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ORIGINAL PAPER

Characterization of antilisterial bacteriocins produced by *Enterococcus faecium* and *Enterococcus durans* isolates from Hispanic-style cheeses

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Abstract Enterococci are often identified as constituents of the indigenous microflora from raw milk artisanal cheeses and are believed to contribute to the unique organoleptic qualities of these products. Many strains of enterococci are also known to produce antimicrobial peptides, enterocins, which may prevent the growth of certain food-born pathogens. In this study 33 enterococcal isolates from Hispanic-style cheeses were screened for the production of bacteriocins. Of the 33 isolates, 5 *Enterococcus faecium* and 1 *Enterococcus durans* isolates inhibited the growth of *Listeria* spp. The antilisterial activity was lost after treatment with pepsin, trypsin, pronase, proteinase K and α-chymotrypsin suggesting the active component was a protein or peptide. The active compounds were heat stable and had molecular weights between 4 and 8 kDa, which is characteristic of Class II enterocins. A PCR screen showed that four *E. faecium* isolates contained nucleic acid sequences for multiple enterocins. Isolate H41K contained *entA* and *entP*; and isolates H51Ca, H51Cb and H41B contained *entA*, *entP* and *entL50AB,* with H41B also containing *entB*. All PCR tests performed were negative for *E. faecium* isolate H41D, suggesting the production of a novel enterocin. The isolates were also screened for susceptibility to antibiotics, with only two showing low-level resistance to vancomycin (8 $\mu g\ ml^{-1}$). However, three isolates were highly resistant to both tetracycline and kanamycin, with two of the isolates also showing high resistance to erythromycin.

Keywords Enterocins · Antilisterial · Raw milk cheese

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Introduction

Enterococci are lactic acid bacteria (LAB) that constitute part of the normal intestinal microflora of mammals and have been identified in a variety of food products, including artisanal cheeses [1, 23, 42]. As a rule, the presence of enterococci in food products is considered a sign of fecal contamination, but more recently enterococci have become accepted as part of the normal flora [27]. In some cases they are considered essential for the development of the organolepic qualities associated with artisanal cheeses due to their protease and esterase activities and their ability to produce diacetyl from citrate metabolism [10, 39, 44, 45]. With regard to Hispanic-style cheeses it has been suggested that *Enterococcus faecium* may be required for the development of the traditional organoleptic qualities associated with Mexican Queso Fresco cheeses [8, 36].

In addition to their role in flavor development, enterococci are also considered desirable due their ability to inhibit the growth of several food-born pathogens including: *Staphylococcus* spp., *Clostridium* spp., *Bacillus* spp., and *Listeria* spp. [24]. The inhibition stems from their production of bacteriocins, which are antimicrobial peptides. Bacteriocins isolated form enterococci are generally referred to as enterocins and have led to the use of enterococci, specifically *E. faecium*, in some commercial probiotic supplements for animals [2, 25, 29]. To date, numerous enterocins have been well characterized and can be grouped into one of four classes: the Class I, lantibiotic enterocins;

Class II, heat stable, linear peptides; Class III, cyclic antibacterial peptides; and Class IV, large proteins [23]. The Class II peptides can be further subdivided into the pediocin-like peptides, which are synthesized with a leader sequence ant the typical 'pediocin box' motif (YGNGVXC); small peptides which are synthesized without the a leader sequence and the pediocin motif; and finally non-pediocin-like peptides synthesized with specific leader peptides [23]. Their potent activity against *Listeria monocytogenes* and the increasing demand for natural products that can improve food safety has driven the search for novel enterocins [22].

