Native production of pediocin PA-1 by *Enterococcus faecium* E16 isolated from goats' cheese

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Summary

Enterococcus faecium E16, a strain isolated from goats' cheese made from unpasteurized milk, showed antibacterial activity, in particular against *Listeria monocytogenes* strains. The active compound was produced at high amounts in de Man Rogosa and Sharpe broth at 37 °C. It was purified to homogenity from culture supernatant by using a simple method consisting of cation-exchange and reversed-phase chromatographies. Mass spectrometry showed that the antibacterial activity was due to a 4 621.32 Da peptide. Its amino acid sequence was analysed using Edman degradation an it showed that the peptide consisted of 44 unmodified amino acids with two disulphide bonds linking cysteine residues at positions 9, 14 and at positions 24, 44. This structure was shown to be identical to pediocin PA-1, a class IIa bacteriocin. The antimicrobial activity of the produced pediocin PA-1 covered a range of bacteria, with strong activity against many species of Gram-positive bacteria, especially the food-borne pathogen *Listeria monocytogenes*, but no activity against Gram-negative bacteria. This study represents the first reported case of natural pediocin PA-1 production by an *Enterococcus* species.

Keywords

bacteriocin; pediocin PA-1; *Enterococcus faecium*; *Listeria monocytogenes*; cheese

Ribosomally synthesized antimicrobial peptides with diverse molecular weights, structures, biochemical characteristics and spectra of activities were identified in a wide range of bacterial strains [1]. These antimicrobials, called bacteriocins, are cationic peptides with less than 70 amino acid residues. They represent a promise for food safety and are proposed to be as human therapeutics, in particular in the context of growing concerns about the development of antibiotic resistance. Many bacteriocins display a high activity against food-spoilage and pathogenic bacteria, such as *Bacillus* spp., *Listeria* spp., *Clostridium* spp., methicillin-resistant *Staphylococcus aureus* strains and vancomycin-resistant enterococci, often at concentrations much lower than those of antibiotics. Unlike antibiotics that target specific enzymes, most bacteriocins kill target cells by pore formation and permeation of the cytoplasmic membrane or inhibition of cell wall biosynthesis, or a combination thereof [2].

Depending on the presence of modified amino acid residues, the bacteriocins are classified as class I (with modification) or class II (no modifi-

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Tab. 1. Antimicrobial activity of pediocin PA-1 produced by *E. faecium* E16.

Indicator species	Strain	*MIC* [$\mu g \cdot l^{-1}$]
Bacillus cereus	CIP 78.3	ND
Bacillus subtilis	ATCC 6633	ND
Enterococcus faecium	LC E10	50
	LC E13	75
	LC E14	75
	LC E15	75
	LC E16	ND
Enterococcus columbae	LC E2	75
Enterococcus faecium	LC E20	50
Enterococcus hirae	LC E22	50
Enterococcus mundtii	LC E23	ND
Enterococcus pseudoavium	LC E24	150
Enterococcus raffinosus	LC E26	15
Enterococcus saccharolyticus	ATCC 43076	175
Enterococcus sulfureus	LC E28	150
Escherichia coli	ATCC 25922	ND
Enterococcus faecalis	ATCC 19433	150
Klebsiella pneumoniae	ATCC 10031	ND
Lactobacillus plantarum	LC L14	ND
	WHE92	ND
	ATCC 10241	125
Lactobacillus sakei subsp. *sakei*	ATCC 15521	25
Lactococcus lactis	LC L17	ND
	LC L21	5
	LC L22	125
Listeria innocua	LC L30	5
	LC L31	25
Listeria monocytogenes 1/2c	LC L35	5
Listeria monocytogenes 3a	LC L36	10
Listeria monocytogenes 3b	LC L37	5
Pediococcus acidilactici	LC P1	ND
Salmonella enterica subsp. *enterica*	LC S1	ND
Salmonella enterica subsp. *enterica*	LC S2	ND
Serratia marcescens	LC S3	ND
	ATCC 13880	ND
Staphylococcus aureus	ATCC 6538	75
	ATCC 6538	ND
Staphylococcus epidermidis	ATCC 12228	200
Staphylococcus xylosus	LC S8	125

CIP – collection of the Pasteur Institute (Paris, France); ATCC – American Type Culture Collection (Manassas, Virginia, USA); LC – laboratory collection; MIC – minimal inhibitory concentration, the lowest bacteriocin concentration yielding no visible growth of indicator microorganisms after 24 h of incubation; ND – no growth inhibition detected at 1 $mg \cdot l^{-1}$.

