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Screening of plantaricin EF and JK in an Algerian *Lactobacillus plantarum* isolate

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ABSTRACT

Objective: To isolate an antagonistic *Lactobacillus* strain from healthy infant feces and estimate its potential against a range of Gram-positive and Gram-negative bacteria.
Methods: Isolation was based on biochemical tests and on 16S rDNA sequences. A PCR based screening of plantaricin bacteriocin encoding genes was conducted using specific primers. Antimicrobial activity was realized using spot on agar and agar well-diffusion tests. Spent culture supernatant was subjected to ultrafiltration. Effect of mitomycin C and UV light, heat treatment, storage, pH and enzymes were tested.
Results: Biochemical tests and 16S rDNA sequences showed that LbM2a belonged to *Lactobacillus plantarum*. Screening of plantaricin genes showed the presence of plantaricin EF and K. Ultrafiltration tests lead to estimate the molecular weight between 3 and 10 kDa. LbM2a showed a broad inhibitory activity, which was stable at pH range of 2.0 to 6.0 and was proteinaceous (inactivation by proteolytic enzymes). Mitomycin C and UV light did not affect the activity.
Conclusions: The paper illustrates that the ability of isolate and its bacteriocins in inhibiting a wide-range of bacteria are great interest for food safety and might have future applications as food preservative.

1. Introduction

Lactic acid bacteria (LAB) represent a heterogeneous group of microorganisms which are naturally present in many kinds of food and in the gastrointestinal and urogenital tract of animals. It has been shown that these microorganisms are able to produce antimicrobial compounds, such as bacteriocins or bacteriocin-like inhibitory substances[1]. Bacteriocins of LAB are defined as ribosomally synthesized proteins or protein complexes usually active against genetically closely related organisms[2].

LAB have a key role in the majority of food fermentation; one of the most important contributions of these microorganisms is to extend shelf life of food fermented products. Growth of spoilage and pathogenic bacteria in these foods is inhibited essentially due to the presence of starter-derived inhibitors such as lactic acid, hydrogen peroxide and bacteriocins[3].

More than 300 different bacteriocins have been described for the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Enterococcus*[1,4,5]. They are generally low molecular weight proteins that reach target cells by binding to cell surface receptors. Their bactericidal mechanisms differ and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rRNA, and inhibition of peptidoglycan synthesis[1,6].

Lactobacillus plantarum (*L. plantarum*) has been found and isolated from a variety of vegetable and fruit sources, meat products and the gastrointestinal tract of humans and animals. The diverse environmental niches occupied by *L. plantarum* are attributed to the strain's ability to ferment a broad range of sugars[7]. *L. plantarum*

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has also been explored as a delivery vehicle for therapeutic compounds[8] and as a probiotic for humans and animals[9].

A number of bacteriocins have been described for *L. plantarum* isolated from various niches, such as fermented milk, cheese, fermented cucumber, fermented olives, pasta, pineapple, grapefruit juice, sorghum beer and barley, molasses, boza, kefir and amasi[10].

L. plantarum strains produce a variety of bacteriocins and often multiple bacteriocins that are encoded by a single strain within a bacteriocin locus located on the chromosome. For example, *L. plantarum* strains C11 and WCFS1 were shown to harbor the genetic determinants for at least three bacteriocin systems, *i.e.* plantaricin N, as well as the two-peptide bacteriocins plantaricin EF (PlnEF) and plantaricin JK (PlnJK)[11,12]. Production of other bacteriocins, such as plantaricin W, NC8 and plantaricin 1.25β by other *L. plantarum* strains has also been reported[13].

Both PlnEF and PlnJK are two-peptide bacteriocins that belong to the large group of small, heat-stable nonlantibiotics termed class II bacteriocins[14]. Plantaricin E, plantaricin F, plantaricin J (PlnJ) and plantaricin K (PlnK) are cationic peptides that consist of 33, 34, 25, and 32 amino acids having molecular weights of 3 703, 3 545, 2 929, and 3 503 Dalton respectively[11].

PlnJ and PlnK are efficient antimicrobials when present together. Manifestly, none of the other combinations of these four peptides enhanced the antimicrobial activity[15]. The amphiphilic structure of these peptides is believed to have a role in pore formation[15].

In this paper, we investigated the antagonistic activity of a *L. plantarum* strain isolated from feces of healthy new born, followed by the screening of presence or not of genes encoding for PlnEF and PlnJK and biochemical characterization of the active spent culture supernatant (SCS).

2. Materials and methods

2.1. Culture conditions

Strain LbM2a, the bacteriocin producer used in this study, was grown in deMan Rogosa Sharpe (MRS) medium and incubated at 37 ˚C[16]. The other strains used as indicator organisms were grown using the appropriate media and temperature as indicated in Table 1.

LAB strains and pathogenic strains were generously provided by Dr. Luis M. Cintas Izarra (CECT: Spanish Collection of Type Cultures), Department of Nutrition and Food Science, Veterinary Faculty, University Complutense of Madrid. The other strains were kindly provided by Dr. Philippe Langella and Dr. Luis Bermudes, Laboratory of Commensals and Probiotics-Host Interactions MICALIS Institute, INRA, Jouy en Josas France. *Listeria* strains BUG 498 and 499 were generously provided by Pr. Pascale Cossart from Pasteur institute, Paris, France (Table 1). All strains were stored at 80 ˚C in culture broth with glycerol (40%) and were subcultured twice before being used in the experiments.

Table 1
Target organisms used for antimicrobial tests.

Indicator organism	[a]Strain	Medium-incubation, Temperature
P. pentosaceus	CECT 4695	MRS, 37 ˚C
L. plantarum	CECT 748	MRS, 37 ˚C
L. acidophilus	CECT 4529	MRS, 37 ˚C
L. sake	LTH673	MRS, 37 ˚C
L. plantarum	CECT 4529	MRS, 37 ˚C
L. rhamnosus	ATCC 7469	MRS, 37 ˚C
L. sakei	K23 INRA	MRS, 37 ˚C
B. cereus	ATCC 9884	BHI, 37 ˚C
S. aureus	ATCC 33862	BHI, 37 ˚C
S. aureus	ATCC 25923	BHI, 37 ˚C
E. coli	ATCC 25122	BHI, 37 ˚C
E. coli	DH10B INRA	BHI, 37 ˚C
S. dysenteria	CECT 457	BHI, 37 ˚C
S. sonnei	CECT 584	BHI, 37 ˚C
C. maltoaromaticum	DSM 20730 INRA	BHI, 37 ˚C
C. maltoaromaticum	DSM 20722 INRA	BHI, 37 ˚C
E. faecalis	EMX2 Our collection	BHI, 37 ˚C
M. luteus	CECT 241	BHI, 37 ˚C
M. luteus	ATCC 10420	BHI, 37 ˚C
L. innocua	BUG498 Pasteur institute	BHI, 37 ˚C
L. innocua	BUG499 Pasteur institute	BHI, 37 ˚C
L. innocua	CECT 910	BHI, 37 ˚C
C. maltoaromaticum	CECT 5808	BHI, 37 ˚C

P. pentosaceus: Pediococcus pentosaceus; *L. acidophilus: Lactobacillus acidophilus*; *L. sake: Lactobacillus sake*; *L. plantarum: Lactobacillus plantarum*; *L. rhamnosus: Lactobacillus rhamnosus*; *L. sakei: Lactobacillus sakei*; *B. cereus: Bacillus cereus*; *S. aureus: Staphylococcus aureus*; *E. coli: Escherichia coli*; *S. dysenteria: Shigella dysenteria*; *S. sonnei: Shigella sonnei*; *C. maltoaromaticum: Carnobacterium maltoaromaticum*; *E. faecalis: Enterococcus faecalis*; *M. luteus: Micrococcus luteus*; *L. innocua: Listeria innocua*; BHI: Brain heart infusion; [a]: ATCC: American Type Culture collection; CECT: Colección Española de Cultivos Tipos; DSM: Deutsche Sammlung von Mikroorganismen.