Despite the benefits associated with enterococci, their potential use in food technology is a controversial topic due to their potential for pathogenicity and role in transferring antibiotic resistance. Enterococci are now recognized as a major cause of nosocomial infections, including surgical wound and urinary tract infections, and bacteremia [26]. Their increasing role as human pathogens is due in part to their high level of resistance to antibiotics, including vancomycin which is one of the few remaining chemotherapeutic agents effective in treating enterococcal infections [6]. In addition, it has been demonstrated that mobile genetic elements, such as conjugative plasmids and transposons, can transfer antibiotic resistance from enterococci to other LAB [37, 40], and other Gram-positive and Gram-negative pathogens, including *Staphylococcus* spp., *Listeria* spp., and *Escherichia coli* [19, 35]. The identification of the same resistance genes in bacteria isolated from unpasteurized cheese and from human patients support the notion that enterococci present in food can either colonize humans or transfer antibiotic resistance genes to other colonizing bacteria [43]. These results support concerns about using enterococci as starter bacteria or probiotic cultures in food products.

Fermented dairy products, specifically raw milk artisanal cheeses, have been shown to serve as a source of novel enterocin producing strains of enterococci [1, 20, 42]. This report summarizes the identification and partial characterization of novel enterocins from enterococci isolated from raw milk Queso Fresco and Mennonite cheeses [8]. The enterococci were screened for antimicrobial activity against several potential pathogens, including *Listeria monocytogenes* that has been reported as the causative agent of infectious outbreaks associated with the consumption of raw milk Hispanic-style cheeses [30].

Materials and methods

Bacterial cultures and media

Enterococcal species were isolated from raw milk Hispanic-style cheeses and identified as previously described [8, 36]. The enterococci screened for antimicrobial activity included (number of isolates): *Enterococcus faecium* (11); *E. faecalis* (13); *E. durans* (4); *E. gallinarum* (4); and *E. casseliflavus* (1). The isolates were passaged regularly in tryptone-yeast extract-lactose (TYL) at 37°C [41], while frozen stocks were stored at −80°C in TYL with 30% glycerol. The following cultures were tested for sensitivity to enterocins: *Listeria innocua*, *L. monocytogenes* Scott and *Pseudomonas fluorescens* 1301 grown in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI); *Escherichia coli* JM109, DH5α, DB11, O157:H7, *Enterobacter sakazakii*, *Shigella sonnei*, *Salmonella infantis*, *Bacillus subtilis* and *Staphylococcus epidermidis* 26 grown in brain heart infusion (BHI, Difco); and *Streptococcus thermophilus* 128 and *Lactococcus lactis* ssp. *lactis* ML3 grown in TYL. All cultures were incubated at 37°C, except *P. fluorescens* 1301 (32°C) and *L. lactis* ML3 (30°C).

Bacteriocin expression from enterococcal isolates

Enterococci were grown overnight in TYL broth at 37°C and tested for bacteriocin production using a well-diffusion technique [17]. Briefly, 40 μl of the overnight enterococcal cultures or cell-free supernatants were loaded into precast wells in agar media inoculated with the target bacteria at a final concentration of 0.5% (v/v). Plates were allowed to equilibrate at 4°C up to 15 h, incubated between 30 and 37°C overnight, and examined for zones of inhibition. Cell-free supernatants from overnight enterococcal cultures were also buffered to pH 6.0–6.3 by the addition of 1 N NaOH to eliminate antimicrobial activity caused by lactic acid production.

Sensitivity of enterocins to proteolytic enzymes

Cell-free supernatants were treated with commercial digestive enzymes (Sigma-Aldrich, St. Louis, MO). Stock solutions (1 mg ml^{-1}) of pronase, proteinase K and trypsin were prepared in 20 mM sodium phosphate buffer (pH 6.0), and pepsin was prepared in distilled water. A 1:10 dilution of enzyme was added directly to the cell-free supernatant and incubated at 37°C for 2–6 h. The untreated and treated supernatants (40 μl) were tested for antimicrobial activity using the well-diffusion assay described previously.

