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ORIGINAL RESEARCH PAPER

Antimicrobial activity of enterocins against *Listeria* sp. and other food spoilage bacteria

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Abstract
Objective To determine bacteriocin producers and the prevalence of structural enterocin genes and to detect the spectrum of activity against foodborne pathogens, from isolates of *Enterococcus faecium* and *Enterococcus faecalis* that were isolated from food and the environment.
Results The *ent*A, *ent*B, *ent*P, *ent*1071 and *ent*X genes, which encode enterocins were the most frequently observed. Enterocins were thermostable, proteinaceous, and resistant to catalase. None of the isolates produced hemolysin, and inhibition resulting from bacteriophage lysis was excluded. The bactericidal effect of enterocins against *L. innocua* 12612 was determined by optical density and colony forming units. For the activity spectrum, elimination of mainly *Listeria* strains, *Bacillus* sp. and clinical enterococci, was observed. Imaging with scanning electron microscopy after treatment with enterocin Efm22 showed irregular rod-shaped cells and loss of cellular integrity.
Conclusions The isolates evaluated in this study are candidates for the production of enterocins that will be used as food biopreservatives, because they have high anti-listerial activity even after 24 h of experimentation, and used in the pharmaceutical area because they inhibit clinical microorganisms.

Keywords Bacteriocins · *Listeria monocytogenes* · *Bacillus* sp. · *Enterococcus* sp. · Food protections · Lactic acid bacteria

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Introduction

Food safety is an important global issue because of increasing foodborne diseases and changes in food consumption habits. Therefore, the need to avoid economic losses resulting from microbial-induced spoilage and the preservation of foods by natural methods may be a satisfactory approach to solve many of the current food-related issues (Kaur and Garg 2013).

The development of biopreservation technologies with lactic acid bacteria (LAB) and/or their metabolites represents an additional hurdle in the protection of food against microbial contamination because these bacteria produce several antimicrobial substances, including organic acids, hydrogen peroxide and bacteriocins (Perin et al. 2013). Many bacteriocin-producing LAB strains have been isolated from milk, plants and fermented dairy, vegetable and meats products, many of which have been identified and

characterized (Zhang et al. 2018). Studies have demonstrated that bacteriocin from LAB has considerable inhibitory activity against pathogenic and spoilage microorganisms in food such as *L. monocytogenes* (Zommiti et al. 2018).

Contrary to other bacteriocins, enterocins (produced by *Enterococcus* sp.) have attracted technological and scientific interest because they exhibit antimicrobial activity against important foodborne pathogens, included Gram positive and negative bacteria, making this peptide of great interest to industry (Schittler et al. 2019). In addition, the search for new enterocin-producing isolates against specific targets or greater action spectra has been constantly highlighted.

Enterocins are found within class I, IIa, IIc and III bacteriocins (Masias et al. 2017), and the cytoplasmic membrane is their primary target. Similar to most bacteriocins, they form pores and thereby deplete transmembrane potential and/or a pH gradient, resulting in cell death. Enterocins show high activity, particularly against *Listeria* species at low concentrations (Schittler et al. 2019). However, low levels of bacteriocins secreted from natural strains do not meet the requirements of industrial-scale production and have limited applications.

The *Listeria* genus can be found as a contaminant in meat, milk and other food processing facilities, and the most important species is *L. monocytogenes*, which causes septicemia, meningitis, encephalitis or death/stillbirth of neonates, especially in high-risk groups in humans (including immunocompromised persons and the elderly) (Khademi and Sahebkar 2019).

Relevant information that must be investigated includes possible bacteriocins that the strains are able to produce, which can be assessed by the identification of specific genes that are related to known bacteriocins and thermal and protease resistance, followed by the inhibitory spectrum (Perin et al. 2013). These data can justify further studies with purified bacteriocins to investigate the diversity of characteristics that allow their use in the food industry as biopreservatives.

In this study, we focused on the isolation and analysis of inhibitory effects of enterocins that are produced by *Enterococcus* spp. isolates that were obtained from food and environment where common foodborne pathogens strains originate. The inhibitory effect of cell-free culture supernatants (CFSs) was tested against several foodborne microorganisms.

