. The OD determination was performed in a Beckman Spectrophotometer (Beckman Instruments, modo DU-640B, Fullerton CA, USA). BLIS production was determined from 3 h of storage by the critical dilution method and the AWDA previously described. Arbitrary units (AU) per ml were calculated as $AU = (1000/v)/d$; being v: volume seeded in the well and d: dilution (Kouakou et al., 2009).

#### 2.8.2. Influence of NaCl concentration

The growth of, and BLIS production by, the collected strains was assessed using MRS broth containing 3.0 or 6.0 g/100 g sodium chloride (Anedra, Argentina). The salt was added to MRS broth, the pH was adjusted to $6.4 \pm 0.2$ with drops of 4 mol equiv/l HCl if necessary. This mixture was placed in tubes and sterilized by autoclaving. Tubes containing 15 ml of the different formulations were inoculated at 1 g/100 g from an overnight culture of the collected strain and incubated at 30 °C. Then, 1 ml aliquots were retrieved after 0, 3, 6, 9, 12, 15, 24 and 36 h to determine bacterial count and the production of bacteriocin during the bacterial growth, as it was previously described. Bacteriocin production was determined from 6 h of storage by the AWDA using *L. innocua* ATCC 33090 and *S. aureus* FBUNT as indicator microorganisms.

#### 2.8.3. Mode of action

Overnight cultures of *L. innocua* ATCC 33090 and *S. aureus* FBUNT (18 h at 30 °C in BHI broth) were treated with CFSs of the selected strains at a ratio of 1:10 in order to yield an initial bacterial count of about $10^7$ cfu/ml. Control sample consisted of bacteriocin-free BHI broth inoculated with the indicator bacteria. For determination of loss of viability of sensitive cells, following incubation at 30 °C (*L. innocua*) and 37 °C (*S. aureus*), samples were withdrawn at regular time intervals for OD measurements (600 nm) and bacterial enumeration by plating samples on BHI agar. Log 10 colony forming units (cfu/ml) against time were used to determine the kinetics of indicator viability loss. A reduction in cfu/ml after the treatment is an indication of cell viability loss (Bhunia, Johnson, & Ray, 1988).

### 2.9. Statistical analyses

All experiments were carried out in duplicate and replicated twice and the data shown are the means of the replicates. Statgraphics Plus for Windows, Version 4.0, was used for the statistical study of the results. A variance analysis (ANOVA) was also applied to establish whether significant differences ($p < 0.05$) existed between the values obtained for the means of every trial conducted. Regarding microbial viable counts, a value of 1 log was chosen for practical significance (Jarvis, 1989).

## 3. Results and discussion

### 3.1. Identification of isolates

A total of 27 strains were randomly isolated from ewes' milk (18 strains) and cheese (9 strains). The isolates were identified as members of the genus *Enterococcus* based on the following criteria (Schleifer & Kilpper-Bälz, 1984): absence of catalase, Gram-positive, chain forming cocci facultatively anaerobic with the ability to grow at 10 and 45 °C in media containing 6.5 g/100 ml NaCl, at pH 9.6, and in BEA medium. The pyrrolidonyl aminopeptidase activity was also tested being positive for all the strains. Further identification to species level was based on carbohydrate fermentation reactions. The isolates were identified as *Enterococcus faecium* (22 isolates) and *Enterococcus faecalis* (5 isolates). The low diversity found in the species level is representative of the ecological niche from which they were isolated. Although scarce microbial information about artisanal ewes' dairy products from Argentina is available, it can be postulated that the described microflora is representative of these products since *E. faecium* and *E. faecalis* together with *Enterococcus durans* comprise the enterococci species most prevalent in artisanal European cheeses (Gomez et al., 2000; Mannu & Paba, 2002; Prodromou, Thasitou, Haritonidou, Tzanetakis, & Litopoulou-Tzanetaki, 2001).