Partial purification and gel electrophoretic analysis of enterocins

A general purification protocol was followed for all enterocins. Overnight cultures (50 ml) were centrifuged at 25,000*g*, 4°C for 30 min. The supernatant was removed, filtered through a 0.22 mm filter (Nalgene), and the pH was adjusted to 4.4. The supernatant was applied to a pre-equlibrated 10 ml SP Sepharose (Invitrogen) cation exchange column with 10 mM phosphate, pH 4.4. The column was washed with five column volumes (CV) of 10 mM phosphate, pH 4.4 to remove non-specifically bound peptides and proteins. The enterocins were eluted using a 108 ml linear gradient of 0–1 M NaCl in 10 mM phosphate, pH 4.4. Fractions were tested for the presence of active material using agar plate bioassays with *L. innocua* as the indicator strain.

Enterocin H41B was further purified by reverse phase high performance liquid chromatography (RP-HPLC) carried out on an Agilent 1200 instrument using a Vydac C18 peptide column (5 mm, 4.6× 250 mm). It was eluted using a gradient of 5–40% B over 5 min, followed by 40–100% B over 35 min (solvent B is 0.086% trifluoroacetic acid in acetonitrile; solvent A is 0.1% trifluoroacetic acid in water). Fractions were collected and activity tested against *L. innocua.* Active fractions were further analyzed by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Active fractions were run on a 12% Bis-Tris gel (Invitrogen). Gel was fixed in 20% isopropanol, 10% acetic acid (100 ml, 2 × 45 min) and washed overnight in distilled water. Washed gel was then placed on an agar plate seeded with *L. monocytogenes* and incubated at 37°C for 8 h [5].

PCR analysis for the presence of known enterocin genes

Enterococcal isolates identified as *E. faecium* were further screened using PCR for the presence genes encoding known enterocins: entA, entB, entP, entQ, L50A and L50B. PCR primers (Integrated DNA Technologies, Coralville, IA) specific for the amplification of these genes were synthesized based on published nucleic acid sequences [14, 33, 34]. PCR was performed using a Biometra thermocycler using the following conditions: 95°C for 5 min, 95°C for 30 s, 50°C for 30 s, 74°C for 1 min, 35 cycles, 74°C for 5 min. *Taq* polymerase (New England Biolabs, Ipswich, MA) was used for amplification and overnight cultures (1 μl) were used as template.

Phenotypic assessment of antibiotic susceptibility

Antibiotic resistance was initially tested using a disc diffusion method on Mueller-Hinton agar (Difco) inoculated with the enterococcal isolates at a final concentration of 0.5%. Each 12.7 mm disc was saturated with one of 14 antibiotics (Sigma) at the following concentrations: ampicillin (Amp, 10 $\mu g\ ml^{-1}$), penicillin (Pen, 10 $\mu g\ ml^{-1}$), tetracycline (Tet, 30 $\mu g\ ml^{-1}$), vancomycin (Van, 30 $\mu g\ ml^{-1}$), chloramphenicol (Chl, 2 $\mu g\ ml^{-1}$, 30 $\mu g\ ml^{-1}$), erythromycin (Ery, 15 $\mu g\ ml^{-1}$), rifampicin (Rif, 5 $\mu g\ ml^{-1}$), streptomycin (Str, 2,000 $\mu g\ ml^{-1}$), kanamycin (Kan, 30 $\mu g\ ml^{-1}$, 2,000 $\mu g\ ml^{-1}$), gentamycin (Gen, 10 $\mu g\ ml^{-1}$, 1,000 $\mu g\ ml^{-1}$), lincomycin (Lin, 10 $\mu g\ ml^{-1}$), spiramycin (Spi, 2.5 $\mu g\ ml^{-1}$), clindamycin (Cli, 2.5 $\mu g\ ml^{-1}$) and tylosin (Tyl, 2.5 $\mu g\ ml^{-1}$). The plates were left at room temperature for 2–3 h to allow the antibiotics to diffuse into the agar and then incubated overnight at 37°C. The plates were then examined for zones of inhibition.