2.2. Isolation of bacteriocin producing strain

Strains were isolated from feces of healthy Algerian new born infants during the first week after birth. The samples were serially diluted in sterile 0.9% (w / v) sodium chloride solution, plated onto MRS agar (Oxoid, England), and incubated anaerobically in an anaerobic flask with an anaerobic reagent (Anaeroculte P, Merck) at 37 ˚C for 48 h.

Gram-positive, catalase-negative bacilli were identified as presumptive lactobacilli; these strains were screened for bacteriocins production using the satb-on-agar test against *M. luteus* ATCC 10420. One bacilli strain which exhibited the strongest antagonistic activity against the indicator strain *M. luteus* ATCC 10420 was retained.

2.3. Stab-on-agar test

The antimicrobial activity of isolates was assayed by a stab-on-agar test. The isolates were stabbed onto MRS plates and incubated at 37 ˚C for 16 h to initiate bacterial growth and bacteriocin production, then, 15 mL of MRS soft agar (0.8% agar) containing about 10^5 CFU/mL of the indicator strains were poured over the plates. Following incubation at 37 ˚C for 18 h, the plates were examined for growth inhibition zones[17].

2.4. Phenotypic identification

Carbohydrate fermentation reactions were recorded by using the API 50 CHL system (BioMerieux, Marcy l'Etoile, France). The API strip was incubated at 30 ℃ and readings were taken twice (after 24 and after 48 h), respectively.

PCR was used to amplify the 16S rRNA gene of bacteriocin-producing strains. The 16S rDNA sequence was determined by direct sequencing. Total DNA was isolated by using Wizard® genomic DNA purification kit (Promega, Madison, USA). Primers used for PCR and DNA sequencing are presented in Table 2. The PCR amplification was performed with the primer pair SPO/SP6 targeted against regions of 16S rDNA[18]. Amplification of DNA was performed in a Thermal cycler, MyCycler personal thermal cycler (Bio-Rad, Brussels, Belgium). PCR conditions included a hot start at 96 ℃ (5 min), 25 cycles consisting of hybridation at 50 ℃ (1 min), polymerisation at 72 ℃ (2 min), denaturation at 96 ℃ (1 min) and a final extension at 72 ℃ (10 min). PCR products were resolved by electrophoresis in 1% (w / v) agarose gel and visualized by ethidium bromide (1 μL/10 mL) staining.

Table 2

Pimers used for PCR and sequencing of 16S rDNA of LbM2a strain.

Technique	Primers	Sequence	Sense	Source
PCR	16SPO	5'-AAGAGTTTGATCCTGGCTCAG-3'	Forward	[18]
	16SP6	5'-CTACGGCTACCTTGTTACGA-3'	Reverse	[18]
Sequencing*	F1	5'-CTGGCTCAGGAYGAACG-3'	Forward	(Sigma-Proligo)
	F2	5'-GAGGCAGCAGTRGGGAAT-3'	Forward	(Sigma-Proligo)
	F3	5'-ACACCARTGGCGAAGGC-3'	Forward	(Sigma-Proligo)
	F4	5'-GCACAAGCGGYGGAGCAT-3'	Forward	(Sigma-Proligo)
	R1	5'-CTGCTGGCACGTAGTTAG-3'	Reverse	(Sigma-Proligo)
	R2	5'-AATCCTGTTYGCTMCCCA-3'	Reverse	(Sigma-Proligo)
	R3	5'-CCAACATCTCACGACACG-3'	Reverse	(Sigma-Proligo)
	R4	5'-TGTGTAGCCCWGGTCRTAAG-3'	Reverse	(Sigma-Proligo)

F: Forward primer; R: Reverse prime; *: Synthetic primers used for sequencing were deduced from alignment of 16S rDNA genes collected from EMBL (Europeen Molecular Biology Laboratory) databases and have been supplied by Sigma-Proligo.

2.5. PCR detection of bacteriocin genes

Using primer pairs of well-known bacteriocins in *L. plantarum*, *P. acidilactici*, and *E. faecalis* were analyzed to determine the presence of bacteriocin cluster genes in *L. plantarum* LbM2a. Primer pairs were designed according to the sequences of PlnEF, PlnJ, PlnK, sakacin P and enterocin P used as negative control (Table 3). These primers were synthesized by Sigma-Proligo (Paris, France). Amplification (Thermal cycler, MyCycler personal thermal cycler; Bio-Rad, Brussels, Belgium) included denaturation at 95 ℃ for 5 min, followed by 30 cycles of denaturation at 95 ℃ for 30 seconds, annealing temperature in Table 3 for 30 seconds, and extension at 72 ℃ for 2 min. A final extension step was performed at 72 ℃ for 7 min. The migration, visualization, and quantification of the reaction products were performed by electrophoresis in 1% (w / v) agarose gels that were visualized by ethidium bromide staining. The 100 bp DNA ladder plus, GeneRuler (Fermentas Life Sciences, St. Leon-Rot, Germany) was used as molecular size standards.

Table 3

Primer sequences used for the amplification and sequencing reactions of bacteriocin gene fragments.

Target	PCR primer	Expected size (bp)	Annealing temperature (℃)	Reference
PlnEF	F: 5'-GGCATAGTTAAAATTCCCCCC-3'	428	53	[19]
	R: 5'-CAGGTTGCCGCAAAAAAAG-3'			
PlnJ	F: 5'-TAACGACGGATTGCTCTG-3'	475	51	[19]
	R: 5'-AATCAAGGAATTATCACATTAGTC-3'			
PlnK	F: 5'-CTGTAAGCATTGCTAACCAATC-3'	469	53	[19]
	R: 5'-ACTGCTGACGCTGAAAAG-3'			
Sakacin P	F: 5'-GAAWTRMMANCAATTAYMGGTGG-3'	120	51	[20]
	R: 5'-GGCCCAGTTTGCAGCTGCAT-3'			
Enterocin P	F: 5'-TGAGAAAAAAATTATTTAGTTTAGCTCTTATTGG-3'	488	60	[21]
	R: 5'-TTAATGTCCCATACCTGCCAAACCAG-3'			

F: Forward primer; R: Reverse primer.