Materials and methods

Bacterial strains, storage conditions and inoculum

Five *Enterococcus faecium* (Efm20, Efm22, Efm24, Efm25 and Ent22) and two *E. faecalis* (Efs27, Efs18) were used. Strains Efm20, Efm22, Efm24, Efm25 and Efs27 were obtained from distinct food (soft cheese) samples over a period of 1 year from 2011 to 2012 as described elsewhere (Furlaneto-Maia et al. 2014). *E. faecium* Ent22 and *E. faecalis* Efs18 were obtained from environmental samples (water). These strains were identified using molecular approaches, as previously described (Furlaneto-Maia et al. 2014). Strains demonstrated in vitro antagonistic activity against the indicator strain of *Listeria innocua* (Ogaki et al. 2016). Other strains were foodborne pathogens and food spoilage (Table 3). Strains belong to the Laboratory of Basic and Applied Microbiology (LAMBA) of the Federal University of Technology—Paraná (Londrina, PR, Brazil) and were maintained at − 80 °C. Before use, frozen stock was inoculated into 10 mL in De Man, Rogosa and Sharpe (MRS) (*Enterococcus* strains) and brain heart infusion (BHI) broth (Neogen Culture Media, USA) and incubated at 37 °C for 24 h.

Genotyping of genes encoding enterocins

The strains selected were submitted to molecular identification of enterocin-producing genes, which included enterocin A (*ent*A), enterocin B (*ent*B), enterocin P (*ent*P), enterocin L50A/B (*ent*L50A/B), enterocin 1071 (*ent*1071), enterocin Q (*ent*Q), mundticin KS (*ent*KS), enterocin X (*ent*X), enterocin 31 (*ent*31) and enterocin AS48 (*ent*AS48), and they were amplified using PCR primers (Table 1). The PCR reactions were performed as previously described in a thermocycler (Esco Technologies, USA). Gene amplification was conducted with an initial denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, matching the oligonucleotide (Thermo Fisher Scientific, USA) (Table 1) for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. Amplicons were analyzed on a 1.0% agarose gel (Merck, Germany).

Table 1 Primer used for the detection of enterocin structural genes

Target gene	Sequence (5′→3′)	Annealing temperature (°C)	Amplicon size (bp)	References
Enterocin A	GGTACCACTCATAGTGGAAA CCCTGGAATTGCTCCACCTAA	55	138	Özdemir et al. (2011)
Enterocin B	CAAAATGTAAAAGAATTAAGTACG AGAGTATACATTTGCTAACCC	56	201	De Vuyst et al. (2003)
Enterocin P	GCTACGCGTTCATATGGTAAT TCCTGCAATATTCTCTTTAGC	55	87	Özdemir et al. (2011)
Enterocin *L*50A/B	ATGGGAGCAATCGCAAAATTA TAGCCATTTTTCAATTTGATC	55	274	Özdemir et al. (2011)
Enterocin 1071 A,B	GGGGAGAGTCGGTTTTTAG ATCATATGCGGGTTGTAGCC	50	243	Martin et al. (2006)
Enterocin 31	CCTACGTATTACGGAAATGGT GCCATGTTGTACCCAACCATT	50	122	Du Toit et al. (2000)
Enterocin AS48	ATATTGTTAAATTACCAA GAGGAGTATCATGGTTAAAGA	50	185	Du Toit et al. (2000)
Enterocin X	CCTCTTAATCATTAACCATAC GTTTCTGTAAAAGAGATGAAAC	50	500	Edalatian et al. (2012)
Enterocin Q	GAAGAAATTTTTTCCCATGGC CTTCTTAAAAATGGTATCGCAA	55	95	Citti et al. (2002)
Mundticin KS	CTACGGTAATGGAGTCTCATG CATCTGCATACAGGCTATACC	50	275	Edalatian et al. (2012)

Production and partial purification of enterocins from cell free supernatant

Cell-free supernatants (CFS) of the isolates were previously selected and assayed as described by Tomé et al. (2009), with modifications. The strains were cultured in MRS medium (Neogen Culture Media, USA) overnight, adjusted to 1.0×10^8 CFU mL^{-1} in MRS (pH 6.2) and maintained at 180 rpm for 18 h at 37 °C. The cultures were centrifuged at 5000 rpm for 15 min, the supernatant was adjusted to pH 6.5 with 1 N NaOH. Partial purification of the enterocins was performed as described by Rocha et al (2019), using 40% saturated ammonium sulfate (Merck). Quantitative determination (UA mL^{-1}) of the partially purified enterocins was conducted as previously described.