### 3.2. Antimicrobial activity

The isolates were evaluated for their inhibitory activity towards selected indicator bacteria (Table 1). Among them, one *E. faecalis* and eight *E. faecium* isolates were found to produce antibacterial substances against *L. innocua* ATCC 33090 and all the *L. monocytogenes* strains. The ability to inhibit growth of *Listeria* spp. is common to most *Enterococcus* bacteriocins (Campos, Rodríguez, Calo-Mata, Prado, & Barros-Velázquez, 2006; Cocolin et al., 2007; Pinto et al., 2009), being this ability related to the close phylogenetic relationship of enterococci and listeriae (Devriese & Pot, 1995; Stackebrandt & Teuber, 1988). Only three *E. faecium* strains (TW15, TW20 and TW22) were capable to inhibit *growth* of *S. aureus* strains (6538 and FBUNT). The rest of the Gram-positive bacteria, including lactic acid bacteria, food pathogens and spoilage microorganisms, and Gram-negative bacteria were not inhibited by the isolates tested (Table 1).

In the view of our results, the three isolates identified as *E. faecium* strains which had inhibitory activity against *L. innocua*, *L. monocytogenes* and *S. aureus* were selected for further studies, including genotypic identification. The fragment of the 16S rRNA gene amplified from strain *E. faecium* TW15 exhibited 100% homology with respect to *E. faecium* strain ATCC 27273 (accession number EU547780.1). Strain *E. faecium* TW20 exhibited 100% homology with respect to *E. faecium* strain ATCC 19434 (accession number DQ411813.1). Strain *E. faecium* TW22 exhibited 100% homology with respect to *E. faecium* strain DSM20477 (accession number AJ276355.1).

The inhibitory compounds produced by the three *E. faecium* isolates selected, namely TW15, TW20 and TW22, were inactivated by treatment with proteolytic enzymes. Moreover, catalase test did not affect the antibacterial activity of strains under investigation. These results suggested that the inhibitory activity could be due to the production of proteinaceous substances and not of hydrogen peroxide or acidity (supernatant adjusted at pH 6.5). In order to simplify the reading of text, BLIS produced by *E. faecium* TW15, *E. faecium* TW20 and *E. faecium* TW22 were termed as BLIS TW15, BLIS TW20 and BLIS TW22, respectively.

### 3.3. Virulence factors

The genus *Enterococcus* is the most controversial group of lactic acid bacteria (Foulquié Moreno et al., 2006). On the one hand, enterococci have been used in many different applications as starters or adjunct cultures, and they are well known for their major role in improving flavour development and quality of cheese (Hugas et al., 2003). On the other hand, the role of enterococci in disease has raised questions on their safety for use in foods or as probiotics since they are important nosocomial pathogens that cause bacteraemia, endocarditis and other infections (Giraffa, 2002). Some strains are resistant to many antibiotics. Vancomycin resistance is of special concern because this antibiotic was considered a last resort for treatment of multiple resistant enterococcal infections. The emergence of vancomycin-resistant enterococci (VRE) in hospitals has led to infections that cannot be treated with conventional antibiotic therapy (Franz et al., 2003). Concerning antibiotic resistance, *E. faecium* TW15, TW20 and TW22 were sensitive to vancomycin (minimum inhibitory concentration $\geq 0.25$ μg/ml). Hosseini et al. (2009) also found that other bacteriocinogenic *Enterococcus* strains showed no resistance to antibiotics. Reported results show a high sensitivity to the antibiotic and indicate that these strains cannot be included in any of the known resistant genotype of enterococci.

The β-haemolysin/bacteriocin or cytolysin (Cyl) is a confirmed enterococcal virulence factor. This is a cellular toxin that enhances virulence in animal models (Gilmore et al., 1994). None of the strains tested display haemolytic activity when grown in human blood agar, which coincides with the results from the PCR analyses that did not show the presence of the *cylB* gen in any of the strains.

Proteases are also believed to be involved in enterococcal pathology. Gelatinase is an extracellular metalloendopeptidase that acts on collagenous material in tissues. Production of gelatinase increased pathogenicity in an animal model (Singh, Coque, Weinstock, & Murray, 1998). Our results showed no degradative activity of the enzyme in any of the strains tested following the agar plate method. Although genetic amplification revealed the presence of the *gelE* gen in *E. faecium* TW15, its presence not necessarily implies the expression of the enzymatic activity. This fact is mentioned by many authors who stated that the prevalence of the gen within the *Enterococcus* genus is not always related to its expression (Eaton & Gasson, 2001; Franz et al., 2001). It has been demonstrated that even when the total transcription of the operon had taken place, the enzymatic activity could not be detected; suggesting that regulation of gelatinase activity can also be post-transcriptional (Silva Lopes, Simões, Tenreiro, Figueiredo Marques, & Barreto Crespo, 2006).