2.6. DNA sequence analysis

The amplicons previously obtained were purified with the Microcon YM-100 kit (Bedford, MA, USA) and sequenced with the same primer used in the amplification or for bacteria identification described in Table 3 and the Big Dye Terminator v3.1 kit (Applied Biosystems, Forster City, CA, USA), was used as specified by the manufacturer. The obtained sequences (Gene Sequencer, ABI PRISM® 3100; ABI, Forster City, CA, USA) were then assembled using the Vector NTI Suite 10 software package and transformed into proteins. The deduced amino acid sequences were aligned with those of bacteriocin precursors proteins collected from the EMBL-EBI database (European Bioinformatics Institute, www.ebi.ac.uk/fasta33) and the GenBank database. Phylogenetic identification was analyzed using the Ribosomal Database Project's Sequence Match (RDP; http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp); the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). GenBank was realized by an alignment of sequence consensus of the 16S rDNA genes collected in an international database. Similarity obtained scores were expressed as percent genetic homology between the submitted sequence and the databases resulting sequences.

2.7. Bacteriocin activity assays

2.7.1. Spot on agar test (SPAT)

The direct antimicrobial activity of culture from LbM2a against indicators was firstly screened by using a SPAT test. Briefly, LbM2a was precultured in MRS broth (OD600 of 2.0) for overnight. Subsequently, amounts of 5 μL of the preculture were spotted onto plates of media and dried for 20 min under a laminar flow hood. These plates were overlaid with MRS or BHI soft agar previously seeded with approximately 1×10^5 CFU/mL of the indicator microorganisms and further incubated at 37 ℃ for 24 h. Therefore, diameters (mm) of growth inhibition zones were measured. After preparation of the supernatant (SCS) from LbM2a strain, antimicrobial activity was secondly confirmed using an agar well-diffusion test (AWDT)[17].

2.7.2. Preparation of the supernatant

The LbM2a was further grown in MRS broth at 37 ˚C. After 16 h of incubation, SCS was obtained by centrifugation at 12 000 r/min at 4 ˚C for 10 min, pH adjustment to 6.2 with 1 mol/L NaOH, supplemented with catalase (Sigma Aldrich, Germany) at a final concentration of 1.0 mg/mL at 30 ˚C for 1 h and filter-sterilization through 0.22 µm-pore-size filters (Millipore Corp., Bedford, Massachusetts, USA) then stored at -20 ˚C until use. Bacteriocin activity of supernatants was determined by an AWDT[17].

2.7.3. AWDT

The antimicrobial activity of the supernatants was determined by the agar well diffusion assay, performed as described by Cintas *et al*[17]. About 50 µL aliquots of SCS were placed in wells (6 mm diameter) cut in cooled soft MRS or BHI agar plates (20 mL) previously seeded (10^5 CFU/mL) with indicator microorganisms above-mentioned. After 2 h at 4 ˚C, the plates were incubated at 37 ˚C for growth of the target organism; after 24 h, the diameters (mm) of the growth inhibition zones were measured.

2.8. Stability of SCS activity during storage

Active SCS was stored at -20, 4 and 37 ˚C. At different time intervals from 0 to 120 min, samples were taken from the stored material to determine bacteriocin activity using AWDT against *M. luteus* ATCC 10420 as target organism[22].

2.9. Effect of mitomycin C on SCS activity

Mitomycin C was added at a final concentration of 1.0 µg/mL to the active SCS then incubated at 30 ˚C. Samples were removed at 20, 60, 90, 100, 120 and 140 min and analyzed by the well diffusion method AWDT against *M. luteus* ATCC 10420.

2.10. Effect of UV light on bacteriocin activity

A 10 mL aliquot of the active SCS was placed in a sterile Petri dish and exposed to short-wave UV light from a 15-W General Electric germicidal bulb at a distance of 30 cm (exposure time ranged from 0 to 60 min). After each time interval, SCS activity was analyzed by the well diffusion method (AWDT) against *M. luteus* ATCC 10420.

2.11. Heat resistance

The SCS was exposed to various heat treatments: 40, 60, 80, 100 and 121 ˚C. Aliquot volumes of each fraction were then removed after 10, 30, 60 and 90 min and assayed by AWDT against *M. luteus* ATCC 10420[22].

2.12. pH sensitivity

SCS (400 µL) were adjusted to pH 2, 3, 4, 6, 8, 10 and 12 with 5 mol/L hydrochloric acid (HCl) and 5 mol/L sodium hydroxide (NaOH), incubated for 4 h at room temperature and similarly assayed against *M. luteus* ATCC 10420[22].

2.13. Ultrafiltration studies

SCS was passed through various filtron membranes, firstly with 10 kDa and secondly with one of 3 kDa cutoffs (Amicon® Ultra-15 Centrifugal Filter Devices, Millipore). Antibacterial activity was determined in both retained and eluted fractions by AWDT from all the fractions against *M. luteus* ATCC 10420 and subsequently, diameters of the inhibition zones were taken[23].

2.14. Statistical analysis

The data from bacteriocin activity assays were analyzed with StatBox 6.40 software (Grimmer logiciels, Paris, France).

3. Results

3.1. Isolation of bacteriocin producing strain

After screening for antibacterial activity, the isolate LbM2a was selected among 10 different colonies on the basis of the production of the largest zone of inhibition against *M. luteus* ATCC 10420. The selected bacterium was catalase negative; the cells presented Gram-positive bacilli.

3.2. Bacterial identification

Phenotypic characterization of the isolated strain was carried out by standard methods using API 50 CHL test kits (BioMérieux SA, Marcy I'Etoile, France). The results were analyzed with the identification system (bioMérieux SA) on apiweb (https://apiweb.biomerieux.com), which used the phenotypic data to predict the species identity of the test strain. Results showed the identification of the LbM2a strain as *L. plantarum* with 98.9% of similarity.

Nucleotide sequences of 16S rDNA were carried out; the determined 16S rDNA sequence was compared directly with the Genbank database. A high level of similarity of 16S ribosomal DNA nucleotide sequences (99% of matches) of the bacilli strain LbM2a isolate was observed with the sequences of *L. plantarum* (Table 4). Genbank databases were deposited under the accession number KF682392. On the basis of 16S rRNA gene sequence of the isolate and their closest phylogenetic neighbours, a phylogenetic tree was performed (Figure 1).

3.3. Nucleotide sequence accession numbers

PlnEF and PlnK nucleotide sequences have been assigned GenBank accession numbers KF802198 and KF802197 respectively (Table 5).