Antimicrobial activity and determination of the arbitrary units

In an agar well diffusion assay (AWDA), 30 µL of the enterocin were deposited in 5 mm wells on BHI agar containing *L. innocua* 12612 (1×10^8 CFU/mL). Finally, the plates were incubated for 24 h at 37 °C. An inhibition halo $\geq$ 2 mm was considered to be a positive result. Each condition was tested in duplicate. The inhibitory activity of enterocin against *L. innocua* 12612 was quantified and expressed as arbitrary units (AU) per milliliter. For this experiment, enterocin at 1:2 (v/v) dilutions were deposited onto microplates using MRS. Then, 100 µL of each dilution were deposited onto a new microplate with 100 µL of the indicator bacterium (10^8 CFU/mL) and incubated at 37 °C for 12 h. Bacterial growth was measured using optical density (OD) in a spectrophotometer (Bio Tek, USA) (600 nm) every 3 h. The arbitrary unit per mL (AU/mL) was defined as the reciprocal of the last dilution that showed growth compared to the control (bacteria without CFS) multiplied by 100 at 6 and 12 h. The OD values were evaluated using one-way ANOVA and Tukey's test, and $p < 0.05$ was considered a statistically significant difference between the antimicrobial activity of an isolate and the control.

Effect of heat and enzymes on enterocin activity

The thermostability of partially concentrated enterocins was evaluated by the treatment at 80 °C for 10 min and 100 °C for 20 min. To determine the sensitivity of the antimicrobial components against proteolytic enzymes, the selected enterocin were treated with α-chymotrypsin (50 mg/mL), protease (50 mg/mL) and trypsin (20 mg/mL) enzymes (Merck) in addition to the catalase enzyme (Merck) at a final concentration of 1 mg/mL. Then, the antagonistic activity of the treated enterocin was evaluated using the AWDA assay against *L. innocua* 12612. Each condition was tested in duplicate.

Mode of inhibition

The antimicrobial effect of partially purified enterocins was evaluated using *L. innocua* 12612, as described by Rocha et al. (2019) with modifications. The indicator bacteria was adjusted to 1.5×10^8 CFU/mL in MRS medium at an OD of 600 nm at 37 °C. Culture medium (10 mL) was placed into tubes, and 0.5 mL of partially purified enterocin was added, except for the control, which only contained ultrapure water (MilliQ). The OD and cell count (CFU/mL) were measured at 0, 1, 2, and 4 h of incubation time. The cell counts were determined on BHI agar medium.

Spectrum of antimicrobial activity

Enterocins selected by the previous experiments against *L. innocua* 12612 were used to evaluate their activity spectrum against 24 indicator bacteria (Table 2). For this experiment, the antagonistic activity of enterocins was evaluated using a diffusion technique in agar as previously described. The antimicrobial spectrum was determined quantitatively against *L. innocua* 12612 and qualitatively against other bacteria.

Hemolytic and bacteriophage activity

The hemolytic activity of enterococci isolates was analyzed as described by Eaton and Gasson (2001) in BHI supplemented with 5% sheep blood (Newprov, Brazil). The bacteriophage activity was analyzed as described by Ogaki et al. (2016). Each condition was tested in duplicate.