Of the four genes screened, *agg*, *gelE*, *cylB* and *cpd*, PCR amplification yielded negative results for the three strains, while *agg* gen was not detected in TW20 and TW22 strains. These results, together with the absence of virulence factors, suggest that the three enterococcal strains analyzed in this study may be regarded as safe to be used for the biopreservation of food.

### 3.4. Detection of enterocin structural genes

The PCR results revealed the presence of structural enterocin A, B, P and LB50B genes in *E. faecium* ETW20 and ETW22 strains, while *E. faecium* ETW15 exhibited the presence of enterocin A and P genes. The presence of one single enterocin gene is the most usually detected case among enterococcus. However, in agreement with previous reports (Strompfová, Lauková, Simonová, & Marcinakova, 2008) we found four different enterocin genes in *E. faecium* TW20 and TW22 and two in *E. faecium* TW15. The detection by PCR amplification of enterocin genes does not imply the production of these peptides. The gen expression depends on environmental conditions or genetics mechanisms like induction or transcription control.

### 3.5. Activity of the BLIS after enzymatic and physicochemical treatments

CFSs from *E. faecium* TW15, *E. faecium* TW20 and *E. faecium* TW22 were treated with lipase and lysozyme. The BLISs from the different strains were not inactivated by these enzymes which suggested that the peptides did not contain or require a lipid moiety for activity. Retention of antimicrobial activity upon treatment with lipase and lysozyme also indicated that the molecule is pure protein rather than a conjugated one (Osmanağaoğlu, 2007).

Heating the CFSs at 100 °C for 5, 15 and 30 min did not affect activity, as activity units did not decrease when compared to an unheated control. However, activity was diminished when treated at 121 °C for 15 or 30 min. The behaviour of the CFSs was identical in all the treatments mentioned. Regardless of the antimicrobial decrease observed after heating at 100 °C and 121 °C, the BLISs were determined to be heat stable since the activity was not completely lost. The thermostability displayed by the enterocin-like substances tested was in keeping with the results found from other bacteriocinogenic *E. faecium* strains (Ben Belgacem et al., 2010; Campos et al., 2006; Ghrairi, Frere, Berjaud, & Manai, 2008).

The results of BLIS stability throughout the refrigerated storage time showed that the maximum inhibitory activity remained constant up to 21 days when the supernatant was stored at 4 °C. In addition, 100% of the initial activity was observed after the three freeze–thaw cycles to which the CFSs were subjected.

The influence that pH exerted on the antimicrobial effectiveness of the CFSs was determined from bacterial cultures grown at 30 °C during 24 h since this condition led to maximum bacteriocin production as it is exposed elsewhere in this work. The antimicrobial activity of the different CFSs is summarized in Table 4. It can be observed that the pH of the growth media exerted a significant influence ($p < 0.05$) on the antimicrobial activity of the CFSs against both indicator microorganisms. Thus, the BLIS-producing strains exhibited a broad pH range of activity against *L. innocua* as well as *S. aureus*. The pH values from 7.5 and up showed to have a negative effect on the BLIS activity for both indicator microorganisms, with the exception of strain TW20 when *S. aureus* was used as indicator.

**Table 4**
Influence of different pH values on bacteriocin activity of free cell supernatants from *E. faecium* strains.