cation). Among the latter, one group (class IIa bacteriocins), with 30 to 58 residues, has spurred the highest interest because of the strong anti-*Listeria* activity that it displays. Elucidation of their amino acid sequences and genetic determinants provided valuable insights into the common characteristics of class IIa bacteriocins, especially their YGNGV/L N-terminal motif, high level of structural similarity, amphiphilic character, as well structure-function relationship [1, 3]. Common producers of class IIa bacteriocins include species of the genera *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc,* as well as *Carnobacterium* [4].

In this work, we report on purification, amino acid sequence analysis and antibacterial activity of pediocin PA-1 produced by *Enterococcus faecium* E16 isolated from French goats' cheese.

MATERIALS AND METHODS

Bacterial strains and cultures

The bacteriocin producer *E. faecium* E16 was isolated from goats' cheese made from unpasteurized milk. It was identified at the species level by both carbohydrate fermentation profiles, partial sequence analysis of the 16S rRNA gene, and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) performed using the Bruker Microflex LT instrument, Biotyper software v. 3.0, and Bruker database v. 3.1.66 (Bruker Daltonik, Bremen, Germany), according to the manufacturer's instructions. *Lactobacillus sakei* subsp. *sakei* ATCC 15521 was selected as the indicator strain to monitor bacteriocin activity during the purification process. Strains used to determine the activity spectrum of the purified bacteriocin are listed in Tab. 1. All cultures were maintained as frozen stocks at –80 °C in Brain heart infusion (BHI) broth with 30% glycerol (Bio-Rad Laboratories, Philadelphia, Pennsylvania, USA). For short time storage, the strains were subcultured on BHI agar (Bio-Rad) and kept at 4 °C. Before experimental use, the strains were cultured twice for 18 h to 24 h in de Man, Rogosa and Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) without aeration.

Antibacterial activity determination

To monitor antibacterial activity during the purification process, fractions were serially diluted and activity was assessed using the spot-on-a-lawn test and expressed in arbitrary units (AU) per millilitre as previously described [4]. The antimicrobial spectrum of pediocin PA-1 was determined as previously described [4] using serial dilutions of the purified bacteriocin in 96-well polystyrene microtiter plates using a Varioskan Flash microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The plates were incubated at 30 °C or 37 °C for 24 h, depending on bacterial

strains. Minimal inhibitory concentration (*MIC*) values were set as the lowest concentration in each row without visible growth, measured by reading the absorbance at a wavelength of 600 nm.

Bacteriocin purification

E. faecium E16 was inoculated in 400 ml of MRS broth to yield an initial count of approximately 10^5 CFU·ml^{-1} and incubated for 24 h at 37 °C. Cells were removed by centrifugation at 4000 ×*g* for 15 min and the supernatant was filter-sterilized (0.45 μm pore size, Durapore mixed cellulose ester filter; Merck Millipore, Darmstadt, Germany). The obtained cell-free culture supernatant was adjusted to pH 6.5 with NaOH and directly applied to a cation exchanger (SP-Sepharose HP; 100 mm length, 26 mm internal diameter; GE Healthcare, Chicago, Illinois, USA). Equilibration and washing were done with sodium acetate 20 mmol·l^{-1} at pH 6.5 (buffer A) and elution was done with 1 mol·l^{-1} NaCl in buffer A, all at a flow rate of 5.0 ml·min^{-1}. The eluted fraction (75 ml) was applied to a semi-preparative C8 reversed-phase HPLC column (Polaris C8-A; 250 mm length, 10 mm internal diameter, 18 nm pore size, 5 μm particle size; Varian, Palo Alto, California, USA). After washing at a flow rate of 4 ml·min^{-1} for 10 min with a mixture containing 80% water with 0.1% trifluoroacetic acid (TFA) (A) and 20% acetonitrile with 0.1% TFA (B) (v/v), elution was done at a flow rate of 4 ml·min^{-1} with a linear gradient of 20–80 % (v/v) B during 40 min. The active fractions were pooled and applied to a Sunfire C18 reversed-phase analytical column (150 mm × 4.6 mm, 5 μm particle size; Waters, Milford, Massachusetts, USA). The separation was achieved at a flow rate of 1 ml·min^{-1} with the following linear gradient: 30–60 % (v/v) B during 20 min. The equipment used was a Dionex Ultimate 3000 HPLC system consisting of WPS3000FC autosampler and fraction collector, 3600 SD dual gradient pump and 3000 (RS) diode array detector (Thermo Fisher Scientific). Detection was performed at wavelengths of 220 nm and 280 nm. The fraction containing the purified bacteriocin was vacuum-dried using a concentrator Speedvac SDP121P (Thermo Fisher Scientific) and stored at –20 °C. Prior to activity testing and structural determination, the obtained powder was re-dissolved in deionized water.