3.4. Identification of genes encoding bacteriocin production

In an attempt to determine whether the *L. plantarum* LbM2a carried genes for the production of some known bacteriocin produced by the same species; PCR analysis using five couples of primers specific for individual bacteriocin genes was used. The PCR

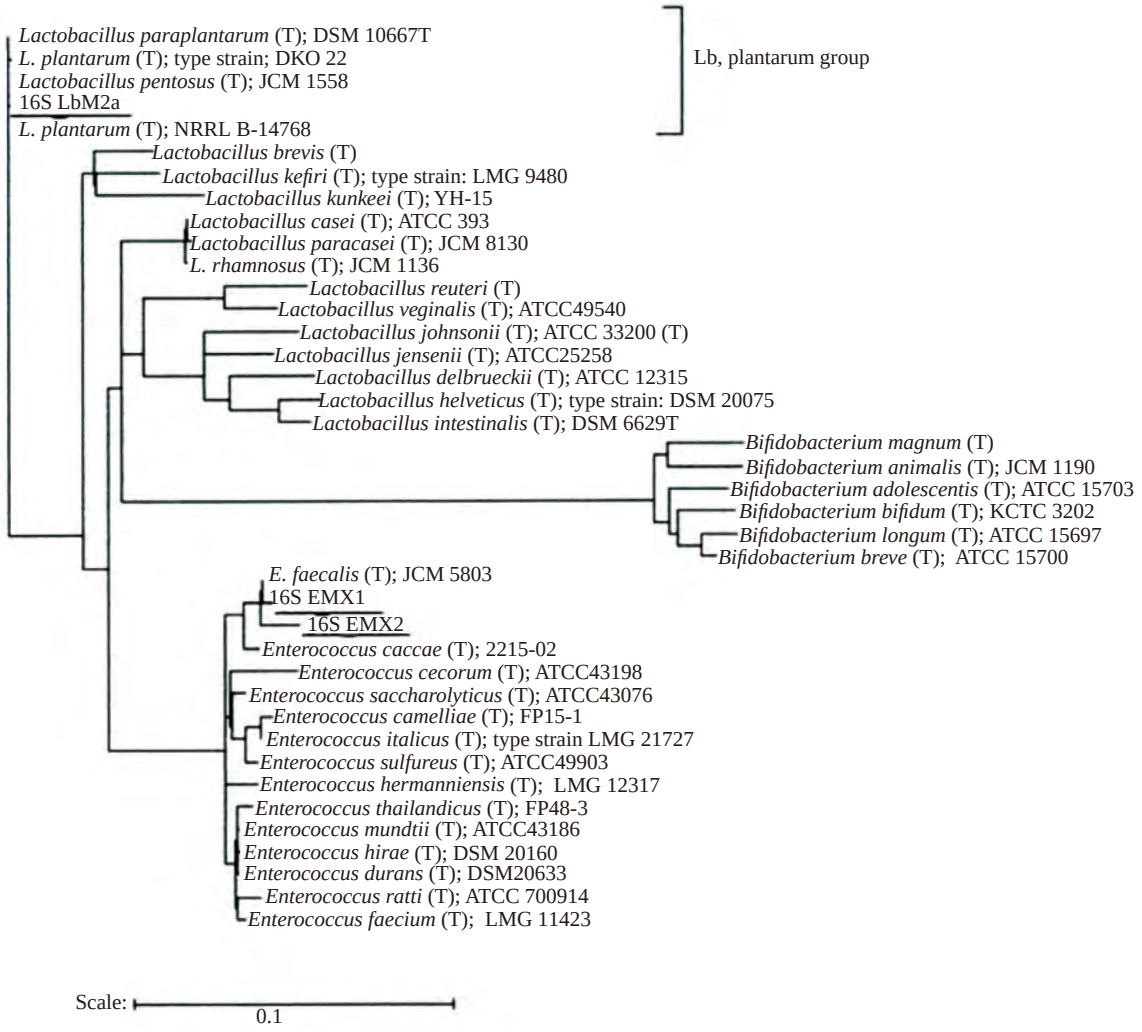

Figure 1. Neighbour-joining phylogenetic tree is based on 16S rRNA gene sequence of the isolate and their closest phylogenetic neighbours.

results showed that approximately 435 and 460 bp product was obtained by primer specific for PlnEF and PlnK respectively, and no DNA fragment was amplified with other three couples of primers (Figure 3).

The amplification product contained PlnEF, PlnK. Sequence analysis, after amplification, showed 99% homology with the structure gene of PlnEF and PlnK from the GenBank. These results suggested that bacteriocins produced by the LbM2a strain were identical to PlnEF and PlnK.

3.5. The inhibitory spectrum

The selected LbM2a strain produced bacteriocin, exerting an important inhibitory activity against one or more of the Gram-positive and Gram-negative target strains (Tables 4 and 6, Figures 4 and 5).

3.6. Sensitivity of the active SCS to temperature, different pH and enzymes

The effects of heat, storage time, pH and enzymes on bacteriocin activity were determined using *M. luteus* ATCC 10420 as indicator organism.

Bacteriocins produced by *L. plantarum* LbM2a remained with constant activity after heating at 100 ˚C for 30 min. Prolongation of this treatment resulted in decrease of this activity.

A constant activity was noted after treatment at 121 ˚C for 10 min followed by subsequent decline. After 90 min, there was no detectable bacteriocin activity (Figure 6).

The effect of time and temperature of storage on bacteriocin activity were also carried out. We observed no change in activity at storage temperature of -80 ˚C for 120 days. It was noted that the bacteriocins produced by the test strain had maintained full stability after storage for 60 days at -20 ˚C; partial stability after storage for 120 days at 4 ˚C, while no activity was detected after storage for 60 to 120 days at 37 ˚C (Figure 7).

Effect of pH on activity of bacteriocin was evaluated. However, it was observed that bacteriocin produced *L. plantarum* LbM2a was stable between pH 2 to 6, with a maximum of activity at pH 6 (Figure 8).

Bacteriocin produced by this strain was tested for its sensitivity to various enzymes. The antimicrobial activity was lost or unstable after treatment with all the proteolytic enzymes (Figure 9), whereas treatment with α–amylase, mitomycin and UV-light did not affect the activity of bacteriocin produced by the LbM2a strain.

The SCS from LbM2a strain was subjected to ultrafiltration

Table 4

Inhibition zone (mm) obtained with LbM2a strain against *M. luteus* ATCC 10420 using AWDT.

Indicator organism	[a]Strain	Medium-incubation temperature	[b]Sensitivity
C. maltaromaticum	DSM 20722	BHI, 37 °C	++++
S. aureus	ATCC 25923	BHI, 37 °C	++++
L. innocua	BUG 498	BHI, 37 °C	++++
C. maltaromaticum	DSM 20730	BHI, 37 °C	++++
C. maltaromaticum	CECT 5808	BHI, 37 °C	++++
M. luteus	CECT 241	BHI, 37 °C	++++
S. sonnei	CECT 584	BHI, 37 °C	++++
L. innocua	BUG 499	BHI, 37 °C	+++
S. aureus	ATCC 33862	BHI, 37 °C	+++
S. dysenteria	CECT 457	BHI, 37 °C	+++
L. innocua	CECT 910	BHI, 37 °C	+++
E. coli	ATCC 25122	BHI, 37 °C	+++
E. coli	DH10B	BHI, 37 °C	+++
M. luteus	ATCC 10420	BHI, 37 °C	+++
E. faecalis	EMX2	BHI, 37 °C	+++
B. cereus	ATCC 9884	BHI, 37 °C	+++
L. sake	LTH 673	MRS, 37 °C	+++
L. sakei	K23	MRS, 37 °C	++
L. rhamnosus	ATCC 7469	MRS, 37 °C	-
L. acidophilus	ATCC 4529	MRS, 37 °C	-
L. plantarum	CECT 748	MRS, 37 °C	-
P. pentosaceus	CECT 4695	MRS, 37 °C	-

[a]: ATCC: American Type Culture collection; CECT: Colección Española de Cultivos Tipos; DSM: Deutsche Sammlung von Mikroorganismen; [b]: Inhibition zone (mm): ++++, > 17 mm; +++, 14-17 mm; ++, 11-14 mm; +, 7-10 mm; -, no inhibition.

