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Enterococcus faecium Isolated from Honey Synthesized Bacteriocin-Like Substances Active against Different *Listeria monocytogenes* Strains

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Four *Enterococcus faecium* strains, isolated from honeycombs (C1 and M2d strains) and feral combs (Mori1 and M1b strains) secreted antimicrobial substances active against fourteen different *Listeria* spp. strains. The antimicrobial compound(s) present in the cell free supernatant were highly thermostable (121°C for 15 min) and inactivated by proteolytic enzymes, but not by α-amylase and lipase, thus suggesting a peptidic nature. Since the structural bacteriocin gene determinants of enterocins A and B were PCR amplified from the four *E. faecium* isolates, only the bacteriocin produced by strain C1 was further characterized: it showed a broad band of approximately 4.0-7.0 kDa in SDS-PAGE and was bactericidal (4 log decrease) against *L. monocytogenes* 99/287. *L. monocytogenes* 99/287R, a clone spontaneously resistant to the enterocin produced by *E. avium* DSMZ17511 (ex PA1), was not inhibited by the enterocin-like compounds produced by strain C1. However, it was inhibited in mixed culture fermentations by *E. faecium* C1 and a bacteriostatic effect was observed. The bacteriocin-producer *Enterococcus* strains were not haemolytic; gelatinase negative and sensitive to vancomycin and other clinically relevant antibiotics.

***Keywords*:** *E. faecium*, honey, *L. monocytogenes*, enterocins, bacteriocin-resistance

The primary sources of the honey microbiota are likely to include pollen, the digestive tract of honeybees, dirt, dust, air, soil, and flowers. The microbiota associated with honey are constituted by osmophile yeasts and sporulated bacteria of the *Bacillus* and/or *Paenibacillus* genera and, in some cases, by *Clostridium* spp. spores (Snowdon and Cliver, 1996; Olaitan *et al*., 2007; Lee *et al*., 2008). Iurlina and Fritz (2005) analyzed and characterized the microorganisms present in different Argentinean honeys, focusing their attention on the detection of bee pathogens such as *Paenibacillus larvae*, the agent of American foulbrood (Williams, 2000) or human pathogens such as *Clostridium botulinum*, *Escherichia coli*, *Salmonella* spp., *Shigella* spp., etc. Snowdon and Cliver (1996) also mentioned the presence of the *Streptococcus*/*Enterococcus* genus associated with bee microbiota. Interestingly, even though Olaitan *et al*. (2007) describe it as a reservoir for microbes, the presence of lactic acid microbiota associated with honey has been scarcely studied. Perhaps, one of the reasons is that selective culture media have not always been used to isolate or detect them. Lactic acid bacteria (LAB) are normal inhabitants of both the human and animal digestive tract (Devriese *et al*., 1987; Murray, 1990; Audisio *et al*., 1999; Franz *et al*., 2003). Besides, different probiotic supplements, not only for human but also for animals, include *Lactobacillus* and *Enterococcus* strains in their composition (Pollmann *et al*., 2005; Tompkins *et al*., 2008; Vankerckhoven *et al*., 2008). Many *E. faecium* strains can also produce bacteriocins (Aymerich *et al*., 1996; Cintas *et al*., 1997; Ennahar *et al*., 2001; Giraffa, 2002; Audisio *et al*., 2005; Foulquié-Moreno *et al*., 2006; Texeira *et al*., 2006; Franz *et al*., 2007; Ghrairi *et al*., 2008). Bacteriocins are ribosomally synthesized, bioactive peptides or proteins with a bactericidal or bacteriostatic effect on closely related species (Jack *et al*., 1995). These peptides are of interest due to their potential application in food presservation since one of the distinctive characteristics of some enterocins is their strong listericidal effect, in particular those belonging to class IIa pediocin-like bacteriocins (Cleveland *et al*., 2001).

In this work, the phenotypic characteristics and safety profile of four bacteriocin-producer *Enterococcus* strains isolated from honey samples from commercial apiaries and feral combs (i.e., wild combs) are described. Furthermore, the preliminary identification of the antilisterial compounds produced by these strains is reported.

Materials and Methods

Honey processing and lactic acid bacteria isolation

Six different honey samples from the Argentinean Northwest towns of Tartagal, Dragone, Morillo and Cachi (Salta), Yuto (Jujuy) and Atamisky (Santiago del Estero) were aseptically gathered by the beekeepers of each place. At the laboratory, equal amounts of each honey sample and sterile distilled water were aseptically mixed and the microbiota were studied by plating directly 100 μl onto de Man-Rogosa-Sharpe agar (MRS, Britania, Argentina), *Streptococcus* selective medium (SSM), prepared in our laboratory as indicated by Audisio *et al.* (2005), and brain-heart infusion agar (BHI, Britania). SSM and

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BHI plates were incubated at 37°C for 24-48 h under aerobic conditions while MRS plates were incubated at 37°C for 48-72 h, either under microaerophilic conditions (about 7% v/v O_2 and 14% v/v CO_2 pressures) or without atmosphere control.

Phenotypic characterization of enterococci strains

White, small, round colonies, recovered from SSm and BHI plates, were purified on BHI agar (1.5% granulated agar; Britania) at 37°C, analyzed by light microscopy (100×) and phenotypically characterized using the classic biochemical tests: nitrate reduction, Voges Proskauer, catalase, arginine hydrolysis, esculine fermentation, and growth at 45°C for 24 h, at 8°C for 5 days, at pH 9.6, and in the presence of 4.5 and 6.5% (w/v) NaCl (Devriese *et al*., 1992). Carbohydrate fermentation patterns were determined with APICH50 (Biomérieux, France). Isolated enterococci were preserved as frozen stocks in BHI broth plus 10% (v/v) glycerol at -20°C.