Scanning electron microscopy (SEM)

The *L. innocua* 12612 and *B. subtilis* cells grown for 18 h in the presence of enterocin were washed twice with PBS and then fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer for 18 h at 4 °C. The samples were carefully washed with 0.1 M sodium cacodylate buffer, and post-fixation was performed for 1 h at 25 °C with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Samples were gently dehydrated in

Table 2 Presence of enterocin structural genes in enterococcal strains

Identification	Strain	Structural genes of enterocins[a]									
PCR		*ent*A	*ent*B	*ent*P	*L50*A/B	*ent*1071	*ent*Q	*ent*KS	*ent*X	*ent*31	*AS48*
E. faecium	Efm20	+	+	+	−	−	−	−	+	−	−
E. faecium	Efm22	+	+	+	−	−	−	−	+	−	−
E. faecium	Efm24	+	+	+	−	−	−	−	+	−	−
E. faecium	Efm25	+	+	+	−	−	−	−	+	−	−
E. faecalis	Efs27	+	+	+	−	−	−	−	+	−	−
E. faecalis	Efs 18	-	−	−	−	+	−	−	−	−	−
E. faecium	Ent 22	-	−	−	−	+	−	−	−	−	−

[a]Enterocin A, *ent*A; enterocin B, *ent*B; enterocin P, *ent*P; enterocin LB50A/B, *entL50*A/B; Enterocin 1071, *ent*1071; Enterocin Q, *ent*Q; Enterocin Mundticin, *ent*KS; Enterocin X, *ent*X; Enterocin 31, *ent*31 and Enterocin AS48, *ent*AS48. (+) indicates the presence of a gene, and (−) indicates the absence of a gene

graded ethanol (50% to 100% ethanol), critical-point-dried in CO_2 (BALTEC DCP 030 Critical Point Dryer), coated with gold (BALTEC SDC 050 Sputter Coater) and viewed in a FEI Quanta 200 Scanning Electron Microscope.

Results and discussion

The presence of ten common structural enterocin genes, singly or in varying combinations, in the genome of *Enterococcus* spp. was tested. All seven enterococci, *E. faecium* 20 (Efm20), *E. faecium* 22 (Efm22), *E. faecium* 24 (Efm24), *E. faecium* 25 (Efm25), *E. faecium* 22 (Ent22), *E. faecalis* 27 (Efs27) and *E. faecalis* 18 (Efs18) harbored at least one enterocin-encoding gene (Table 2), of which 71% harbored *ent*A, *ent*B, *ent*P and *ent*X genes, concomitantly. Strains comprising single or multiple-enterocin-encoding genes may possess unique combinations of beneficial and desirable biotechnological properties, particularly antimicrobial activity.

The structural enterocin genes *ent*A, *ent*B, *ent*P and *ent*X were the most frequently observed. *E. faecium* isolates that had the most complex enterocin gene profile (*entA*/*entB*/*entP*/*entX*) were capable of causing weak inactivation of *Listeria* strains, corroborating the data from Rocha et al. (2019). Vandera et al. (2018) have observed the genomes of *E. faecalis* and *E. faecium*, which indicated the presence of *ent*A, *ent*B and *ent*P genes. The genes *ent*A and *ent*B are used by the same carrier responsible for externalizing enterocin, and they are controlled by the same regulatory system and commonly found together (Hassan et al. 2012). Differently, genes encoding for enterocin L50A/B, Q, K, 31 and AS48 were not found in any of the enterococci evaluated.

Enterocins are commonly classified as class II bacteriocins and are characterized as small, non-lantibiotics with a strong anti-listerial effect, which are desired characteristics for their use in foods (Masias et al. 2017); genes *ent*A and *ent*P are related to anti-listerial activity with the presence of class IIa bacteriocins (Avcı and Özden 2017). Hassan et al. (2012) observed the presence of the *ent*1071 and *ent*L50A/B genes, which differs from the results in this study. The presence of four enterocin genes in several strains (Table 2) indicates their potential to produce various enterocins.

In this study enterocins were characterized as proteinaceous in nature, with the inhibition of hydrogen peroxide activity through the use of catalase, because of the lost of antibacterial activity after treatment with α-chymotrypsin, protease and trypsin. In addition, the studied enterocins were thermally stable at both evaluated temperatures since they had the same halo measurements relative to the untreated control.

None of the strains showed hemolytic activity or bacteriophage activity. These characteristics were observed by other authors (Tomé et al. 2009; Ben Braïek et al. 2018). Studies to evaluate the strain safety, especially the inability to cause hemolysis using sheep blood, have been conducted successfully for *Enterococcus* spp. (Zhang et al. 2016; Vandera et al. 2020). Hemolysin production may increase the risk of enterococcal infections (Schittler et al. 2019).