Table 5

GenBank accession numbers of isolates and plantaricin.

Sequence	GenBank accession numbers	Reference from NCBI database	Percentage similarity (%)
16S_LbM2a	KF682392	*L. plantarum*	99
16S_EMX1	KF682393	*E. faecalis*	99
LbM2a_plnK	KF802197	PlnK	99
LbM2a_plnE	KF802198	PlnE	99

Table 6

Inhibition zones (mm) obtained with LbM2a strain against *M. luteus* ATCC 10420 using SPAT.

Indicator organism	[a]Strain	Medium-incubation temperature	[b]Sensitivity
L. innocua	BUG499	BHI, 37 °C	++++
L. innocua	CECT 910	BHI, 37 °C	++++
S. sonnei	CECT 584	BHI, 37 °C	++++
S. aureus	ATCC 25923	BHI, 37 °C	++++
S. aureus	ATCC 33862	BHI, 37 °C	++++
M. luteus	CECT 241	BHI, 37 °C	++++
S. dysenteria	CECT 457	BHI, 37 °C	++++
L. innocua	BUG498	BHI, 37 °C	++++
C. maltaromaticum	DSM20722	BHI, 37 °C	++++
M. luteus	ATCC 10420	BHI, 37 °C	++++
C. maltaromaticum	DSM20730	BHI, 37 °C	++++
C. maltaromaticum	CECT5808	BHI, 37 °C	++++
B. cereus	ATCC 9884	BHI, 37 °C	++++
E. coli	ATCC 25122	BHI, 37 °C	++++
E. coli	DH10B	BHI, 37 °C	++++
E. faecalis	EMX2	BHI, 37 °C	++++
L. sake	LTH 673	MRS, 37 °C	++++
L. sakei	K23	MRS, 37 °C	++++
L. rhamnosus	ATCC 7469	MRS, 37 °C	-
L. acidophilus	ATCC 4529	MRS, 37 °C	-
L. plantarum	CECT 748	MRS, 37 °C	-
P. pentosaceus	CECT 4695	MRS, 37 °C	-

[a]: ATCC: American Type Culture collection; CECT: Colección Española de Cultivos Tipos; DSM: Deutsche Sammlung von Mikroorganismen; [b]: Inhibition zone (mm): ++++ > 17 mm; +++, 14-17 mm; ++, 11-14 mm; +, 7-10 mm; -, no inhibition.

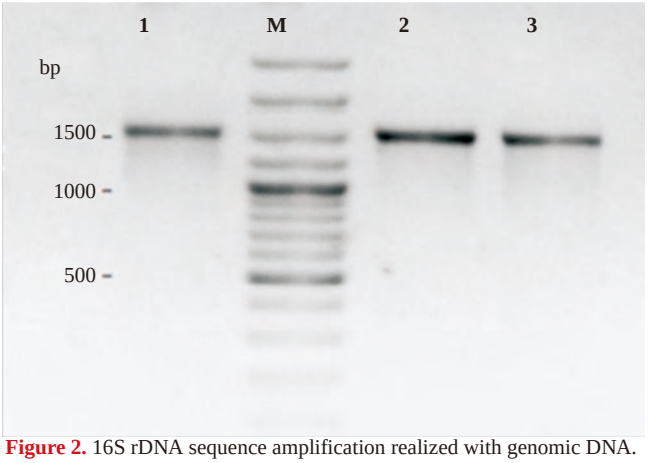

Figure 2. 16S rDNA sequence amplification realized with genomic DNA. 1: *Enterococcus* EMX1; 2: *Enterococcus* EMX2; 3: LbM2a. Using primer pair SP0/SP6, a GeneRuler 100-bp DNA ladder plus (Fermentas) was used as nucleic acid molecular size marker (Lane M).

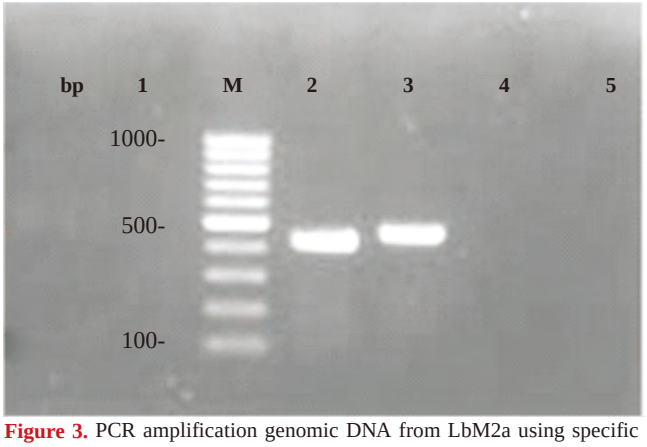

Figure 3. PCR amplification genomic DNA from LbM2a using specific primer pairs of sakacin P (Lane 1), PlnEF (Lane 2), PlnK (Lane 3), PlnJ (Lane 4) and enterocin P as negative control (Lane 5).
A GeneRuler 100 bp DNA ladder (Fermentas) was used as nucleic acid molecular size marker (Lane M).

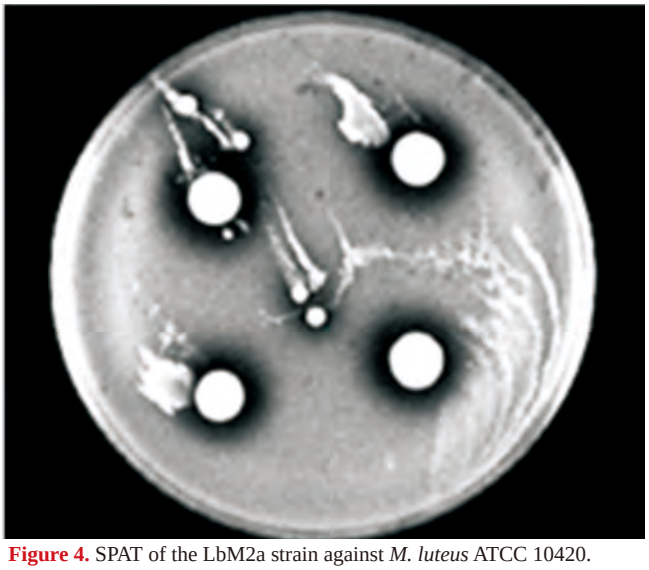

Figure 4. SPAT of the LbM2a strain against *M. luteus* ATCC 10420.