Susceptibility to seven antibiotics was further characterized by determining the minimal inhibitory concentration (MIC) in Mueller-Hinton broth. Serial two-fold dilutions were prepared for each antibiotic within the following concentrations: Tet (0.5–32 $\mu g\ ml^{-1}$), Van (1–128 $\mu g\ ml^{-1}$), Pen (0.5–32 $\mu g\ ml^{-1}$), Chl (1–64 $\mu g\ ml^{-1}$), Ery (0.5–60 $\mu g\ ml^{-1}$), rifampicin (0.5–32 $\mu g\ ml^{-1}$) and Kan (128–4,112 $\mu g\ ml^{-1}$). MIC values were determined by measuring turbidity at OD_{600} using a Beckman DU650 spectrophotometer (Beckman Coulter Inc., Fullerton, CA). MIC values were compared to MIC breakpoints described by the Clinical and Laboratory Standards Institute [16].

Results and discussion

Enterococccal isolates possessing antimicrobial activity

The enterococci used in this study were isolated from both raw and pasteurized milk Hispanic-style cheeses obtained from Mexico: Queso Fresco, obtained from the state of Sonora (6 samples); and Mennonite cheese, from the state of Chihuahua (12 samples). The enterococci were selected on kanamycin azide aesculin (KEA) agar, and species determination was based on 16S rDNA sequence analysis [8, 36]. The predominant enterococcal species were identified as *E. faecalis* and *E. faecium*, with four Queso Fresco and nine Mennonite cheeses possessing *E. faecalis*; and five Queso Fresco and six Mennonite cheeses possessing *E. faecium*. *Enterococcus durans* was present in two Queso Fresco and two Mennonite cheeses, *E. casselflavus* was present in one Mennonite cheese and *E. gallinarum* were present in four Mennonite cheeses. Of these enterococcal species, *E. faecalis*, *E. faecium* and *E. durans* have been studied extensively for their ability to contribute to cheese quality. Strains of all three species have been shown to possess proteolytic and lipolytic activities and have the ability to metabolize citrate, with *E. faecalis* having the highest levels of activity [38].

The 33 enterococcal isolates were screened for antimicrobial activity since they may be useful to control the growth of food-born pathogens due to the production of enterocins [23]. Of the 33 enterococcal isolates screened, 6 had antilisterial activity against *L. innocua* and *L. monocytogenes* Scott (Fig. 1, column C). The isolates having antilisterial activity included two strains of *E. faecium* (H51Ca,

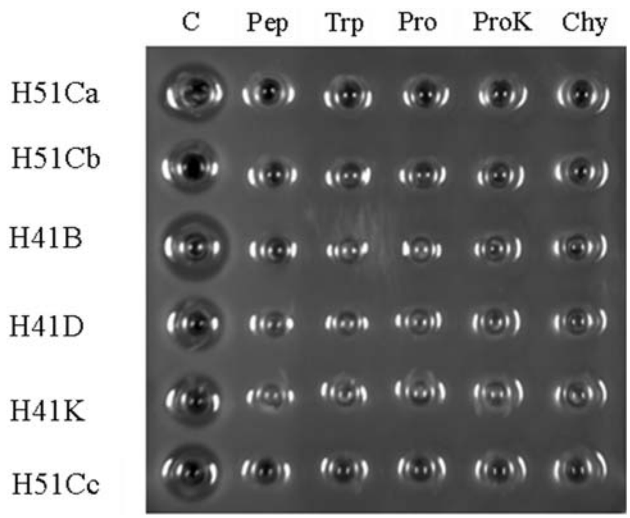

Fig. 1 Characterization of antilisterial compounds by digestion with proteolytic enzymes. Tyrptic soy agar was inoculated with *L. innocua* and precast wells were filled with cell-free culture supernatants from overnight cultures of *E. faecium* H51Ca, H51Cb, H41B, H41D and H41K; and *E. durans* H51Cc. *C* control; *Pep* pepsin; *Trp* trypsin; *pro* pronase; *ProtK* proteinase K; *Chy* α chymotrypsin