The activity spectrum of enterocins that were characterized and selected against *L. innocua* 12612 was evaluated against 23 other indicator bacteria (Table 3). The enterocins showed anti-listerial activity against *L. innocua*, *L. monocytogenes*, *L. ivanovii*, *E. faecalis* 29112 and *Bacillus* strains. The anti-listerial activity of CFSs from *E. faecalis* and *E. faecium* has been observed in reports by several authors, including Hassan et al. (2012), Jaouani et al. (2014) and Vandera et al. (2018).

These enterocins were observed to more efficiently inhibit *Listeria* and *Enterococcus* sp. isolates; *B. subtilis* was also sensitive to the action of enterocins Efm20, Efm22 and Efm25, with inhibitory activity against 62% of foodborne strains.

Although *L. monocytogenes* is well known for its pathogenicity, *L. ivanovii* has been well characterized by infection in ruminants, and it is considered a sporadic occurrence, an infection caused by *L. ivanovii* in immunocompromised humans has been observed (Khademi and Sahebkar 2019). In addition, no inhibitory activity of enterocins against Gram-negative bacteria was observed, which corroborated with the observations of Tomé et al. (2009) and Ben Braïek et al. (2018), and is likely due to the presence of the outer membrane that hinders the entry of enterocins. Notably, there was no inhibition of bacterial that produced enterocin, showing that there is immunity in the producing cells (data not shown). Immunity of bacteriocin-producing cells is conferred by a

Table 3 Inhibitory spectrum of enterocins against indicator bacteria

Indicator bacteria	*Enterococcus* strains						
	$E_{fm}20$	$E_{fm}22$	$E_{fm}24$	$E_{fm}25$	$E_{nt}22$	$E_{fs}27$	$E_{fs}18$
L. innocua CLIP 12612	+	+	+	+	+	+	+
L. innocua CLIST 2050	+	+	+	+	+	+	+
L. innocua CLIST 2052	+	+	+	+	+	+	+
L. monocytogenes 2032	+	+	+	+	−	+	+
L. monocytogenes CLIST 2044	+	+	+	+	−	+	+
L. monocytogenes CLIST 2048	+	+ +	+	+	+	+	+
L. monocytogenes CDC 4555	+	+	+	+	+	+	+
L. ivanovii CLIST 2056	+ +	+ +	+ +	+ +	+	+ +	+
E. faecalis 29212	+	+	+	+	−	+	−
E. faecalis 10766	+	+	+	+	−	+	−
*Enterococcus*14524	+	+	+	+	+	+	+
E. faecium 4c	+	+	+	+	−	+	−
B. licheniformis	−	−	−	+	−	−	−
S. aureus 25925	−	−	−	−	−	−	−
B. sporothermodurans	+	−	−	−	−	−	−
B. pumilus	−	+	−	−	−	−	−
B. circulans	−	−	−	−	−	−	+
B. borstelensis	−	−	−	−	−	−	−
B. subtilis	+	+	+	+	−	+	−
B. cereus	−	−	−	−	−	−	−
E. coli Lon 164	−	−	−	−	−	−	−
E. coli 49 LT	−	−	−	−	−	−	-
S. typhimurium 14028	−	−	−	−	−	−	−

Inhibitory activity: no inhibitory activity (−) $x \leq 1$ mm; (+) $2 \leq x \leq 10$ mm; (+ +) $10 \leq x \leq 18$ mm

peptide expressed along with bacteriocin (Benz and Meinhart 2014; Ogaki et al. 2016).

For determination of the arbitrary unit activity (AU), partially purified enterocins produced by all seven enterococci strains were used. As shown in Fig. 1a, high AU values were observed, indicating that the enterocins identified in this study had high bactericidal activity. There was an increase in the AU values from 6 to 12 h (400 to 6400 AU/mL, respectively) for most of enterocins that were tested.

These data are a good indication of the action of these enterocins over time because their main attribute as food biopreservatives is to remain active as long as possible for the safety of food products. In addition, their susceptibility to proteases demonstrates that they are easily digested by enzymes in the gastrointestinal tract without affecting the normal microbiota.