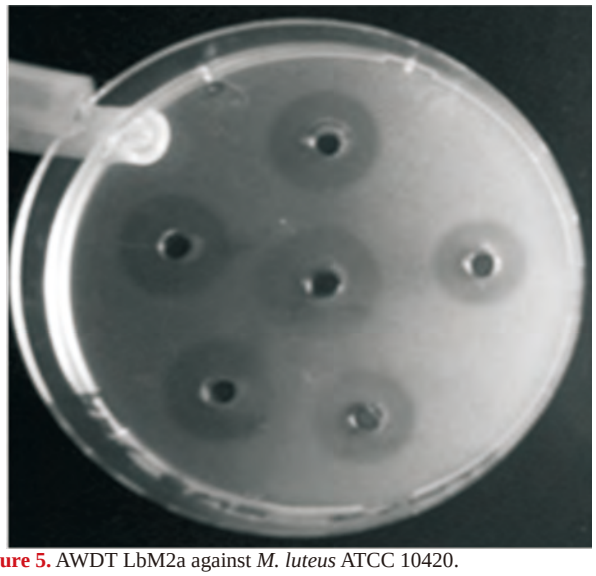

Figure 5. AWDT LbM2a against *M. luteus* ATCC 10420.

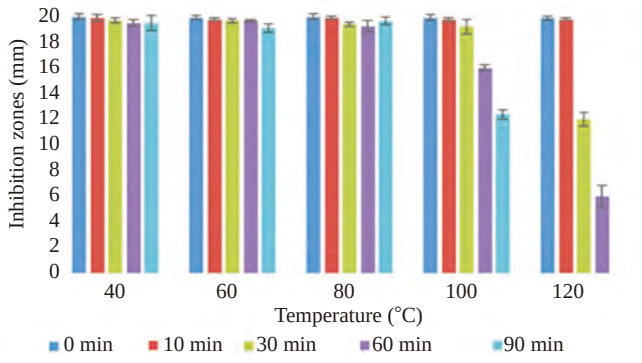

Figure 6. Effect of heat treatment on SCS activity of the LbM2a strain using *M. luteus* ATCC 10420 as indicator strain.

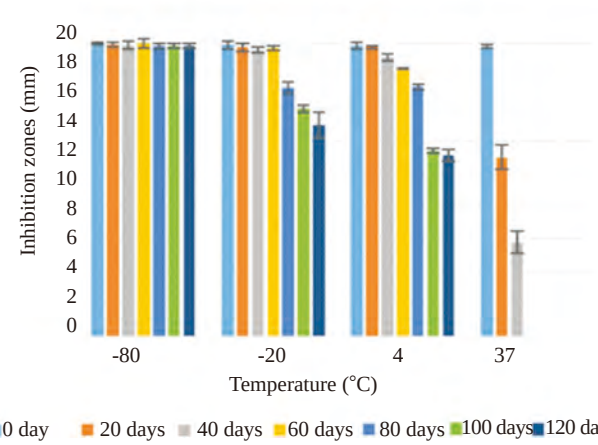

Figure 7. Effect of storage time and temperature on SCS activity of the LbM2a strain using *M. luteus* ATCC 10420 as indicator strain.

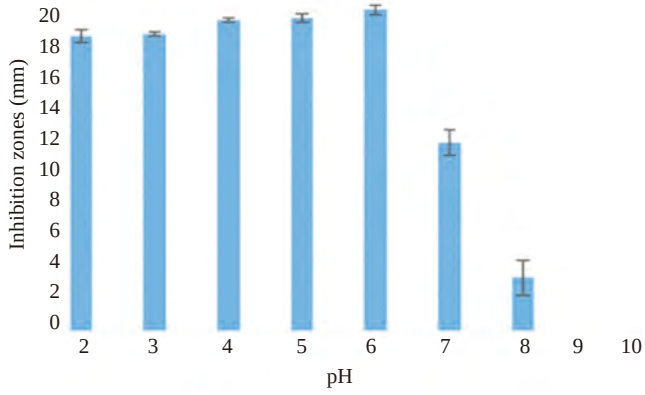

Figure 8. Activity of SCS of the LbM2a strain in different range of pH using *M. luteus* ATCC 10420 as indicator strain.

using various filtron membranes. The eluted and retained fractions were collected and assayed for bacteriocin activity. When filtered through membrane with 10 000 kDa molecular weight cut off, the bacteriocins were able to pass through cellulose membranes, but filtration was not achieved with 3 000 kDa molecular weight cut off.

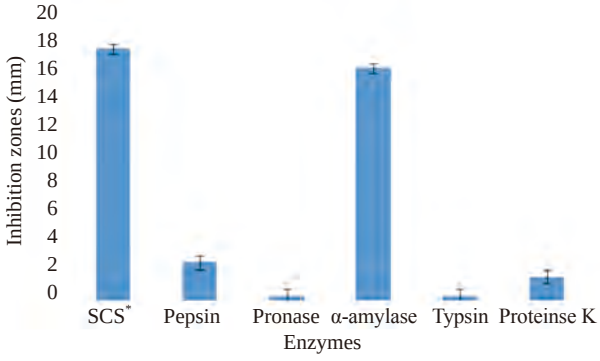

Figure 9. Effect of enzymes on activity (mm) of SCS of the LbM2a strain using *M. luteus* ATCC 10420 as indicator strain.
SCS*: Spent culture supernatant without treatment.

4. Discussion

The human gastrointestinal tract contains a complex and dynamic microbiota. The gastrointestinal tract of each individual has a unique microbiota that varies according to age, health, and lifestyle[24]. The microbial balance provides a barrier against pathogens and harmful food substances and has important protective functions promoting beneficial effects in the host[25].

Results of the phenotypic identification reveal that the selected isolate LbM2a from healthy infant feces belongs to *L. plantarum*. Kılıç *et al*. isolated twenty *L. plantarum* strains from the fecal samples of humans[26]. These strains were investigated *in vitro* for their characteristics as potential new probiotic strains.

A clear identification of species, especially within the genus *Lactobacillus*, based on fermentation patterns, may sometimes be difficult, due to an increasing number of lactic acid bacteria species which vary on a small number of biochemical traits[27]. For this reason, the fermentation profile should be combined with genotypic techniques.

The nucleotide sequences of 16S rDNA confirm the appurtenance of the bacilli strain LbM2a to *L. plantarum* species.

Sequence analysis after amplification using five couples of primers specific for individual bacteriocin showed 99% homology with the structure gene of PlnEF and PlnK from the GenBank and therefore confirms the implication of plantaricin in the antagonistic action.

These results suggested that bacteriocins produced by were identical to PlnEF and PlnK.

Bacteriocin activity exerted by the LbM2a is not limited by the extremely narrow antibacterial spectrum as reported for some bacteriocins of some lactic acid bacteria. In a similar study, Sankar

et al. reported the bacteriocin producing *L. plantarum* strain isolated from raw cow's milk samples and showed broad range of antibacterial activity against food borne pathogens[28].