Mass analysis and amino acid sequencing

The monoisotopic mass of the purified bacteriocin was measured on an electrospray quadrupole-time-of-flight mass spectrometer (Synapt G2 HDMS, Waters) equipped with an automated chip-based nanoESI source (Triversa Nanomate, Advion Biosciences, Ithaca, New York, USA) operating in the positive ion mode. External calibration was performed with the multiple charged ions produced by a 2 μmol·l^{-1} horse heart myoglobin solution diluted in a 1:1 (v/v) water/acetonitrile mixture acidified with 1% (v/v) formic acid. Data analysis was performed with MassLynx 3.5 (Waters).

Amino acid sequence determination based on Edman degradation was performed using an gas-phase sequencer Model 492 (Applied Biosystems, Foster City, California, USA). Phenylthiohydantoin amino acid derivatives generated at each sequence cycle were identified and quantified online with an Applied Biosystems Model 140C HPLC system using the data analysis system for protein sequencing from Applied Biosystems (Procise PC version 2.1 software). The phenylthiohydantoin-amino acid standard kit from Perkin-Elmer (Waltham, Massachusetts, USA) was used and reconstituted according to the manufacturer's instructions. The procedures and reagents used were as recommended by the manufacturer. Chromatography was used to identify and quantify the derivatized amino acid removed at each sequence cycle. Retention times and integration values of peaks were compared to the chromatographic profile obtained for a standard mixture of derivatized amino acids.

RESULTS

The bacteriocin produced by *E. faecium* E16 was purified and biochemically characterized. Fig. 1A shows the obtained bacteriocin peak following semi-preparative C8 reversed-phase chromatography. The active fraction was collected and re-chromatographed on an analytical C18 reversed-phase as many times as necessary until a single chromatographic peak was obtained (Fig. 1B). The bacteriocin was eluted at around 46% acetonitrile at a retention time of 10.9 min.

Deconvolution of the bacteriocin spectrum led to a monoisotopic mass of the purified bacteriocin of 4621.32 Da (Fig. 2). The amino acid sequence revealed a medium-sized bacteriocin made up of 44 unmodified amino acids, which contained a class IIa YGNGV N-terminal motif (Tab. 2) [5–17]. The calculated mass of this sequence was 4625.06 Da, which was by 4 Da higher than the mass obtained experimentally for the purified bacteriocin, suggesting the presence of two disulphide bridges between the two cysteine residues at positions 9, 14 and at positions 24, 44. This was further