| Indicator microorganisms | pH treatment | *E. faecium* strains | | |
|---|---|---|---|---|
| | | TW15 | TW20 | TW22 |
| *L. innocua* ATCC 33090 | 2.50 | $14.0 \pm 0.3^a$ | $13.0 \pm 0.1^a$ | $13.0 \pm 0.1^a$ |
| | 3.50 | $16.5 \pm 0.2^b$ | $16.0 \pm 0.1^b$ | $17.0 \pm 0.4^b$ |
| | 4.50 | $16.0 \pm 0.2^b$ | $16.0 \pm 0.1^b$ | $17.0 \pm 0.3^b$ |
| | 5.50 | $16.0 \pm 0.3^b$ | $16.0 \pm 0.1^b$ | $16.5 \pm 0.4^b$ |
| | 6.50 | $16.5 \pm 0.2^b$ | $16.0 \pm 0.1^b$ | $17.0 \pm 0.1^b$ |
| | 7.50 | $11.0 \pm 0.1^c$ | $11.0 \pm 0.4^c$ | $10.0 \pm 0.4^c$ |
| | 8.50 | $12.0 \pm 0.1^d$ | $12.0 \pm 0.3^d$ | $11.0 \pm 0.3^c$ |
| | 9.50 | $12.0 \pm 0.1^d$ | $12.0 \pm 0.3^d$ | $11.0 \pm 0.2^c$ |
| *S. aureus* FBUNT | 2.50 | $12.0 \pm 0.1^a$ | $13.0 \pm 0.1^a$ | $13.0 \pm 0.2^a$ |
| | 3.50 | $15.0 \pm 0.5^c$ | $13.0 \pm 0.2^a$ | $12.5 \pm 0.1^b$ |
| | 4.50 | $13.0 \pm 0.2^b$ | $12.0 \pm 0.1^b$ | $11.5 \pm 0.1^c$ |
| | 5.50 | $12.0 \pm 0.2^a$ | $12.0 \pm 0.1^b$ | $11.5 \pm 0.1^c$ |
| | 6.50 | $13.0 \pm 0.1^b$ | $12.0 \pm 0.1^b$ | $11.5 \pm 0.1^c$ |
| | 7.50 | $10.0 \pm 0.1^d$ | $13.0 \pm 0.1^a$ | $10.0 \pm 0.1^d$ |
| | 8.50 | $10.0 \pm 0.1^d$ | $13.0 \pm 0.2^a$ | $10.0 \pm 0.3^d$ |
| | 9.50 | $10.0 \pm 0.1^d$ | $13.0 \pm 0.2^a$ | $10.0 \pm 0.4^d$ |

Data shown correspond to the diameters (mm) of the inhibition zones displayed in the Agar Well Diffusion Assay.
Superscript letters indicates no statistical difference

Maximum antimicrobial activity values against *L. innocua* were recorded within the pH range 3.5–6.5, showing no significant differences between them ($p > 0.05$). Although high acidity (pH 2.5) lowered the bacteriocin activities of all the strains tested against *L. innocua*, the decrement was less compared to alkaline pH values (7.5 and up). *E. faecium* TW15 CFS showed a curious behaviour towards pH changes since a maximum antibacterial effect against *S. aureus* was recorded at pH 3.5 and then exhibiting a decrease as pH increases. No significant differences were detected for *E. faecium* TW20 CFS against *S. aureus* within the whole pH range assayed. This pH stability accounts for the robustness of the BLIS produced by this strain. Considering the wide pH range covered by this trial, it is remarkable that all the *E. faecium* strains showed an effective antimicrobial activity against both indicator microorganisms. The broad range pH stability of these enterocin-like substances is in keeping with enterocin 900 activity (Franz, Schillinger, & Holzapfel, 1996). As expected, non-inoculated MRS broth whose pH values were adjusted within the range 2.5–9.5 showed no antimicrobial activity.

### 3.6. Mode of action of BLIS

Addition of BLIS from each of the three *E. faecium* strains at the early exponential phase of growth of *L. innocua* and *S. aureus* cell suspensions resulted in a deep decrease in OD (Fig. 1, panels A and B). This rapid fall in OD indicates a bactericidal effect towards target microorganisms. Bacterial counts of the indicator microorganisms showed a decrease in viable cells from $1 \times 10^{10}$–$3 \times 10^{12}$ cfu/ml at the moment of adding the BLIS to $4 \times 10^{8}$–$2 \times 10^{10}$ cfu/m at the end of the trial (data not shown). As differences of one order of magnitude are generally regarded as being of microbial significance (Gill & Holley, 2000; Jarvis, 1989), the data collected herein highlight the bactericidal character of the antimicrobial substances produced by the *E. faecium* strains tested. In the untreated (control) samples, no repression or inhibition of growth was observed. Bacteriocins activity depends on concentration and on the nature and the physiological stage of the target strain. Enterocins, as most bacteriocins, have the cytoplasmic membrane as their primary target. They form pores in the cell membrane, thereby depleting the transmembrane potential and/or the pH gradient, resulting in the leakage of indispensable intracellular molecules (Cleveland, Montville, Nes, & Chikindas, 2001).

The bactericidal effect showed by the BLISs produced by the three *E. faecium* strains towards *L. innocua* ATCC 33090 is similar to that reported by Tomé, Gibbs, and Teixeira (2008) who studied the antimicrobial activity of *E. faecium* ET05 isolated from cold smoked salmon against *L. innocua* 2030c. Similar results were achieved by Foulquié Moreno, Callewaert, Devreese, Van Beeumen, and De Vuyst (2003) when the activity of six *E. faecium* strains isolated from different origins was compared.