Indicator microorganisms

The indicator microorganisms (Table 1) were grown in BHI broth at 37°C without a special atmosphere, with the exception of *Paenibacillus larvae* strains which were grown in MPYGP broth at 37°C under microaerophilic conditions (Dingman and Stahly, 1983). All indicator strains were kept at -20°C in both skim milk (9%, w/v) with the addition of yeast extract (0.5%, w/v), and in BHI broth with glycerol (10%, v/v). It is important to note that *L. monocytogenes* 99/287R is a spontaneously resistant clone to the bacteriocin-like substances produced by *E. avium* DSMZ17511 (ex PA1), another lactic acid bacterium studied in our laboratory (Audisio *et al*., 2005). This clone was detected as satellite colonies in the inhibition halo when analyzing antimicrobial production of *E. avium* DSMZ17511 by the well diffusion assay using *L. monocytogenes* 99/287 as a sensitive strain.

Antimicrobial spectra screening by the well diffusion assay

Cell-free supernatants (CFS) of the isolated microorganisms, grown in BHI broth at 37°C for 6 h, were adjusted to pH 6.0 with sterile NaOH 2N, filter sterilized (0.45 µm) and kept at 4°C until used. Their effects on the different indicator microorganisms were studied using the well diffusion assay as described by Audisio *et al*. (2005). Briefly, 20 µl of the CFS under study were sieved in 5 mm wells made in BHI agar plates inoculated with ca. 10^7 CFU/ml of the sensitive strain. Plates were incubated at 30°C for 12-24 h and examined for inhibition halos.

16S rDNA gene sequencing

The 16S rDNA gene sequence of strain C1, selected by its antimicrobial spectrum, was determined (Macrogen Inc., Korea). Total genomic DNA was extracted by alcaline lysis from an overnight BHI culture and PCR reactions were carried out according to Martínez and Siñeriz (2004) with universal eubacterial primers S-D-BACT-1494-A-S-20 (GTCGTAACAAGGTAGCCGTA) and L-D-BACT-0035-A-A-15 (CAAGGCATCCACCGT) (Daffonchio *et al*., 1998), to amplify the 16S rDNA gene sequences. Control reaction mixtures lacking a DNA template were also included in each experiment. On-line similarity searches of strain C1 16S rDNA sequences were performed with the BLAST program (http://www.ncbi.nlm.nih.gov). The 16S rDNA nucleotide sequence data of *E. faecium* C1 has been deposited in GenBank under the accession number EU428011.

Enterococci safety profile

Haemolytic activity was tested in fresh cultures of the enterococci isolates, streaked on Columbia agar plates containing 5% (v/v) sheep blood (Laborkit, Argentina), and incubated for 48 h at 37°C. Subsequent clearing around the colonies indicated β-haemolysin production. In the gelatinase assay, enterococci were inoculated on Todd-Hewitt agar (Britania) supplemented with gelatin (30 g/L) and incubated for 48 h at 37°C. Then the surfaces of the plates were covered with the developing solution (15% $HgCl_2$ in 20% HCl). After cooling at 4°C, turbidity loss around the colonies was monitored.

Tests for antibiotic susceptibility were performed by the agar diffusion method, according to the guidelines of the Clinical and Laboratory Standards Institute for all the strains isolated. The antibiotics tested were ampicillin (AMN 10 µg), chloramphenicol (CMP 30 µg), ciprofloxacin (CIP 5 µg), clindamycin (CLI 2 µg), erythromycin (ERY 15 µg), penicillin G (PEN 10 IU), rifampicin (RFA 5 µg), teicoplanin (TEI 30 µg), tetracycline (TET 30 µg), oxacilin (OXA 1 µg), furazolidone (FUR 100 µg); minocycline (MIN 30 µg); cephotaxime (CTX 30 µg) and vancomycin (VAN 30 µg). Fresh cultures of enterococcal strains, grown in BHI, were diluted to 0.5 of McFarland scale (close to 1×10^8 CFU/ml) and spread on Mueller-Hinton agar (Britania). Inhibition zone diameters were measured after overnight incubation of the plates at 37°C. The inhibition results were evaluated according to the manufacturer's instructions and to the break points recommended by the NCCLS antimicrobial susceptibility testing standards M2-A7 (NCCLS document, 2000).

Physicochemical characterization of antimicrobial substances

Heat resistance was studied after each CFS was heated at 70°C for 30 min, 100°C for 15 min and 121°C for 15 min (in an autoclave), cooled and tested for antimicrobial activity by the well diffusion assay using *L. monocytogenes* 99/287 as the indicator strain. An aliquot without thermal treatment was used as a control.

The effect of trypsin, proteinase K, pronase E, α-chymotrypsin, pepsin, α-amylase, lipase, and catalase (Sigma) was also determined on each CFS at pH 6.0. All enzyme solutions (except for lipase which is provided supported in a solid), were prepared in a phosphate buffer (pH 7.00, 0.05 M) and put in contact with the respective CFS to get a final enzyme concentration of 1 mg/ml in the reaction media. After 1 h, the reaction system was heated at 100°C for 1 min to stop enzyme effect and the antimicrobial activity was determined by the well diffusion assay. *L. monocytogenes* 99/287 was used as an indicator strain. A control of each CFS without enzyme treatment was performed.