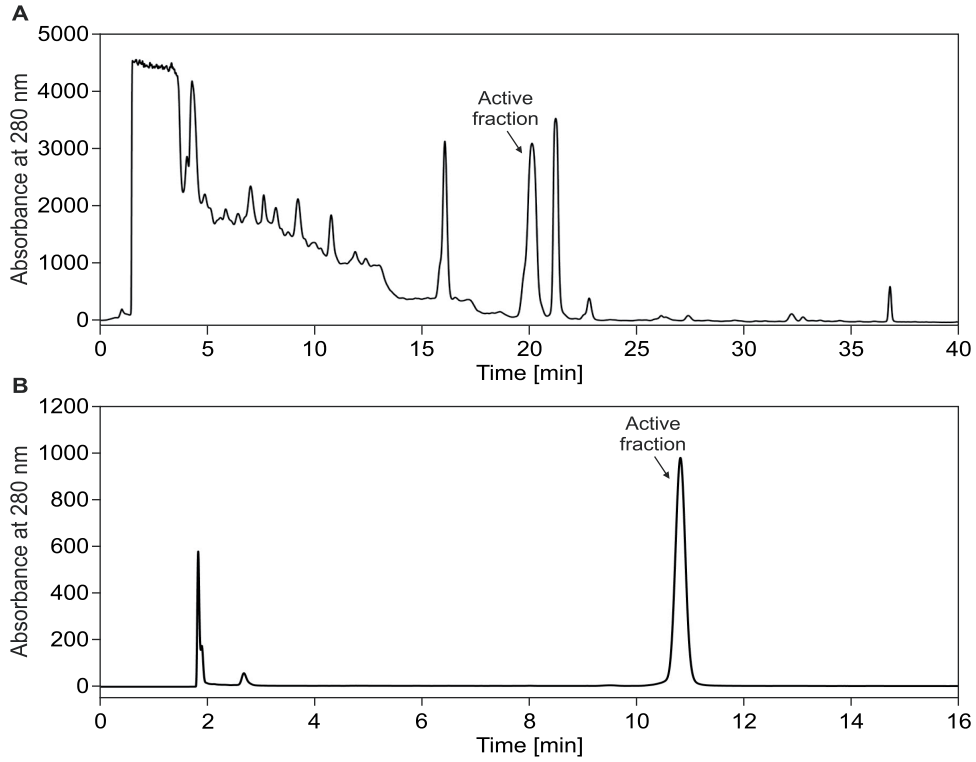

Fig. 1. Reversed-phase HPLC of *E. faecium* E16 extract.

A – semi-preparative C8 chromatography, B – analytical C18 chromatography of the active fraction.

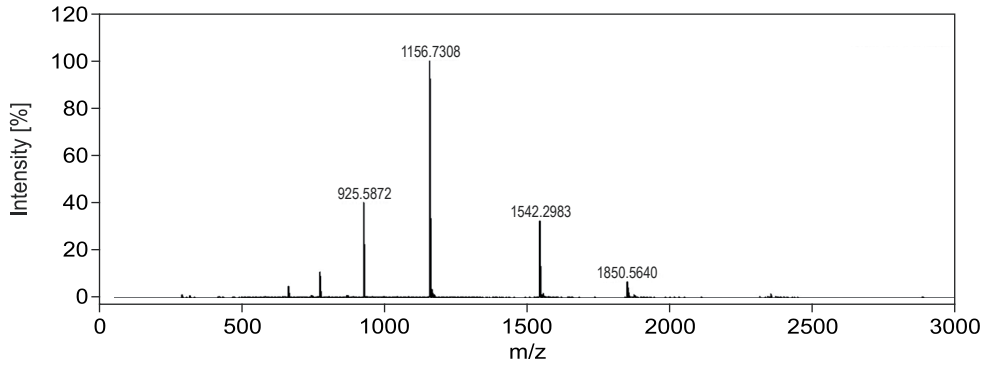

Fig. 2. Mass spectrum obtained by electrospray ionization mass spectrometry analysis of purified pediocin PA-1.

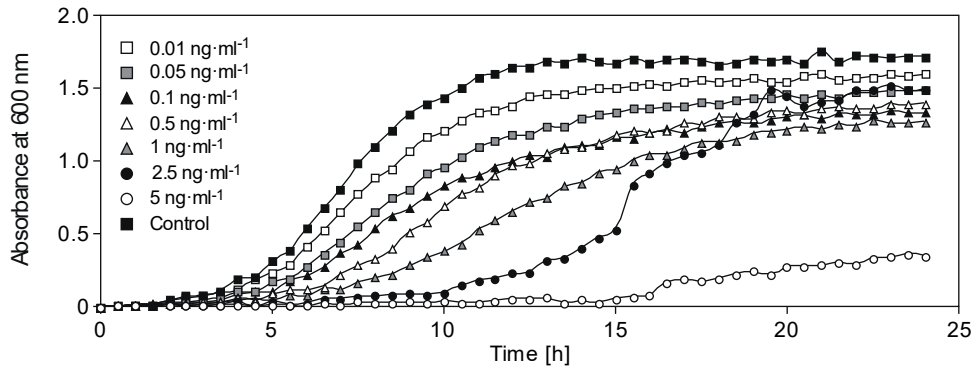

Fig. 3. Kinetics of antimicrobial effect of purified pediocin PA-1 on indicator *L. monocytogenes* 3a at various concentrations.

confirmed by a reduction-alkylation experiment, which yielded a mass of 4 849.30 Da corresponding to the peptide with four iodoacetamide-alkylated cysteines. The estimated isoelectric point of this bacteriocin was 8.52 and the overall charge at pH 7.0 was 3.5. Comparison with known class IIa bacteriocins showed that the determined structure corresponded to pediocin PA-1 [1].