### 3.7. Bacteriocin production

#### 3.7.1. Determination of enterococcal growth for maximum bacteriocin production

BLISs investigated in the current study were secreted into the culture medium in the early exponential phase of growth. The detectable levels of BLISs were recorded after 3 h of growth for TW15 and TW22 and after 9 h for TW20 (Fig. 2, section A from panels 1–3). Since production of BLIS against *S. aureus* was considerably less than against *L. innocua*, Fig. 2 only shows NaCl effect against the latter bacteria. Mentioned results could indicate that the peptides are primary metabolites as reported for enterocins produced by *E. faecium* ALP7 (Pinto et al., 2009) and *Enterococcus mundtii* ST15 (De Kwaadsteniet, Todorov, Knoetze, & Dicks, 2005). In all three cases, the maximum levels of antimicrobial activity were found at different stages of the stationary phase of growth, depending on both the producer strain and the indicator microorganism. Among the *E. faecium* strains tested, TW20 exhibited the maximum inhibitory activity (6400 AU/ml) against *L. innocua* after 36 h of incubation at 30 °C, which corresponded to the stationary phase (Fig. 2, panel 2, section A). Likewise, maximum inhibitory activity (800 AU/ml) against *S. aureus* was detected at the same stage and time of incubation. In both cases, after time mentioned no further increase of inhibitory activity was found (data not shown). No differences in maximum bacteriocin production against *S. aureus* were recorded among the other two *E. faecium* strains, namely TW15 and TW22. In these cases, 400 AU/ml was the highest antimicrobial activity achieved by the latter strains against *S. aureus* antimicrobial activity against *L. innocua* was 3200 AU/ml for *E. faecium* TW15, while it was half of that value for *E. faecium* TW22 (Fig. 2, panels 2 and 3, section A).

Coincidently, the three *E. faecium* strains produced the highest bacteriocin titles against *L. innocua* and the lowest ones against *S. aureus.* This fact could be attributed to the inherent sensitivity of each indicator microorganism towards the bacteriocins and was reported previously by several authors (Campos et al., 2006; Castro, Palavecino, Herman, Garro, & Campos, 2011; Herman, Cayré, Vignolo, & Garro, 2008).

Environmental conditions that lead to maximum antimicrobial activity of BLIS comprised the culture medium composition (MRS broth), its initial pH ($6.2 \pm 0.1$) and temperature of incubation (30 °C). Similar results, even same bacteriocin concentrations, were reported by Franz et al. (1996) for the bacteriocin-producing strain *E. faecium* BFE 900, isolated from black olives, growing in the same medium.

#### 3.7.2. Influence of NaCl concentration on enterocin production

Enterococcal growth showed significant decreasing values with increasing NaCl concentrations. These results could be observed in Fig. 2, panels 1–3, section B, and are in agreement with those

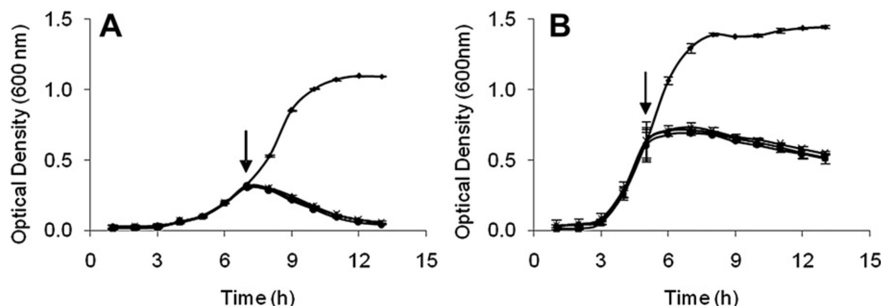


**Fig. 1.** Mode of action of the enterocins. Growth Inhibition produced by *E. faecium* TW15 (●), *E. faecium* TW20 (▲) and *E. faecium* TW22 (×) against: *L. innocua* ATCC 33090 (section A); *S. aureus* FBUNT (section B). Control without added bacteriocin (◆). Arrows show the moment when the enterocins were added to the inoculated media.