The effect of enterocin activity as bactericidal substances was evaluated against *L. innocua* 12612 (Fig. 2). Our data showed that bacteriocins of enterococci were effective at decreasing *L. innocua* cell viability, in the 0–4 h period, except for the enterocin that was obtained from Efs18 h, and this effect was observed after 2 h. The bactericidal characteristic was confirmed because there was a decrease in OD and viability measured by CFU. *L. innocua* was previously deemed to be a suitable biological indicator for *L. monocytogenes* (Rocha et al. 2019) and the strain showed a similar sensitivity to the bacteriocin as eight other *L. monocytogenes* isolates that were tested. It was used as a pathogen surrogate throughout the study.

Although several enterocins differ in their molecular structures, the mode of bactericidal action is similar. Contrary to Jaouani et al. (2014), in this study, there was a correlation between the presence of

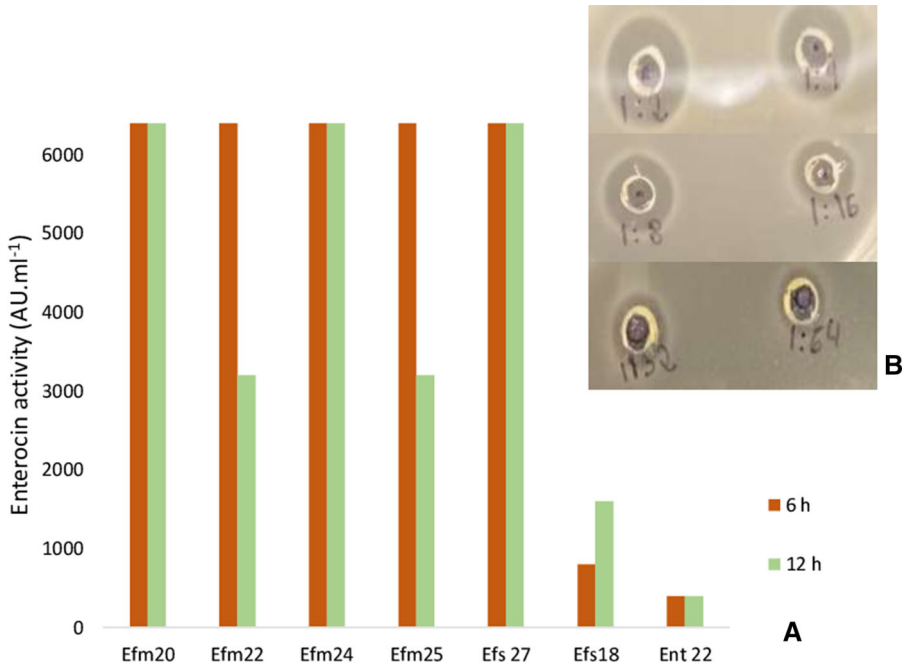

Fig. 1 **a** Anti-listerial activity of enterocins expressed as arbitrary units (AU/mL). **b** Inhibitory activity of enterocins against *Listeria innocua* 12,612, at 12 h incubation, using an agar well diffusion assay

enterocin structural genes as well as the values of arbitrary units and the activity spectrum of bacteriocinogenic strains.

The enterocins that were evaluated in this study showed characteristics that were of interest as well as potential activity against clinical isolates and species that are known as contaminants and pathogens in food, which make them potential food biopreservatives. This can help to increase the shelf life of food as well as the food supply and human safety.

Proper evaluation of the potential application of these isolates and their antimicrobial peptides in food processing should be based on their activity and mode of action under conditions that reproduce those used in food products, as performed by Pingitore et al. (2012) and Mogoşanu et al. (2017). However, Maia et al. (2019) showed that the conditions of food products that did not affect the antagonistic action of enterocins Efm20 and Efm22.