Pure pediocin PA-1 was dried and then serially diluted in culture broths, which were inoculated with various indicator bacteria with the aim of assessing their growth using an automated turbidometer. Sensitive strains had their growth significantly slowed down as a result of the elimination of a part of the initial population as shown in Fig. 3 with *L. monocytogenes* 3a. The data for all indicator bacteria are summarized in Tab. 1, which presents the obtained *MIC* values corresponding to the lowest bacteriocin concentrations leading to total growth inhibition for up to 24 h of incubation. Unsurprisingly, the activity spectrum of pediocin PA-1 was a one typical for medium-range class IIa bacteriocin, targeting Gram-positive bacteria, including *Listeria* sp. The antibacterial sensitivity was highly strain-dependent, as is common for bacteriocins in general. All tested *L. monocytogenes* and *L. innocua* strains were highly sensitive to pediocin PA-1. Interestingly, *MIC* values were as low as 5 $\mu g \cdot l^{-1}$ against *Listeria* strains (Tab. 1). The bacteriocin was also active against strains of *Staphylococcus aureus* and *Staph. epidermidis,* but at much higher *MIC* values (75 $\mu g \cdot l^{-1}$ and 200 $\mu g \cdot l^{-1}$, respectively). On the other hand, a number of lactic acid bacteria strains were also affected. As far as Gram-negative bacteria (*Escherichia coli, Klebsiella pneumonia, Salmonella enterica* subsp. *enterica* and *Serratia marcescens*) are concerned, no antimicrobial activity could be detected at concentrations up to 1 $mg \cdot l^{-1}$, which is a common feature of the vast majority of bacteriocins from lactic acid bacteria.

DISCUSSION

In this study, we isolated a bacteriocin-producing *E. faecium* strain (E16) from retail French goats' cheese made from unpasteurized milk. This strain, which displayed high antilisterial activity, was identified by using Bruker's MALDI-TOF MS Biotyper system. The antimicrobial peptide was purified by using a simple two-step method consisting of cation-exchange and reversed-phase chromatographies. Amino acid sequencing showed that the produced bacteriocin was identical to pediocin PA-1, a member of the class IIa

Tab. 2. Amino acid sequence of pediocin PA-1 from *E. faecium* E16 and its alignment with sequences of similar class IIa bacteriocins.

Bacteriocin	Sequence	Ref.
Pediocin PA-1	KYYGNGVTCGKHSCSVDWGKATICINNG–AMAWATGGHQGNHKC	[5]
Coagulin	KYYGNGVTCGKHSCSVDWGKATTCINNG–AMAWATGGHQGTHKC	[6]
Bavaricin A	KYYGNGVHXGKHSXTVDWGTAIGNIGNNA–AANXATGXGGNGG	[7]
Sakacin P	KYYGNGVHCGKHSCTVDWGTAIGNIGNNA–AANWATGGGWNNK	[8]
Mundticin L	KYYGNGLSCNKKGCSVDWGKAIGIGNNS–AANLATGGGWKSK	[9]
Mundticin KS	KYYGNGVSCNKKGCSVDWGKAIGIGNNS–AANLATGGGWKSK	[10]
Mundticin	KYYGNGVSCNKKGCSVDWGKAIGIGNNS–AANLATGGGWSSK	[11]
Avicin A	TYYGNGVSCNKKGCSVDWGKAISIGNNS–AANLATGGGWKSK	[12]
Enterocin HF	KYYGNGVSCNKKGCSVDWGKAIGIGNNA–AANLTTGGKAGWKGG	[13]
Piscicolin 126	KYYGNGVSCNKNGCTVDWSKAIGIGNNA–AANLTTGGAAGWNKG	[14]
Listeriocin 743A	KSYGNGVHCNKKCWVDWGSAISTIGNNS–AANWATGGAAGWKSK	[15]
Piscicocin CS526	KYYGNGLSXNKKGXTVDWGTAIGIGNNA–AANXATGGAAGXNKK	[16]
Weisselin A	KNYGNGVYCNKHKCSVDWATFSANIANNS–AMAGLTGGNAGNK	[17]

bacteriocin family, typically produced by *Pediococcus acidilactici* strains [18]. Also, the observed antibacterial activity was typical of a class IIa bacteriocin, with a relatively narrow spectrum of activity and *Listeria* sp. strains being particularly sensitive, which is generally ascribed to the highly conserved YGNGV/L motif [1].