H51Cb) from Queso Fresco; three *E. faecium* strains (H41B, H41D, H41K) from Mennonite cheese; and one *E. durans* (H51Cc) isolate from Queso Fresco. All of the bacteriocin producing isolates were recovered from raw milk cheeses. The two active *E. faecium* strains from Queso Fresco were isolated from the same cheese sample; however, they displayed phenotypic differences with regard to aesculin hydrolysis on KEA plates. None of the enterococcal isolates screened had activity against the other potential pathogens targeted, including *E. coli* O157:H7, *E. sakazakii*, *P. fluorescens*, *S. sonnei*, *S. infantis*, and *S. epidermidis*. The non-pathogenic *E. coli* strains JM109, DB11 and DH5α were also resistant to the putative enterocins. These results are in agreement with previous reports showing most enterocin producing enterococci displayed antilisterial activity [23, 24], while a smaller fraction also showed broad spectrum activity against *Staphylococcus* spp., *Bacillus* spp. and *Clostridium* spp. [15, 18]. In addition, an antilisterial bacteriocin-like inhibitory substance was recently identified in *E. faecium* UQ31, an isolate from a raw milk panela cheese from Mexico [1]. The observed antilisterial activity supports a potential role of these indigenous enterococci in preventing outbreaks of listeriosis, which have been reportedly associated with the consumption of raw milk Hispanic-style cheeses [30]. Since enterocins have been proposed for use in food preservation, activity against common dairy starter cultures *Lactococcus lactis* ssp. *lactis* ML3 and *S. thermophilus* ST128 was tested. The *E. durans* H51Cc strain exhibited activity against *L. lactis* ssp. *lactis* ML3 but not against *S. thermophilus* ST128. The remaining enterococcal isolates showed no activity against these starter bacteria.

In comparison with other LAB, enterococci are not considered strong acidifying agents [31] however, the six enterococcal strains lowered the culture pH from 6.5 to 4.6–4.8, following overnight growth at 37°C. To ensure this drop in pH was not responsible for the observed antimicrobial activity, cell-free culture supernatants were buffered with NaOH to pH 6.0–6.5. The buffered culture supernatants of all six isolates inhibited both *L. innocua* and *L. monocytogenes* Scott to the same extent as the unbuffered supernatants.

Characterization of antilisterial activity

The antilisterial activity observed for the five *E. faecium* isolates (H41B, H41D, H41K, H51Ca, H51Cb) and the *E. durans* isolate (H51Cc) was further characterized by treating the cell-free supernatants with various proteolytic enzymes including: pepsin, trypsin, pronase, proteinase K, and α chymotrypsin. Treatment with all of the proteolytic enzymes tested inactivated the inhibitory agents (Fig. 1). The cell-free culture supernatants were also incubated at 100°C for up to 2 h without the loss of antilisterial activity, indicating that the putative bacteriocins produced were heat stable proteins or peptides. These results supported the notion that the cheese isolates are enterocin producing enterococci. Since *E. faecalis* is the only enterococcal species known to produce a Class I lantibiotic (cytolysin) [7], it appears that the enterocins produced by these six isolates could be Class II enterocins [23].

Peptides were partially purified using a sepharose cation exchange column and collected fractions were tested for antilisterial activity (data not shown). The active fractions were analyzed by SDS gel electrophoresis, and staining with Coomassie blue revealed the presence of a band between 4 and 7 kDa for each isolate (Fig. 2a). However, it is not possible to determine if the bands observed represent a single peptide or multiple peptides with similar molecular weights. The peptide bands exhibited antilisterial activity, as zones of inhibition were closely aligned with them when a SDS gel was washed and overlayed on TSA inoculated with *L. monocytogenes* (Fig. 2b). The partially purified fraction from *E. faecium* H41B (Fig. 2a, lane 6) was further purified by reverse phase-HPLC and analyzed by MALDI-TOF MS, revealing a peptide with a molecular weight of 4,832 Da that corresponds to enterocin A [3]. Another peak with a molecular weight of 5,497 Da was detectable, which may represent enterocin B with a reported molecular weight of 5,465 Da [9].