The antimicrobial activity of the bacteriocins exerted by the LbM2a strain in this study was not due to hydrogen peroxide or acidity, as the activity was not lost after treatment with catalase or adjustment of pH to 6.2.

Lactic acid bacteria synthesize bactericidal agents that vary in their spectra of activity. Many of these agents are bacteriocins substances of protein structure[2].

This strain inhibited 18 out of 22 indicator strains including *C. maltaromaticum*, *S. aureus*, *Listeria*, *M. luteus*, *E. faecalis*, *S. sonnei*, *E. coli*, *B. cereus* and *L. sake*. However, no activity was detected against *P. pentosaceus*, *L. acidophilus* and *L. rhamnosus*. Mills *et al*. demonstrates that *L. plantarum* LMG P-26358 isolated from a soft french artisanal cheese produces a potent class IIa bacteriocin with 100% homology to plantaricin 423 and bactericidal activity against *L. innocua* and *Listeria monocytogenes* (*L. monocytogenes*)[29].

Gong *et al*. observed that plantaricin MG produced by *L. plantarum* KLDS1.0391 which was isolated from ''Jiaoke"[30]. A traditional naturally fermented cream from Inner Mongolia in China showed a broad inhibitory activity against Gram-positive and Gram-negative bacteria including *L. monocytogenes*, *S. aureus*, *M. luteus*, *Clostridium perfringens*, *B. cereus*, *Bacillus subtilis*, *E. coli*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Salmonella typhimurium*, but showed low activity against most *Lactobacillus* spp.

In another research Hu *et al*. demonstrated that plantaricin 163, a novel bacteriocin produced by *L. plantarum* 163 which was isolated from traditional Chinese fermented vegetables exerts a broad-spectrum inhibitory activity[31]. This activity was not only against LAB but also against other Gram-positive and Gram-negative bacteria (*S. aureus*, *L. monocytogenes*, *Bacillus pumilus*, *B. cereus*, *M. luteus*, *Lactobacillus thermophilus*, *L. rhamnosus*, *E. coli*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens*). These findings seemed to be in total agreement with our observations.

Bacteriocins produced by *L. plantarum* LbM2a strain appear to be resistant after heating at 100 ˚C for 30 min, stable at pH range from 2 to 6, with a proteinaceous nature and conserve full stability after storage for 60 days at -20 ˚C. All of these characteristics are advantageous for the application of these bacteriocins in food industry. These observations are in accordance with the guidelines outlined by Tagg *et al*[32].

Heat stability of antibacterial substances produced by *Lactobacillus* spp. has been well established[33]. Bacteriocin MBSa1 isolated from *L. sakei* MBSa1 was heat-stable and showed also a stability of activity at pH 2 to 6[34]. Todorov *et al*. reported that *L. plantarum* ST31 bacteriocin exhibited the maximum of activity at pH 5.0-5.5[35]. Bovicin HC5 was at least 10 fold more active at pH 5.5 than at pH 7, which indicates its ability to form a pore in the cell membrane is much greater at acidic pH condition[36].

The antimicrobial activity was lost or unstable after treatment with all the proteolytic enzymes, whereas no changes in the activity was observed after treatment with α–amylase, mitomycin and UV-light. Similar results have been reported for other proteinaceous inhibitors on bacteriocin of *L. plantarum* bacST202Ch and bacST216Ch[37].

Ultrafiltration studies showed that the antagonistic activity was detected at molecular weight comprised between 10 000 and 3 000 kDa, which corresponds to the molecular weight range in which the plantaricins PlnE, PlnF, PlnJ, and PlnK are included[11]. However, partial loss of bacteriocin activity was observed during ultrafiltration which might be due to the membrane absorption of the bacteriocin. The same observation was noted by Mojgani *et al*[38].

In conclusion, the bacteriocins produced by *L. plantarum* LbM2a strain showed globally high stability to various factors as well as a broad antibacterial spectrum. Our results highly suggest that strain LbM2a isolated from the stools of Algerian babies or its bacteriocins might have future applications as food preservative to inhibit the growth of spoilage and pathogenic bacteria as well as the possibility of its use as therapeutic molecules.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1] Todorov SD. Bacteriocins from *Lactobacillus plantarum* production, genetic organization and mode of action. *Braz J Microbiol* 2009; **40**: 209-22.

[2] Zacharof MP, Lovitt RW. Bacteriocins produced by lactic acid bacteria a review article. *APCBEE Procedia* 2012; **2**: 50-6.

[3] Maurya AP, Thakur RL. Inhibition spectrum, purification and characterization of bacteriocin from *Leuconostoc* NT-1. *Curr Sci* 2012; **103**(12): 1405-7.

[4] Cetinkaya S, Osmanağaoğlu O, Cökmüş C. Bacteriocin diversity in *Bacillus sphaericus*. *Folia Microbiol* 2003; **48**: 157-61.

[5] Osuntoki AA, Gbenle GO, Olukoya DK. Evidence for chromosomal determination of fungicidal activity in strains of *Lactobacillus brevis* and *Lactobacillus fermentum* isolated from fermented foods. *Folia Microbiol* 2003; **48**: 56-8.

[6] Todorov SD, Rachman C, Fourrier A, Dicks LM, van Reenen CA, Prévost H, et al. Characterization of a bacteriocin produced by *Lactobacillus sakei* R1333 isolated from smoked salmon. *Anaerobe* 2011; **17**: 23-31.

[7] Prins WA, Botha M, Botes M, Kwaadsteniet M, Endo A, Dicks LM. *Lactobacillus plantarum* 24, isolated from the marula fruit (*Sclerocarya birrea*), has probiotic properties and harbors genes encoding the production of three bacteriocins. *Curr Microbiol* 2010; **61**: 584-9.

[8] Daniel C, Roussel Y, Kleerebezem M, Pot B. Recombinant lactic acid bacteria as mucosal biotherapeutic agents. *Trends Biotechnol* 2011; **29**: 499-508.

[9] Parente E, Ciocia F, Ricciardi A, Zotta T, Felis GE, Torriani S. Diversity

of stress tolerance in *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum*: a multivariate screening study. *Int J Food Microbol* 2010; **144**: 270-9.

[10] Todorov SD, Prévost H, Lebois M, Dousset X, LeBlanc JG, Franco BDGM. Bacteriocinogenic *Lactobacillus plantarum* ST16Pa isolated from papaya (*Carica papaya*)-from isolation to application: characterization of a bacteriocin. *Food Res Int* 2011; **44**: 1351-63.

[11] Diep DB, Straume D, Kjos M, Torres C, Nes IF. An overview of the mosaic bacteriocin pln loci from *Lactobacillus plantarum*. *Peptides* 2009; **30**: 1562-74.

[12] Rogne P, Haugen C, Fimland G, Nissen-Meyer J, Kristiansen PE. Three dimensional structure of the two-peptide bacteriocin plantaricin JK. *Peptides* 2009; **30**(9): 1613-21.