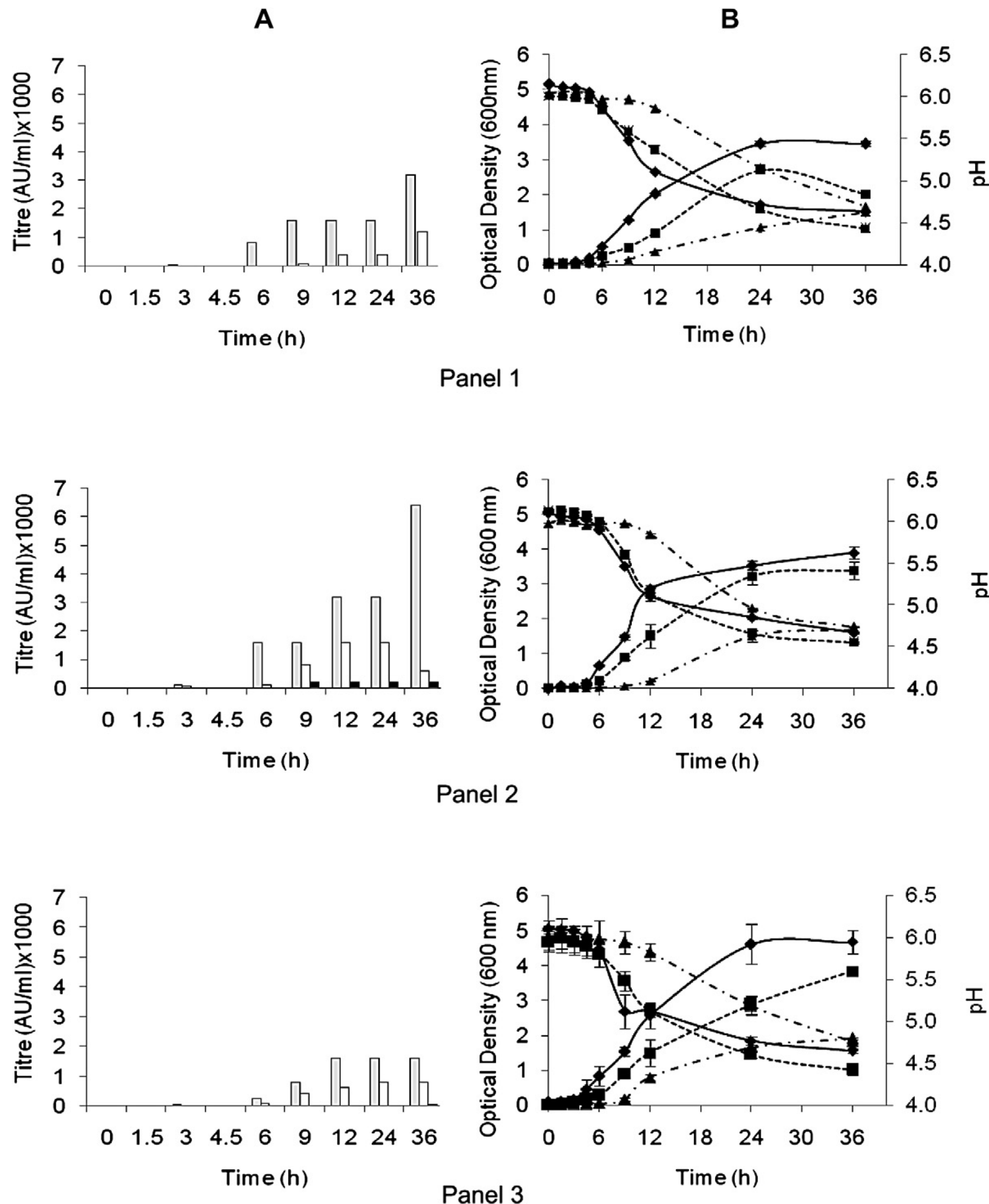


**Fig. 2.** Section (A) NaCl effect on BLIS production against *L. innocua* ATCC 33090. Grey column: no NaCl added. White column: 3 g/100 g NaCl. Black column: 6 g/100 g NaCl. Section (B) Kinetics of growth and acidification profile of the systems containing different levels of NaCl. Symbols: no NaCl (◆); 3 g/100 g NaCl (■) and 6 g/100 g NaCl (▲). Panel 1 *E. faecium* TW15; panel 2 *E. faecium* TW20; panel 3 *E. faecium* TW22.