SDS-PAGE analyses

To determine the molecular size of bacteriocin-like substances the peptides present in the CFS of the different *E. faecium*, without any previous concentration and/or purification steps, were separated by Tricine-SDS-PAGE as described by Schägger (2006). The polyacrylamide concentration on the separating gel was 16% (w/v). Electrophoresis was set at a constant voltage of 50 V for 1 h, 75 V for 2 h and finally 150 V for 1 h. A low rainbow molecular weight marker was used, with sizes ranging from 3.5 to 38 kDa (Amersham Pharmacia, Germany). The gel was fixed with an isopropanol (20%, v/v) - acetic acid (10%, v/v) solution, exhaustively washed with distilled water, and overlaid on BHI agar inoculated with *L. monocytogenes* 99/287, which had been previously prepared from a 16 h culture of the pathogen in BHI broth to give a final concentration of *L. monocytogenes* ca. 1×10^7 CFU/ml.

Bacteriocin-like compounds kinetic production

The production of bacteriocin-like compounds by enterococci was analyzed on BHI broth at 37°C. Samples were taken at different times

Table 1. Antimicrobial activity spectra of cell-free supernatant of the different *E. faecium* isolated from honey determined by the well-diffusion assay

Indicator strain	Source	C1	Mori1	M1b	M2d
Paenibacillus larvae IV	INTA	-	-	-	-
Paenibacillus larvae Azul	INTA	-	-	-	-
Paenibacillus larvae II	INTA	-	-	-	-
Enterococcus hirae 8043	ATCC	+	+	+	+
Enterococcus avium 137/96	Malbrán	+	+	+	+
Enterococcus faecium CA	CA	+	+	+	+
Enterococcus faecium 1385	CRL	-	-	-	-
Enterococcus avium 17511	DSMZ	-	-	-	-
Enterococcus faecium C1	CA	-	-	-	-
Enterococcus faecium Mori1	CA	-	-	-	-
Enterococcus faecium M1b	CA	-	-	-	-
Enterococcus faecium M2d	CA	-	-	-	-
Enterococus faecium CA12	CA	-	-	-	-
Enterococcus faecium SM7	CA	-	-	-	-
Salmonella enterica serovar Typhimurium	INTA	-	-	-	-
Salmonella enterica serovar Enteritidis	INTA	-	-	-	-
Pseudomonas aeruginosa 27853	ATCC	-	-	-	-
Staphylococcus aureus 29213	ATCC	-	-	-	-
Bacillus subtilis subsp. *niger* C4	CA	-	-	-	-
Bacillus spp. M1d	CA	-	-	-	-
Bacillus spp. Cachi2	CA	-	-	-	-
Bacillus subtilis Mori2	CA	-	-	-	-
Listeria monocytogenes 99/287	Malbrán	+	+	+	+
Listeria monocytogenes 01/198	Malbrán	+	+	+	+
Listeria monocytogenes 00/288	Malbrán	+	+	+	+
Listeria monocytogenes 00-3/364	Malbrán	+	+	+	+
Listeria monocytogenes 01/200	Malbrán	+	+	+	+
Listeria monocytogenes 99/320	Malbrán	+	+	+	+
Listeria monocytogenes 01/155	Malbrán	+	+	+	+
Listeria monocytogenes 99/267	Malbrán	+	+	+	+
Listeria spp. 01/61	Malbrán	+	+	+	+
Listeria spp. 00/282	Malbrán	+	+	+	+
Listeria spp. 99/316	Malbrán	+	+	+	+
Listeria spp. 00/360	Malbrán	+	+	+	+
Listeria spp. 99/128	Malbrán	+	+	+	+
Listeria monocytogenes 99/287R	CA	-	-	-	-
Fusarium sporotrichoides	LC	-	-	-	-
Aspergillus niger	LC	-	-	-	-
Penicilium viridicatum	LC	-	-	-	-

American Type Culture Collection; CRL, Centro de Referencias para Lactobacilos; Malbrán, Instituto de Microbiología "Dr. Carlos Malbrán", Bs. As, Argentina; DSMZ, German Resource Centre for Biological Material; INTA, Instituto Nacional de Tecnología Agropecuaria, Balcarce, Bs. As., Argentina; LC, Leonor Carillo, Fac. Cs. Agrarias, Universidad Nacional de Jujuy, Argentina; CA, Carina Audisio, INIQUI-CONICET, Universidad Nacional de Salta, Argentina.
(+): halo diameter > 5 mm, (–): no zone of inhibition.

to record bacteriocin synthesis and changes in cell viability. The titer of bacteriocin-like substances was determined by a serial two-fold dilution and expressed in arbitrary units per milliliter, AU/ml, with *L. monocytogenes* 99/287 as the indicator strain (Daba *et al*., 1991). AU/ml were calculated as follows: $1000/(V_s \times D)$ were V_s: bacteriocin volume tested (µl) and D: highest dilution still inhibiting sensitive strain growth. Viable indicator cells were determined by plating in duplicate using BHI (1.5%, w/v) agar. These plates being incubated at 37°C for 24 h.