Class IIa bacteriocins are known to be produced by a wide range of lactic acid bacteria, with similar structures often being associated with particular species or genera. As far as pediocin PA-1 is concerned, studies reported the natural spread of bacteriocins structurally similar to pediocin PA-1 (pediocin PA-1 like) among lactic acid bacteria, in particular *Lactobacillus casei, Lb. paracasei* and *Lb. rhamnosus* [19–21]. However, so far, pediocin PA-1 has been found only among pediococci. The only exception remained *Lb. plantarum* WHE92 isolated from Munster cheese, which was found to be a pediocin PA-1 producer [22].

Pediocin PA-1 has been associated with strong activity against *L. monocytogenes*, a food-borne pathogen of concern in cheese. Since pediococci have not suitable technological properties, it is of interest to discover a natural producer of this class of bacteriocins, representing other genus. With this regard, our previous study [22] that led to the discovery of *Lb. plantarum* WHE92 had a significant importance regarding the fight against *L. monocytogenes* in the cheese manufacturing industry [23, 24]. This strain, which is available commercially as *Lb. plantarum* ALC-01 (Anti-Listeria Culture, Danisco, Denmark), is used as a potent measure to combat *Listeria* in industrial cheese production lines.

In the present study, *E. faecium* E16 is reported as the second natural intergeneric pediocin PA-1 producer after *Lb. plantarum* WHE92. The ability of enterococci to produce anti-*Listeria* bacteriocins is well known [25], which is often explained by the close phylogenetic relationship of enterococci and listeriae [26]. Enterocins are found within all bacteriocin classes, including class IIa. Therefore, the production of pediocin PA-1 by *E. faecium* is not surprising after all.

Enterococci are widely distributed in the environment, principally inhabiting the human and animal gastrointestinal tract. Due to their association with human infections and with resistance to antibiotics, enterococci are certainly the most controversial group of lactic acid bacteria. Yet, as natural gut inhabitants, enterococci are nowadays used as probiotics. Also, they represent a major bacterial entity of many endogenous dairy and meat products, as well as fermented food products [27]. Studies on the microbiota of many traditional cheeses in the Mediterranean countries indicated that enterococci play an important role in the ripening of these cheeses, probably through proteolysis, lipolysis and citrate breakdown, hence contributing to their typical taste and flavour [25]. Enterococci are also present in other fermented foods, such as sausages and olives [25].

The resistance of enterococci to pasteurization temperatures, and their adaptability to different substrates and growth conditions (low and high temperature, extreme pH, salinity) imply that these bacteria could withstand usual conditions of food production and that they possess interesting biochemical and technological properties [25]. As a consequence, there has been an increased interest in enterococci as starter cultures or co-cultures in cheese products and, to a lesser extent, in meat products.

For the above-mentioned reasons the use of bacteriocin-producing enterococci as starter cultures, co-cultures or protective cultures in fermented food products (especially in cheese products) is a point of great interest amongst fermentation technologists. Therefore, given the demonstrated biopreservation potential of pediocin PA-1, *E. faecium* E16 could represent a viable and efficient alternative to deliver pediocin PA-1 to fermented foods, and cheese in particular. However, this strain has to go through a thorough examination before it can be used in food production, in order to exclude the possibility of the presence of virulence factors [28].

CONCLUSIONS

A bacteriocin-producing strain *E. faecium* E16 was isolated from French goats' cheese made from unpasteurized milk. This strain showed strong anti-*Listeria* activity via a peptide, which was purified by a simple two-step method consisting of cation-exchange and reversed-phase chromatographies. Amino acid sequencing showed that the produced peptide was a bacteriocin identical to pediocin PA-1. Naturally occurring intergeneric, and even interspecies production of bacteriocins is a relatively rare phenomenon. As far as pediocin PA-1 is concerned, to the best of our knowledge, this is the first report on the presence of pediocin PA-1 in another species and another genus outside *Lb. plantarum*. Although pediocin PA-1 exhibited a strong activity against *L. monocytogenes*, a food-borne pathogen of concern in cheese, pediococcal strains have limited technological properties and applications in dairy products. Enterococcal producers are, in this regard, of highest technological

interest. This study gives new insight in the natural occurrence of pediocin PA-1 among lactic acid bacteria.

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