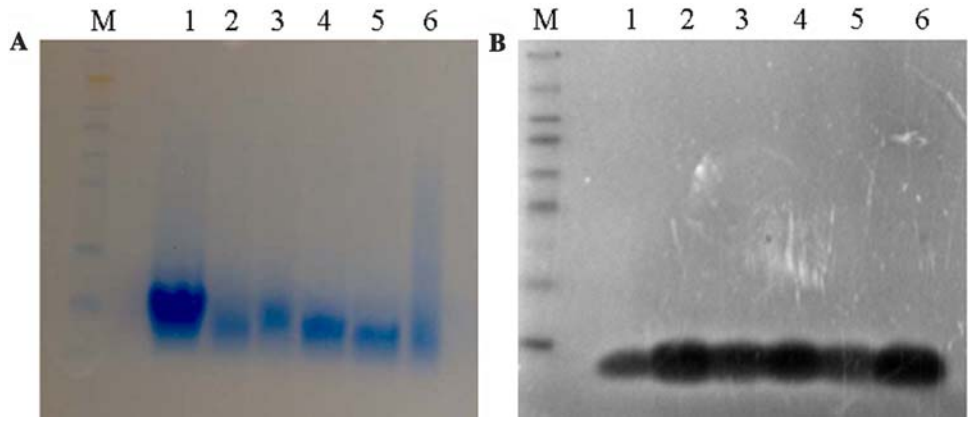

Fig. 2 Expression of bacteriocins from enterococcal isolates. **a** SDS gel electrophoresis of partially purified enterocins; **b** overlay of SDS gel on TSA inoculated with *L. monocytogenes*. Lanes: *M* marker; *1* H41 K; *2* H51Ca; *3* H51Cb; *4* H51Cc; *5* H41D; *6* H41B

Genetic screen for Class II enterocin genes

The *E. faecium* isolates were further screened by PCR for the presence of genes encoding known Class II enterocins including enterocins A, B, P, Q, L50A and L50B. *E. faecium* isolates H51Ca and H51Cb showed bands corresponding to the *entA* (137 bp), *entP* (86 bp), entL50A (105 bp) and *entL50B* (120 bp) (Fig. 3a). A second band, approximately 274 bp in length, was observed when attempting to amplify *entL50B* (Fig. 3a, lanes 6 and 12), this band corresponds to the amplification of both the *entL50A* and *entL50B* genes which reside in the same operon [13]. The forward primers used to amplify these genes differ by only one base pair, allowing for the larger product to be amplified. *E. faecium* isolate H41B apparently produced *entA* (Fig. 3b, lane 1), *entP* (lane 3) and *entL50A* (lane 5) and *entL50B* (lane 6). The individual PCR products were very faint for *entL50A* and *entL50B*, but the 279 bp product incorporating both genes was clearly present (Fig. 3b, lane 6). The 279 bp fragment was further analyzed by nucleic acid sequencing analysis and confirmed that the *entL50AB* genes were present in the H41B isolate (data not shown). In addition, isolate H41B also showed a 200 bp band corresponding to *entB* (lane 2). Both enterocins A and P are considered ClassII.1 or pediocin-like bacteriocins, produced as prepeptides with a leader sequence and containing the YGNGVXC motif at their N-terminus [3, 12]. Both enterocins have been reported to exhibit a broad spectrum of activity against *Listeria* spp., *Bacillus* spp., and *Clostridium* spp. [9, 12]. Enterocins L50A and L50B are considered ClassII.2 enterocins implying that they are synthesized without a leader peptide [11]. The peptides do not undergo any posttranslational modifications, and although they are active independently, they have a broad spectrum synergistic effect when expressed together [13]. Enterocin B is considered a Class II.3 enterocin, synthesized with a leader peptide but lacking the characteristic pediocin motif at the N-terminus. It displayed a similar antibacterial spectrum as enterocin A, the latter appeared to have higher