PCR amplification of enterocin genes

Chromosomal DNA was isolated from 5 ml of *E. faecium* cultures

grown in BHI broth for 16 h, using the method described by Pospiech and Neumann (1995). The presence of enterocin-encoding genes was studied by PCR amplification, with the same primers as described by du Toit *et al*. (2000) for the following well-known enterococcal bacteriocins: enterocin A (Aymerich *et al*., 1996); enterocin B (Casaus *et al.*, 1997); enterocin P (Cintas *et al.*, 1997); enterocin 31 (Tomita *et al.*, 1996) and enterocin L50A/B (Cintas *et al.*, 1998). PCR reactions were carried out with a final volume of 25 µl, containing 10 mM Tris-HCl, 4 mM $MgCl_2$, 0.2 mM each dNTPs, 0.8 µM each primer, ≈ 100 ng of genomic DNA and 2.5 U Taq-polymerase (Invitrogen). After an initial denaturation at 94°C for 5 min, the samples were amplified for 30 cycles at 94°C for 1 min, at 50°C (enterocin B, P, 31), 52°C (enterocin L50) or 56°C (enterocin A) for 1 min and at 72°C for 1 min; a final extension at 72°C for 5 min was also completed. PCR products were analyzed by electrophoresis (85 V for 1 h 15 min) on 2% (w/v) agarose gels in 1× TAE buffer. These gels were stained with GelRed (Invitrogen) and observed under UV light. The sizes of the amplified fragments were determined using 100-bp DNA Ladder (Invitrogen) as a molecular weight marker.

Analysis of the response of *L. monocytogenes* strains, bacteriocin-resistant or sensitive, to the bacteriocin-like substances synthesized by *E. faecium* C1

Two different cultures of *L. monocytogenes* 99/287 were used for these experiments: *L. monocytogenes* 99/287 (sensitive to all bacteriocin-like compounds assayed in this work) and *L. monocytogenes* 99/287R, a resistant-mutant to the bacteriocin/s produced by *E. avium* DSMZ17511, as indicated above. Strain response to *E. faecium* C1 bacteriocin-like compounds was analyzed using microplate direct contact and by mixed culture growth with the bacteriocin producer strain.

Microplate direct contact: *Listeria* cells from overnight cultures grown in BHI broth were harvested by centrifugation at 5,000×g for 10 min at 4°C, washed twice in phosphate buffer 0.05 M, pH 7.0, then diluted in the same buffer in order to obtain a suspension ca. 10^9 CFU/ml. The effect of the *E. faecium* C1 enterocin-like compound on each *Listeria* viability was analyzed at three different concentrations: 12,800, 6,400, and 3,200 AU/ml. Ninety-six well microplates were used and the different CFS's were put in direct contact with the indicator strains' suspensions at a 1/10 ratio at 37°C for 0.5, 1, 1.5, and 2 h. Viable indicator cells were determined by plating in duplicate using BHI (1.5%, w/v) agar. The plates were incubated at 37°C for 24 h.

Mixed culture fermentations of *E. faecium* C1 with *L. monocytogenes* 99/287s and *E. faecium* with *L. monocytogenes* 99/287R were carried out in BHI broth with an inoculum of 1% (v/v) of each strain. A pure control culture of each *Listeria* strain was also analyzed in BHI broth in the same concentration. The *Listeria* viable number was determined in duplicate by plating on BHI agar cells from the pure culture and on *Listeria* Oxford selective agar (Merck) cells from mixed culture with *E. faecium* C1. All plates were incubated at 37°C for 24 h.

Results

Pheno- and genotype characteristics of the isolated enterococci

The microbiota of six different honey samples from the Argentinean Northwest were analyzed. Two came from feral combs (Morillos and Dragone) and four from commercial apiaries (Tartagal, Yuto, Galpón, and Atamisky). It is important to mention that these places are, in general, more than 150 km from each other. Bacterial growth was not detected on MRS agar from the honey samples analyzed. Twenty colonies with typical lactic bacteria characteristics were recovered from both *Streptococcus* Selective Medium agar (10 colonies) and BHI agar (10 colonies). All of them were transferred to BHI broth and, after 24 h of incubation at 37°C, their cellular morphology was determined under the microscopy (100×). Only those colonies coming from the selective agarized medium were catalase negative and Gram-positive cocci and were phenotypically characterized as *Enterococcus* spp. due to their answers to classic biochemical tests for this genus. Four of them (C1, M1b, M2d, and Mori1), selected for their antilisterial activity (see below), belong to the *faecium* species according to their fermentation profile (API CH50 galleries). Furthermore, the *E. faecium* C1 16S rDNA sequence exhibited 99% DNA sequence identity to database entries associated with known *Enterococcus* species.

Antimicrobial activity of isolated enterococci strains according to the well diffusion assay

A screening for the presence of antimicrobial compounds in the cell-free supernantants (CFS) of the ten isolated enterococci, was performed by the well-diffusion assay. Four of them inhibited *E. hirae* ATCC 8043, *E. avium* 137/96, and all the *Listeria monocytogenes*' strains tested. However, they failed to inhibit *Paenibacillus larvae* or Gram-positive bacteria such as *Lactobacillus* spp., *Staphylococcus aureus*, *Bacillus subtilis*, *E. faecium* CRL1385 (ex-J96), and *E. avium* DSMZ17511, the last two being bacteriocin producer strains (Audisio *et al*., 1999, 2005). Gram-negative pathogens like *Salmonella*, *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* and the different molds were not inhibited by these compounds either. The results obtained for the strains with positive anti-*Listeria* effects are presented in Table 1. Besides, CFS of the four selected cocci (C1, Mori1, M1b, M2d) inhibited *L. monocytogenes* 99/287, but they did not inhibit *L. monocytogenes* 99/287R by this technique (Fig. 1).