SEM analyses revealed that exposure of *L. innocua* and *B. subtilis* to bacteriocin Efm22 resulted in a coarse, collapsed surface of the cell with surface protuberances that may indicate cellular leakages (Fig. 3b and d). According to Masias et al. (2017) class-IIa bacteriocins bind to bacterial cell envelope associated mannose phosphotransferase system, leading to pore formation. Our results suggest that bacteriocins produced by enterococci represent potential character for promising future applications to control pathogenic *Listeria* species. The SEM images showed that non-treated *Listeria* and *Bacillus* cells had typical rod shapes with smooth surfaces (Fig. 3a and c), while enterocin-treated cells displayed alterations resembling cell membrane damage and the presence of extracellular material (Fig. 3b and d).

The results obtained provide new insights on antibacterial activity produced by *E. faecium* and *E. faecalis* strains against food borne and spoilage bacteria, concomitantly.

Conclusions

The antimicrobial capacity of enterococci has been well studied because of the search for alternative forms of antimicrobials and food biopreservatives in

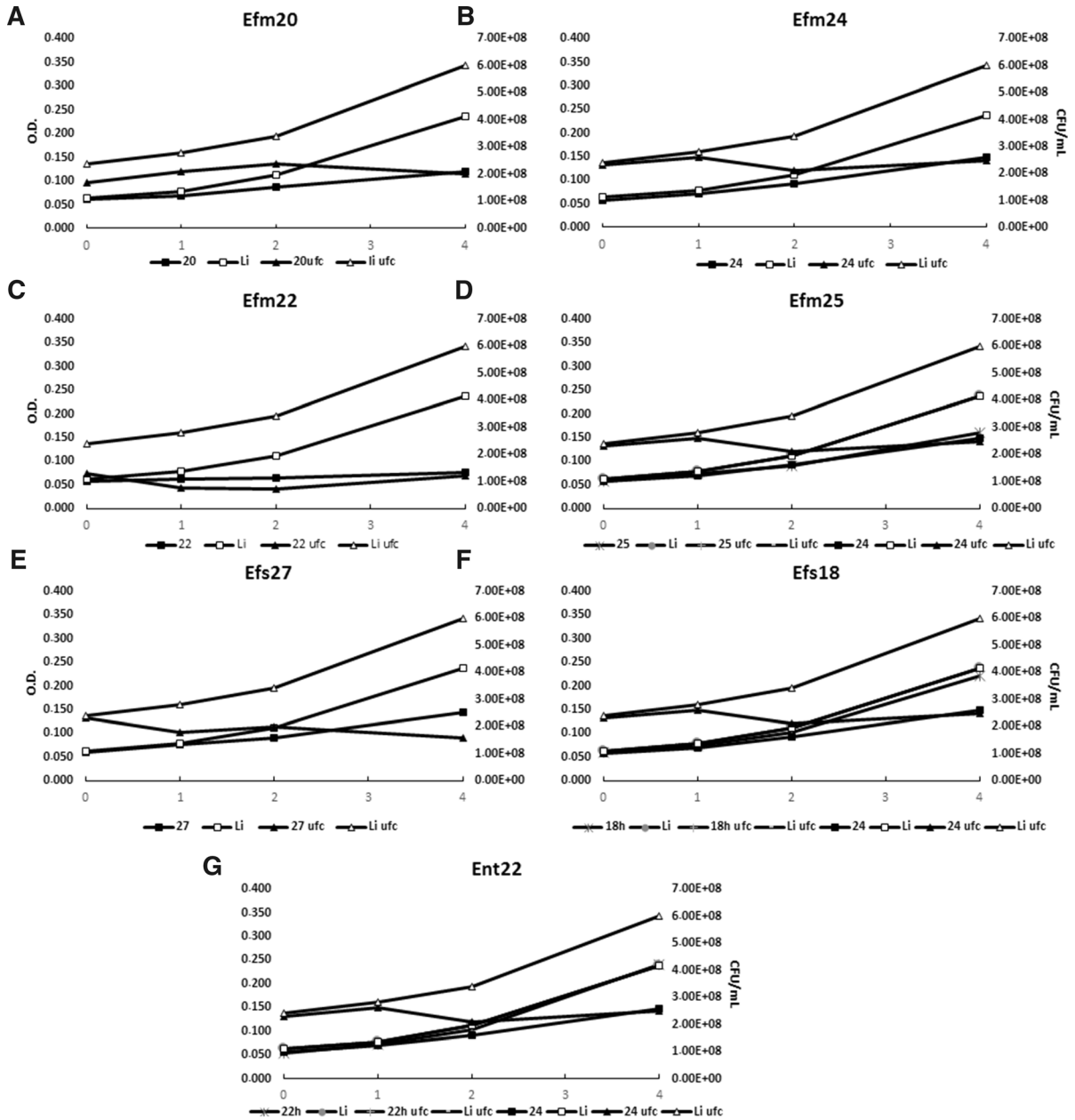