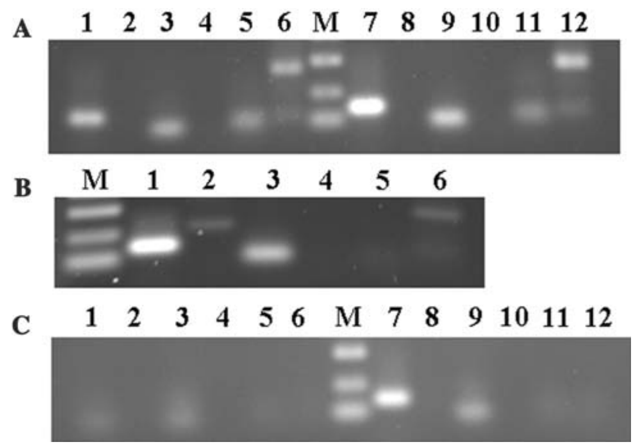

Fig. 3 PCR screen for genes encoding known enterocins from *E. faecium* isolates. **a** H51Ca (lanes 1–6) and H51Cb (lanes 7–12); **b** H41B; **c** H41D (lanes 1–6) and H41 K (lanes 7–12). M 100 bp marker; Lane 1/7 *entA*; lane 2/8 *entB*; lane 3/9 *entP*; lane 4/10 *entQ*; lane 5/11 *entL50A*; lane 6/12 *entL50B*

activity against *Listeria* spp. [9]. In this study, the H51Ca, H51Cb and H41B isolates showed activity only against *Listeria* spp. suggesting all of the enterocins identified may not be expressed. This could be due to growth conditions, since the expression of some enterocins is temperature dependent including enterocin L50AB, which is preferentially expressed between 16 and 32°C [14]. Variations in growth conditions may increase the spectrum and levels of antimicrobial associated with the enterococci isolates used in this study.

The remaining two *E. faecium* isolates, H41D and H41K, appeared to possess *entP* (Fig. 3c, lane 3); and *entA* (Fig. 3c, lane7) and *entP* (lane 9), respectively. The *entP* band observed for isolate H41D was faint and diffuse but appeared to be the correct size (~86 bp); however the band could not be analyzed further by nucleic acid sequence analysis, suggesting that it may represent a primer dimer. The other diffuse bands observed for H41D (lanes 1 and 5) were not of the expected sizes and most likely are the result of primer dimers. These results suggest that *E. faecium*

Table 1 Susceptibility of enterococci isolated from Hispanic-style cheeses to select antibiotics

Enterococcal isolates	Antibiotic MIC values (μg ml^{-1})						
	Tet	Van	Pen	Chl	Em	Rif	Kan
E. faecium H41B	256	8	16	32	>30	16	>2,048
E. faecium H41D	128	2	16	16	0.9	32	>2,048
E. faecium H41K	128	4	16	16	>30	<2	>2,048
E. faecium H51Ca	4	4	8	>32	0.9	<2	512
E. faecium H51Cb	4	4	8	>32	0.9	<2	512
E. durans H51Cc	<0.5	8	4	16	7.5	4	256

H41D may produce a novel Class II enterocin, or one similar to other identified *E. faecium* enterocins possessing antilisterial activity, including: enterocins 81 [20], I [21], SF 68 [22], JCM 5804 [34] or UQ31 [1].

E. durans H51Cc, was not initially analyzed by PCR due to the lack of genetic information known for durancins with antilisterial activity [28, 46]. However, the comparison of deduced amino acid sequences show that bacteriocins A5–11A and B isolated from *E. durnas* exhibit a high degree of similarity with enterocins I, L50A and L50B isolated from *E. faecium* [4]. When *E. durans* H51Cc was used as template with the above primers, bands corresponding to *entA*, *entP* and *entL50AB* were obtained, suggesting that the bacteriocin(s) produced by this strain share some nucleic acid sequence homology with known enterocins from *E. faecium* (data not shown).