Physicochemical characterization

The anti-*Listeria* activity observed in the CFS of the four selected strains, filtered and adjusted to pH 6.0 with NaOH, disappeared after treatment with 1 mg/ml of the following proteolytic enzymes: pepsin, α-chymotrypsin, trypsin, pronase E, and proteinase K. Lipase, catalase, and α-amylase did not affect their inhibitory effects. The compounds were highly

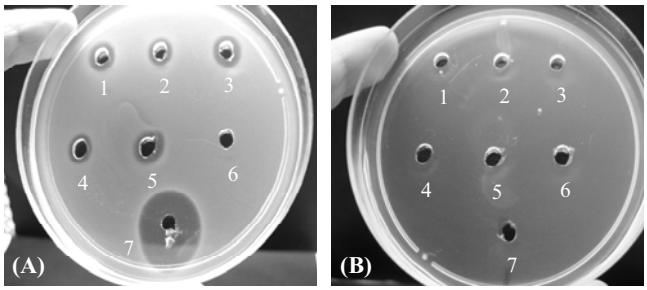

Fig. 1. Antimicrobial effect of the different *E. faecium* CFS on (A) *L. monocytogenes* 99/287 and (B) *L. monocytogenes* 99/287R, bacteriocin-resistant mutant, by the well diffusion assay (1, C1; 2, Mori1; 3, M2d; 4, M1b; 5, J4b; 6, control of culture medium; 7, *E. avium* DSMZ17511 CFS)

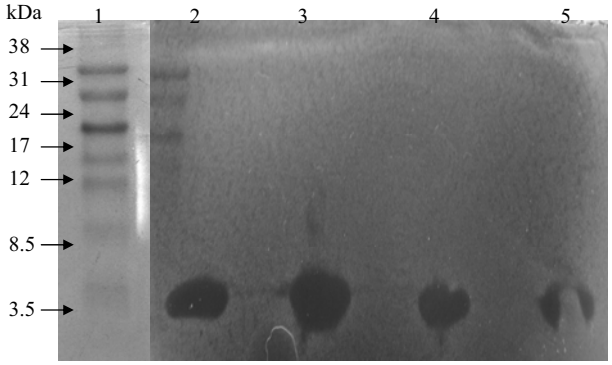

Fig. 2. Tricine-SDS-PAGE gel showing the zone of growth inhibition of *L. monocytogenes* 99/287 by the band of the CFS of the different *E. faecium*. strains isolated from honey. Lanes: 1, molecular weight marker; 2, C1; 3, Mori1; 4, M2d; 5, M1b.

thermostable because they retained their antagonistic activity not only after 30 min at 70°C and 15 min at 100°C, but also after autoclave sterilization (121°C for 15 min). After the three different thermal treatments, no significant differences were detected in halo diameter with reference to the controls. The analysis of the CFS proteins by Tricine-SDS-PAGE, and further antimicrobial assays against *L. monocytogenes* 99/287, showed a similar profile for the four entecococci. As Fig. 2 shows for the CFS of *E. faecium* C1, Mori1, M2d, and M1b, with 51200, 25600, 12800, and 25600 UA/ml titers respectively, a broad band with a molecular weight of approximately 4.0-7.0 kDa, was detected on *L. monocytogenes* 99/287 lawn.

Bacteriocin-like compounds production

E. faecium C1, M1b, M2d, and Mori1 reached the late exponential phase after 6 h at 37°C in BHI broth, and the number of viable microorganisms was similar for all enterococci (cell count 8.5±0.3 log CFU/ml). Bacteriocin-like compounds synthesis occurred together with bacterial growth (Fig. 3). The anti-*Listeria* activity, determined by the well-diffusion assay against *L. monocytogenes* 99/287, was detected after 2 h of incubation in BHI broth at 37°C (Fig. 3). The four strains were able to grow at low temperature; after 4 days at 8±2°C (with an initial inoculum of *ca*. 1×10^6 CFU/ml) a cell sediment was observed at the bottom of the tube. Bacteriocin-like activity against *L. monocytogenes* 99/287 was also measured in