Fig. 2 Mode of inhibition of enterocin at an optical density (filled square, OD) of 600 nm and the number cells (filled triangle, CFU/mL) of *Listeria innocua*. Controls (open symbols)

the pharmaceutical and food industries, respectively. The antimicrobial activity that was observed in the supernatants from enterococcal cultures against bacteria that are important in the contamination and pathogenicity of food and against bacteria of clinical origin makes these isolates promising candidates in an alimentary and/or pharmaceutical context.

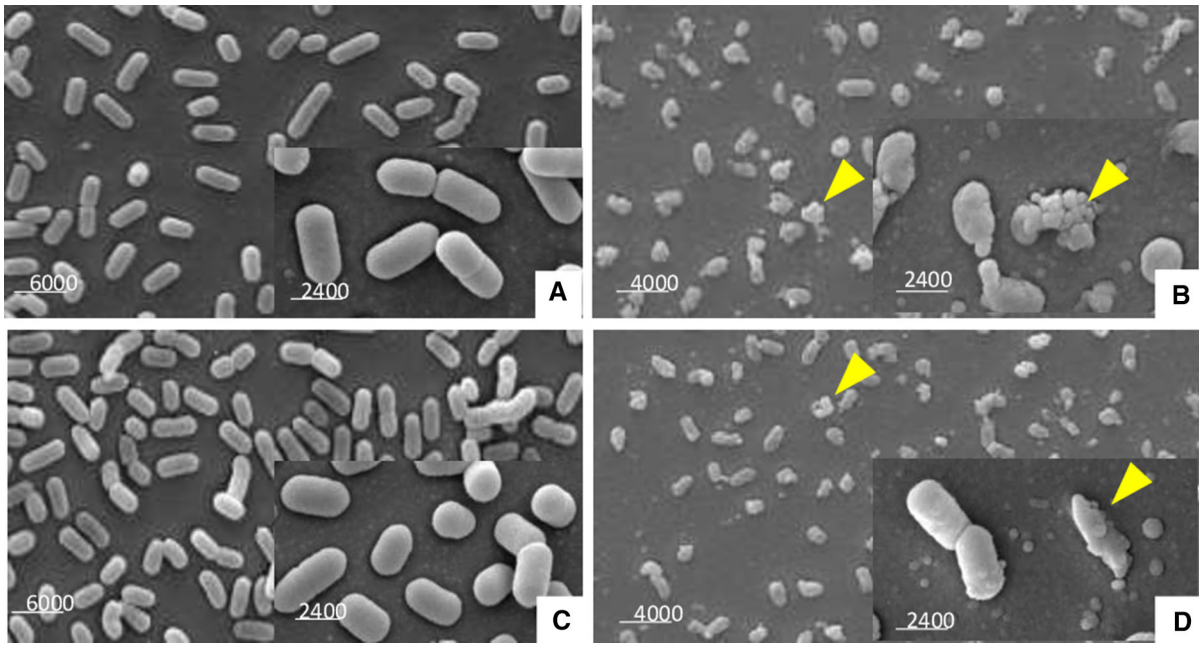

Fig. 3 Scanning electron micrographs of *Listeria innocua* and *Bacillus subtilis* cells after 18 h growth (control) (**a**, **c**), respectively and effect of enterocin Efm22 on cells (**b**, **d**). Cell membrane damage and flocculant extracellular material in the presence of Efm22 are indicated by yellow arrows heads

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. The founding sponsors had no role in the design of the study, nor in the data collection, analyses, or interpretation of data, the writing of the manuscript, nor the decision to publish the results.

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