reported by Vermeiren, Devlieghere, and Debevere (2004) and Delgado, Brito, Peres, Arroyo-López, and Garrido-Fernández (2005) for lactic acid bacteria. BLISs production had also been diminished, showing titles significantly lower than the ones produced in MRS broth without added salt (Fig. 2, panels 1–3, section A). Most bacteriocins have been referred to as primary metabolites (Lejeune, Callewaert, Crabbé, & De Vuyst, 1998; Leroy & De Vuyst, 1999a, 1999b; Motta & Brandelli, 2003) mainly based on the parallelism between growth and production curves (bioactivity accumulation in broth). However, that assumption could be questioned in certain cases since several bacteriocins are continuously synthesized throughout the stationary phase after long incubation periods (Delgado et al., 2005; Pinto et al., 2009). The time extension of the trials performed in this study becomes a limiting factor when elucidating the regulation of bacteriocin production as well as its relationship to growth. Nevertheless, it could be possible to determine that the stress caused by the presence of sodium chloride affected BLIS production. Nilsen, Nes, and Holo (1998) suggested that the decrease in bacteriocin production in the presence of salt is due to interference of sodium chloride molecules with

binding of the induction factor to its receptor, which is essential for bacteriocin production. Although BLIS TW15 was active in the presence of NaCl (3.0 g/100 g), this BLIS kept only 33% of its activity against *L. innocua* and 25% of it against *S. aureus* compared to BLIS activity when no NaCl was present (Fig. 2, panel 1, section A). The addition of 6.0 g/100 g NaCl inhibited BLIS production during the period of time considered for this study since no antimicrobial activity was detected against none of the indicator microorganisms. The production of BLIS TW20 was reduced four times in the presence of 3.0 g/100 g NaCl for both microorganisms whereas 6.0 g/100 g NaCl only permitted the production of 200 AU/ml against *L. innocua* (Fig. 2, panel 2, section A). Regarding BLIS TW22, the presence of 3.0 g/100 g NaCl halved its production against indicator microorganisms compared to the values obtained without the salt. Moreover, 6.0 g/100 g NaCl lead to a significative decrease in bacteriocin production (50 AU/ml), showing antimicrobial activity towards *L. innocua* but not *S. aureus*. This is in keeping with the results from Himelbloom, Nilsson, and Gram (2001), which showed decreasing bacteriocin production together with increasing NaCl concentrations. Delgado et al. (2005) stated that bacteriocin production in the presence of sodium chloride is closely related to the number of growing cells. Thus, if 6.0 g/100 g NaCl conditioned bacterial growth, it could be expected that it would also affect bacteriocin production. Nevertheless, it has to be highlighted that the type of control that NaCl exerts on bacteriocin production is apparently dependent on the strain (Delgado et al., 2007).

Sections B from Fig. 2 not only show the kinetics of growth but also the acidification profiles of *E. faecium* strains. As media acidification is bond to bacterial growth, it can be noticed that NaCl increasing concentrations gave smoother curves than the ones without salt in both cases, i.e. acidification and growth. Maximum specific production has been registered mostly in the pH range 4.5–5.5, coinciding with what was reported by several authors (Calderón-Santoyo, Mendonza-García, García-Alvarado, & Escudero-Abarca, 2001; Klostermaier, Scheyhing, Ehrmann, & Vogel, 1999; Krier, Revol-Junelles, & Germain, 1998).

## 4. Conclusions

These *Enterococcus* strains, lacking undesirable traits (such as antibiotic resistance or haemolytic and gelatinase activity), and displaying inhibitory spectrum towards *Listeria* and *Staphylococcus* would be interesting as protective culture and could be considered as additional bio-preservative hurdles being of practical use in food industry. The fact that our enterocin-like substances are stable at low refrigeration temperatures suggests their feasible use in minimally processed refrigerated or frozen foods to prevent the growth of pathogenic and spoilage psychotropic microorganisms as an alternative to traditional chemical preservatives. In addition, pH stability shown by these bacteriocins may be advantageous in food preservation where the product is acidified, or where the pH of the food is lowered by growth of naturally occurring LAB (e.g. fermentations). However, more experiments must be carried out to study bacteriocin production *in situ*, namely “the food product”, and to elucidate chemical structure of the molecules produced to facilitate massive production and purification procedures.

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