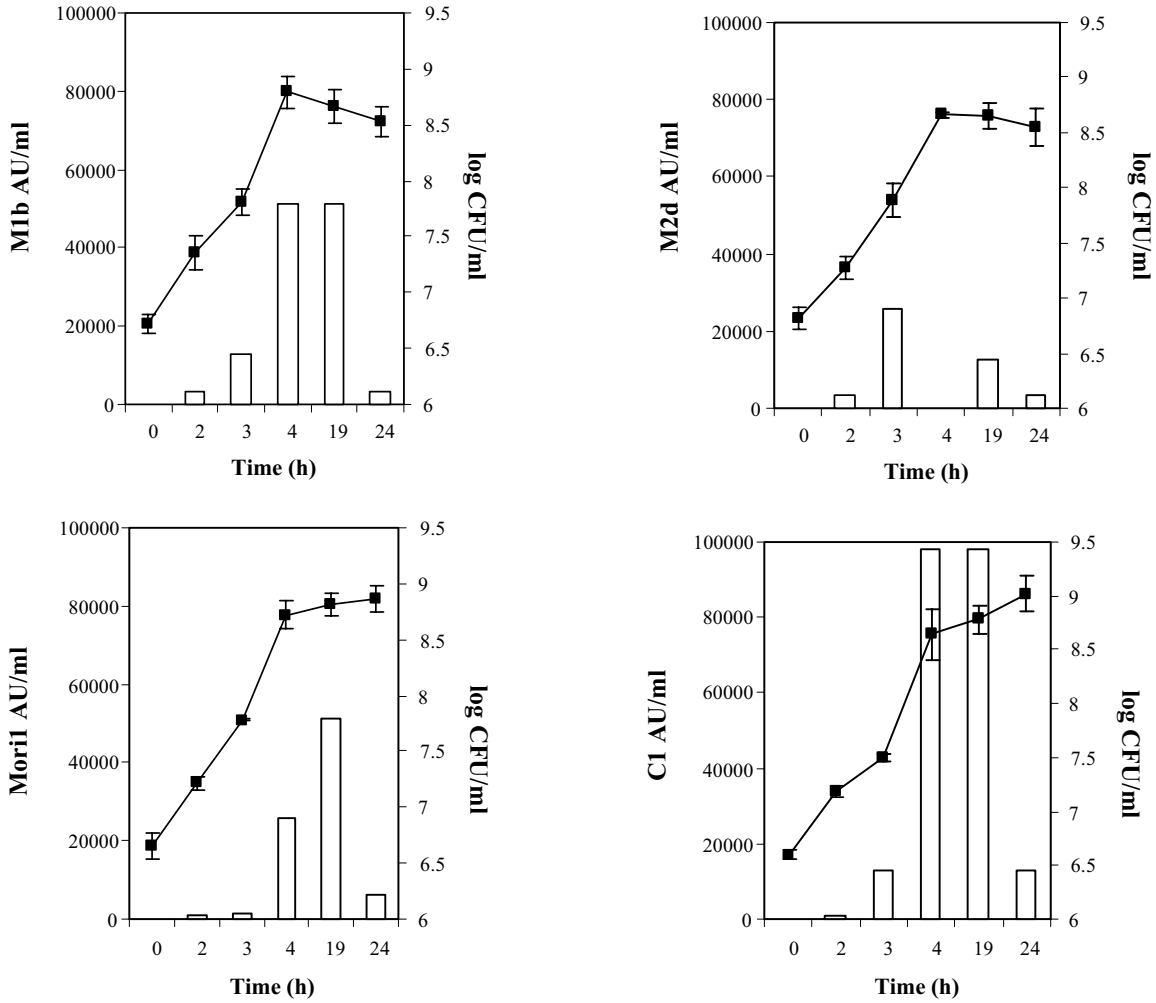

Fig. 3. Kinetics of culture growth (■) and bacteriocin-like compounds synthesis (bars) by the different *E. faecium* strains isolated from honey. *L. monocytogenes* 99/287 was used as the indicator microorganisms.

Table 2. Antibiogramme profile of the different honey-associated *E. faecium* strains

Antibiotic	Strain			
	C1	M1b	M2d	Mori1
VAN (30 µg)	S	S	S	S
RFA (5 µg)	S	S	I	I
MIN (30 µg)	S	S	S	S
CHP (30 µg)	S	S	S	S
TEI (30 µg)	S	S	S	S
PEN (10 U)	S	S	S	S
OXA (1 µg)	R	R	R	R
ERY (15 µg)	S	S	S	S
CLI (2 µg)	R	R	R	R
TET (30 µg)	S	S	S	S
AMP (10 µg)	S	S	S	S
FUR (100 µg)	I	I	I	I
CIP (5 µg)	S	S	S	S
CTX (30 µg)	R	R	R	R

S, sensitive ≥18 mm; I, intermediate 13-17 mm; R, resistant ≤12 mm

the CFS of the enterococci cells grown at refrigerator temperature (around 8°C). In this case, the titers detected were 60% lower than those of CFS from cultures grown for 6 h at 37°C.

Safety aspects of the *E. faecium* isolates

The potential safety of the different *E. faecium* isolates was evaluated by hemolysin and gelatinase production and by antibiotic susceptibility testing. Even though the presence of genes encoding potential virulence factors was not evaluated by PCR assays, the four selected strains were found to be phenotypically negative for haemolysin and gelatinase. These *E. faecium* strains were sensitive to vancomycin, tetracycline, erythromycin, ampicillin, penicillin G, chloramphenicol, ciprofloxacin, and teicoplanin. They were resistant to clindamycin and oxacillin and presented a moderate susceptibility to furazolidone (Table 2).

Presence of enterocins structural genes in *E. faecium* strains

The study for the presence of different structural enterocin genes in *E. faecium* C1, M2d, Mori1, and M1b showed that all strains contained both entA and entB enterocin genes. Specific PCR fragments of 126- and 162-bp, corresponding respectively to enterocins A and B, were amplified in all these enterococci (Fig. 4). Moreover, no specific PCR fragments were observed with the set of primers for bacteriocins P, L50A/B, and 31.

CFS bactericidal and bacteriostatic effects of strain C1

An immediate bactericidal effect on *L. monocytogenes* 99/287 viability was always detected when C1 bacteriocin-like compounds were analyzed by the microplate technique. Cell viability decreased 3 or 4 orders, independently of the CFS titer (12800, 6400, and 3200 AU/ml, Fig. 5A). No *L. monocytogenes* 99/287R inhibition was found when similar titers of the CFS were mixed with the mutant strain (Fig. 5B).