Phenotypic characterization of antibiotic resistance in enterocin producing isolates

The disc diffusion method initially showed that all enterococcal isolates used in this study appeared sensitive to lincomycin, clindamycin, spiramycin, tylosin, and streptomycin. All of the enterococci showed resistance to a low level of Gen (10 μg ml^{-1}) but appeared sensitive to higher concentration of 1,000 μg ml^{-1}. All isolates also showed resistance to Amp at 10 μg ml^{-1}. Differences in susceptibility were observed for the isolates with regard to the remaining antibiotics tested, thus MIC values were determined to more accurately define the differences (Table 1). Resistance to vancomycin is of great concern as the number of vancomycin resistant enterococci (VRE) continues to increase [32]. With the susceptible breakpoint for vancomycin defined as 4 μg ml^{-1} [16], only *E. faecium* H41B and *E. durans* H51Cc would be considered to have intermediate resistance with MIC values of 8 μg ml^{-1}.

The isolates recovered from the Mennonite cheeses, H41B, H41D and H41K all appeared resistant to Tet (30 μg ml^{-1}), Chl (30 μg ml^{-1}), and Kan (2,000 μg ml^{-1}) according to the disc diffusion test. MIC values supported these results with regard to Tet, with MIC values of 128 μg ml^{-1} (H41D and K) and 256 μg ml^{-1} (H41B); and Kan, with MIC values greater than 2,048 μg ml^{-1} for all three isolates. These values were well above the susceptible breakpoints defined for enterococci, 4 μg ml^{-1} for Tet and 512 μg ml^{-1} for Kan. With regard to Chl, only H41B had a MIC value above the designated resistant breakpoint of 32 μg ml^{-1} [16], the other two isolates had MIC values of 16 μg ml^{-1}, which would classify them as having intermediate susceptibility. It appeared that H41D was weakly inhibited by Pen in the disc test, however all three isolates showed MIC values of 16 μg ml^{-1} which would classify them as resistant. Isolates H41B and H41K were also determined to be highly resistant to Ery with MIC values above 30 μg ml^{-1} [16].

The disc diffusion test showed that the Queso Fresco isolates were susceptible to Tet (30 μg ml^{-1}), Ery (15 μg ml^{-1}) and Kan (2,000 μg ml^{-1}); MIC values confirmed that all isolates would be considered susceptible to Tet and Ery, but, only *E. durans* H51Cc, with a MIC value of 256 μg ml^{-1}, would be considered susceptible to Kan [16]. Although clear zones were not observed around the Pen discs, all three isolates showed susceptible MIC values of 4 or 8 μg ml^{-1}. The *E. faecium* isolates from Queso Fresco showed the highest level of resistance to Chl with MIC values greater than 32 μg ml^{-1}, but the *E. durans* isolate showed an intermediate level of susceptibility [16].

In conclusion, it appears that *E. faecium* isolates H51Ca and H51Cb, isolated from the same Queso Fresco sample, are the same strain since they were identical with regard to the presence of enterocin genes (Fig. 3a) and antibiotic susceptibility (Table 1). These isolates, along with *E. faecium* H41B and H41K, apparently possess multiple enterocin genes. However, further analysis is required to determine which of these peptides may be responsible for the antilisterial activity associated with the cell-free supernantants. More interestingly, isolate H41D did not test positive for any of the Class II enterocins, thus it may produce a novel antilisterial enterocin. The *E. durans* isolate, H51Cc, also showed positive results when screened for the presence of *E. faecium* enterocin genes, suggesting that it may also produce more than one type of durancin. All strains tested showed reasonable susceptibility to vancomycin, but they also showed higher levels of resistance to other antibiotics tested suggesting they would not serve as GRAS starter or probiotic adjunct cultures. Nevertheless, their indigenous presence as constituents of raw milk cheeses may contribute to the safety of the cheeses by controlling the level of *Listeria* contamination.

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