[13] Ehrmann MA, Remiger A, Eijsink VGH, Vogel RF. A gene cluster encoding plantaricin 1.25β and other bacteriocin-like peptides in *Lactobacillus plantarum* TMW1.25. *Biochim Biophys Acta* 2000; **1490**: 355-61.

[14] Nissen-Meyer J, Rogne P, Oppegård C, Haugen HS, Kristiansen PE. Structure-function relationships of the non-lanthionine-containing peptide (class II) bacteriocins produced by Gram-positive bacteria. *Curr Pharm Biotechnol* 2009; **10**: 19-37.

[15] Hauge HH, Mantzilas D, Eijsink VG, Nissen-Meyer J. Membrane-mimicking entities induce structuring of the two-peptide bacteriocins plantaricin E/F and plantaricin J/K. *J Bacteriol* 1999; **181**: 740-7.

[16] De Man JC, Rogosa M, Sharpe ME. A medium for the cultivation of lactobacilli. *J Appl Microbiol* 2008; **23**(1): 130-5.

[17] Cintas LM, Rodriguez JM, Fernandez MF, Sletten K, Nes IF, Hernandez PE, et al. Isolation and characterization of pediocin L50, a new bacteriocin from *Pediococcus acidilactici* with a broad inhibitory spectrum. *Appl Environ Microbiol* 1995; **61**: 2643-8.

[18] Ventura M, Elli M, Reniero R, Zink R. Molecular microbial analysis of *Bifidobacterium* isolates from different environments by the species-specific amplified ribosomal DNA restriction analysis (ARDRA). *FEMS Microbiol Ecol* 2001; **36**: 113-21.

[19] Rojo-Bezares B, Sáenz Y, Navarro L, Zarazaga M, Ruiz-Larrea F, Torres C. Coculture-inducible bacteriocin activity of *Lactobacillus plantarum* strain J23 isolated from grape must. *Food Microbiol* 2007; **24**: 482-91.

[20] Kouakou P, Dortu C, Dubois-Dauphin R, Vandenbol M, Thonart P. Plasmid-associated bacteriocin production by *Lactobacillus* LMG21688 suppresses *Listeria monocytogenes* growth rebound in a food system. *FEMS Microbiol Lett* 2010; **306**: 37-44.

[21] Gutiérrez J, Criado R, Citti R, Martín M, Herranz C, Nes IF, et al. Cloning, production and functional expression of enterocin P, a sec-dependent bacteriocin produced by *Enterococcus faecium* P13, in *Escherichia coli*. *Int J Food Microbiol* 2005; **103**: 239-50.

[22] ten Brink B, Minekus M, van der Vossen JM, Leer RJ, Huis in't Veld JH. Antimicrobial activity of lactobacilli: preliminary characterization and optimization of production of acidocin B, a novel bacteriocin produced by *Lactobacillus acidophilus* M46. *J Appl Bacteriol* 1994; **77**: 140-8.

[23] Lee NK, Park YL, Park YH, Kim JM, Nam HM, Jung SC, et al. Purification and characterization pediocin SA131 produced by *Pediococcus pentosaceus* SA131 against bovine mastsis pathogens. *Milchwissenschaft-Milk Sci Int* 2010; **65**: 19-21.

[24] Tiihonen K, Ouwehand AC, Rautonen N. Human intestinal microbiota and healthy ageing. *Ageing Res Rev* 2010; **9**: 107-16.

[25] Jankovic I, Sybesma W, Phothirath P, Ananta E, Mercenier A. Application of probiotics in food products-challenges and new approaches. *Curr Opin Biotechnol* 2010; **21**: 175-81.

[26] Kılıç GB, Kuleaşan H, Sömer VF, Akpınar D. Determining potential probiotic properties of human originated *Lactobacillus plantarum* strains. *Biotechnol Bioprocess Eng* 2013; **18**: 479-85.

[27] Marroki A, Zúñiga M, Kihal M, Pérez-Matínez G. Characterization of *Lactobacillus* from Algerian goat's milk based on phenotypic, 16S rDNA sequencing and their technological properties. *Braz J Microbiol* 2011; **42**: 158-71.

[28] Sankar NR, Priyanka VD, Reddy PS, Rajanikanth P, Kumar VK, Indira M. Purification and characterization of bacteriocin produced by *Lactobacillus plantarum* isolated from cow milk. *Int J Microbiol Res* 2012; **3**(2): 133-7.

[29] Mills S, Serrano LM, Griffin C, O'Connor PM, Schaad G, Bruining C, et al. Inhibitory activity of *Lactobacillus plantarum* LMG P-26358 against *Listeria innocua* when used as an adjunct starter in the manufacture of cheese. *Microb Cell Fact* 2011; **10**(Suppl 1): S7.

[30] Gong HS, Meng XC, Wang H. Plantaricin MG active against Gram-negative bacteria produced by *Lactobacillus plantarum* KLDS1.0391 isolated from "Jiaoke", a traditional fermented cream from China. *Food Control* 2010; **21**: 89-96.

[31] Hu M, Zhao H, Zhang C, Yu J, Lu Z. Purification and characterization of plantaricin 163, a novel bacteriocin produced by *Lactobacillus plantarum* 163 isolated from traditional Chinese fermented vegetables. *J Agric Food Chem* 2013; **61**: 11676-82.

[32] Tagg JR, Dajani AS, Wannamaker LW. Bacteriocins of Gram-positive bacteria. *Bacteriol Rev* 1976; **40**(3): 722-56.

[33] Suma K, Misra MC, Varadaraj MC. Plantaricin LP84, a broad spectrum heat-stable bacteriocin of *Lactobacillus plantarum* NCIM 2084 produced in a simple glucose broth medium. *Int J Food Microbiol* 1998; **40**(1-2): 17-25.

[34] Barbosa MS, Todorov SD, Belguesmia Y, Choiset Y, Rabesona H, Ivanova IV, et al. Purification and characterization of the bacteriocin produced by *Lactobacillus sakei* MBSa1 isolated from Brazilian salami. *J Appl Microbiol* 2014; **116**: 1195-1208.

[35] Todorov S, Onno B, Sorokine O, Chobert JM, Ivanova I, Dousset X. Detection and characterization of a novel antibacterial substance produced by *Lactobacillus plantarum* ST31 isolated from sourdough. *Int J Food Microbiol* 1999; **48**: 167-77.

[36] Houlihan AJ, Mantovani HC, Russell JB. Effect of pH on the activity of bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5. *FEMS Microbiol Lett* 2004; **231**: 27-32.

[37] Todorov SD, Ho P, Vaz-Velho M, Dicks LM. Characterization of bacteriocins produced by two strains of *Lactobacillus plantarum* isolated from Beloura and Chouriço, traditional pork products from Portugal. *Meat Sci* 2010; **84**: 334-43.

[38] Mojgani N, Sabiri G, Ashtiani M, Torshizi M. Characterization of bacteriocins produced by *Lactobacillus brevis* NM 24 and *L. fermentum* NM 332 isolated from green olives in Iran. *Int J Microbiol* 2009; **6**(2). doi: 10.5580/1596.