In mixed culture with the lactic acid bacterium, the bacteriocin-sensitive *Listeria* strain showed the same inhibition behavior as with the microplate technique. However, the *Listeria* bacteriocin-resistant mutant was inhibited, and after

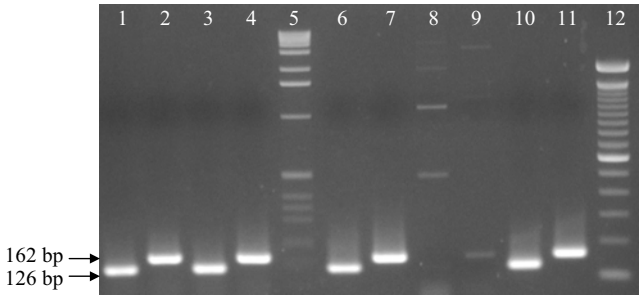

Fig. 4. Amplification of structural enterocin genes from DNA of the different *E. faecium* honey associated strains. Lanes: 1, M2d (Ent A); 2, M2d (Ent B); 3, Mori1 (Ent A); 4, Mori1 (Ent B); 5, 1 kb ladder (invitrogen); 6, C1 (Ent A); 7, C1 (Ent B); 8, negative control (Ent A); 9, negative control (Ent B); 10, M1b (Ent A); 11, M1b (Ent B); 12, 100 bp ladder (Invitrogen).

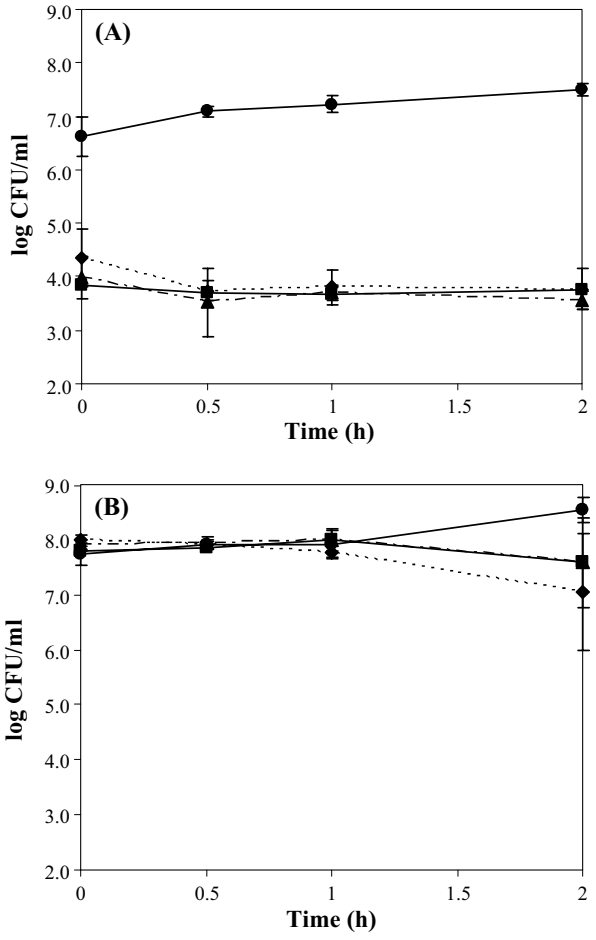

Fig. 5. Viability of (A) *L. monocytogenes* 99/287 and (B) *L. monocytogenes* 99/287R in direct contact with different concentrations of *E. faecium* C1 CFS using the microplate technique. (●) control in BHI broth, (■) CFS 12400 AU/ml, (▲) CFS 6400 AU/ml, (◆) CFS 3200 AU/ml.

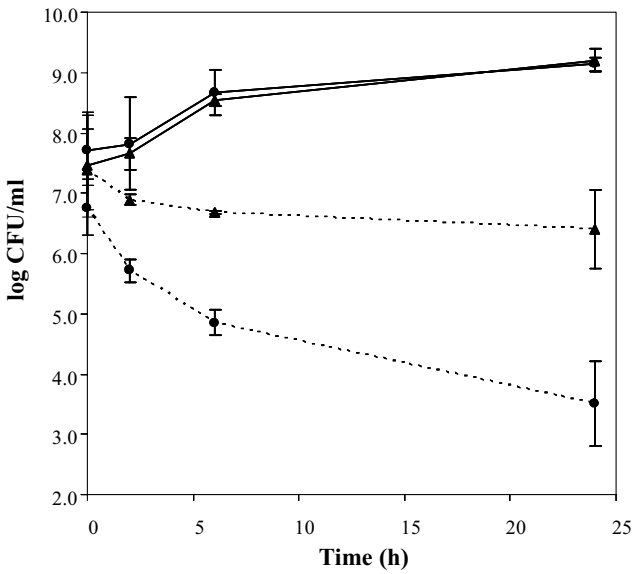

Fig. 6. Viability of *L. monocytogenes* 99/287 (sensitive) (●) and 99/287R (resistant-mutant) (▲) in mixed culture with *E. faecium* C1. Full lines, control; dotted lines, co-culture.

1 h of mixed culture at 37°C, a decrease of more than 1 log of viable cells was detected. Twenty four h later, the number of viable cells dropped to 3.0 and 6.4 log CFU/ml for the sensitive and resistant strains respectively, while controls reached 9.0 and 9.1 log CFU/ml (Fig. 6). These results confirm *E. faecium* C1 bactericidal effect observed by the microplate technique on *L. monocytogenes* 99/287 and suggest that C1 bacteriocin-like compound could control mutant strain 99/287R growth by means of a bacteriostatic mechanism.

Discussion

In this work, the possible presence of lactic acid bacteria associated with honey from different places of the Argentinean Northwest (NOA) was analyzed. The idea of studying this bacterial group in honey arose from the fact that scientific references about the subject were not too many. No *Lactobacillus* spp. colonies were detected at all on MRS agar; but ten different colonies grew on SSM agar Petri dishes inoculated with feral comb (4 colonies) and commercial apiary honey samples (6 colonies). The enterococcal strain isolation from honey might be due to the high resistance several species of this genus display in adverse environmental conditions, as reported by other author. Besides, enterococci are known to have some insects, plants and/or flowers as their habitat (Mundt, 1963; Franz *et al*., 2003).

As enterococci were mainly isolated and *E. faecium* bacteriocin-like compound producer strains had already been studied in our laboratory, we decided to determine their antimicrobial spectra and to ascertain if these strains could inhibit the growth of *P. larvae*, the American foulbrood etiological agent. This microorganism is a rod-shaped, sporulated, Gram-positive bacterium that causes an extremely contagious disease in honeybee larvae (Williams, 2000), and its control is extremely important for honey producers. Even though these enterococci failed to inhibit this bacterium, four of them presented an important anti-*Listeria monocytogenes* activity. They were phenotypified as *E. faecium*, and strain C1 was also genotypically characterized by the analyses of its 16S rDNA which, combined with classical biochemical tests, enabled us to confirm that it would belong to the *faecium* species (EU428011). This strain was selected because it presented the greatest antilisterial effect against the pathogens tested during the CFS antimicrobial screening. Eight different *L. monocytogenes* and six *Listeria* spp. strains were inhibited by the CFS of C1, M2d, Mori1, and M1b. With the exception of *E. avium* 137/96, *E. faecium* CA and *E. hirae* ATCC 8043, these substances did not inhibit other LAB. They did not inhibit each other or exert any antagonistic effect on other bacteriocin-producing enterococci such as *E. faecium* CRL1385 or *E. avium* DSMZ17511. This situation was observed before by other authors who informed that different class lla bacteriocins synthesized by lactic acid bacteria could present a similar anti-*Listeria* activity although they might exhibit a particular antimicrobial spectrum against related producer strains (Eijsink *et al*., 1998). The anti-*Listeria* compounds studied in this work were synthesized at 37°C during bacterial growth. They were small and highly thermostable and remained active without previous purification steps. Besides, the products of the amplification of bacteriocin structural genes evidenced that all four *E. faecium* strains could synthesize enterocins A and B. Thus, these properties also lead to stating that these molecules share some common features with class IIa bacteriocins (Ennahar *et al*., 2000; Drider *et al*., 2006; Franz *et al*., 2007). Many authors have reported that the occurrence of enterocin structural genes seems to be widespread among enterococci isolated from different hosts (Ennahar *et al*., 2001; Poeta *et al*., 2006). Strompfová *et al*. (2008) also observed that the enterocin A gene was exclusively detected among *E. faecium* strains, while the genes of enterocins P, B, and L50B were detected in strains of both *E. faecium* and *E. faecalis* species. Our results would agree with these observations, and they constitute the first report of this situation in *E. faecium* associated with honey. In this sense, only two articles have informed the presence in honey of different bacterial strains that displayed antimicrobial properties (Magnusson *et al*., 2003; Lee *et al*., 2008). Magnusson *et al*. (2003) delved deeper into the analysis of the bacteria found in honey and identified them, but they only isolated one *Enterococcus durans* strain with antifungal properties.

L. monocytogenes is known to be a very difficult pathogen to control in food. Although the incidence of listeriosis is relatively low, it presents a mortality rate of around 20-30% (Jurado *et al*., 1993; Tauxe, 2002; Ghandi and Chikindas, 2007). In Argentina and other countries, regulatory authorities have set a "zero tolerance" policy for ready to eat (RTE) foods that have an extended shelf-life and can support the growth of *Listeria* (FAO/OMS, 2004; ANMAT, 2006). Moreover, some authors have reported the emergence of *Listeria* strains that are resistant to nisin and to some class IIa bacteriocins (Davies and Adams, 1994; Naghmouchi *et al*., 2007). In this work, all fourteen *Listeria* strains tested were inhibited by the bacteriocin-like compounds produced by the four selected *E. faecium.* However, the effect of these molecules on a bacteriocin-resistant-mutant isolated in our group depended

on the detection technique used to measure such action. The agar diffusion method and the microplate technique revealed negative results. In co-culture with *E. faecium* C1, this bacteriocin resistant-mutant was inhibited and a bacteriostatic effect was observed. This result would suggest a sinergistic effect between the bacteriocin-like compound and the lactic acid produced by strain C1 on *Listeria* cells.

New bacteriocin-producing strains from different sources open interesting possibilities for the design of new bioprotectors. Bioprotectors could be metabolites (bacteriocins) or the producer strains (cells) and an integral knowledge of these microorganisms would increase the range of their possible applications. Hence, preliminary safety tests were performed on enterococci since they are not considered "Generally Recognized As Safe" (GRAS) like other lactic acid bacteria (Ogier and Serror, 2008). All four enterococci were haemolysin and gelatinase negative by phenotypical studies. Besides, they showed no resistance to vancomycin and they were susceptible to most clinically relevant antibiotics.

The current study shows that *E. faecium* from honey exhibit an inhibitory activity against different *Listeria monocytogenes* strains that may be either sensitive or resistant to bacteriocin substances. Thus, having new bacteriocin producer strains would be of strategic importance, especially if the pathogen does not present resistance against such bacteriocins *in vitro* assays.

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