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
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 newborn 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 LbM 2a, 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, J ove 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

| Indicator organism | 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. sake             | K23 INRA | MRS, 37 °C                     |
| R. 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             | DH108 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         | EMX 2 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      | CECT 4695 | MRS, 37 °C                     |
| L. acidophilus       | CECT 748  | MRS, 37 °C                     |
| L. sake              | LTH673   | MRS, 37 °C                     |
| L. plantarum        | CECT 4529 | MRS, 37 °C                     |
| L. rhamnosus         | ATCC 7469 | MRS, 37 °C                     |
| L. sake              | K23 INRA | MRS, 37 °C                     |
| S. aureus            | 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              | DH108 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          | EMX 2 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                     |

2.2. Isolation of bacteriocin producing strain

Strains were isolated from feces of healthy Algerian newborn 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 sabt-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 10¹³ 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 °C 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 °C (5 min), 25 cycles consisting of hybridation at 50 °C (1 min), polymerisation at 72 °C (2 min), denaturation at 96 °C (1 min) and a final extension at 72 °C (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
Primers used for PCR and sequencing of 16S rDNA of LbM2a strain.

| Technique | Primers | Sequence | Sense | Source |
|-----------|---------|----------|-------|--------|
| PCR       | 165P0   | 5'-AAGAAGTTTGTACCTGGCTCAG-3' | Forward | [18]   |
|           | 165P6   | 5'-CTACGGCTACCTTGTTACGA-3' | Reverse | [18]   |
| Sequencing | F1     | 5'-CTGGCTCAGAAYAAGACG-3' | Forward | Sigma-Pro ligol | 475 60 [21] |
|           | F2     | 5'-GAGGCGGACGATRGGGAAT-3' | Forward | Sigma-Pro ligol | 478 54 [20] |
|           | F3     | 5'-ACACCARTGGCGAAGACG-3' | Forward | Sigma-Pro ligol | 478 54 [21] |
|           | F4     | 5'-GCAAACCGGYYGGGACAT-3' | Forward | Sigma-Pro ligol | 478 54 [21] |
|           | R1     | 5'-CTCGTGACACGTAGTATAT-3' | Reverse | Sigma-Pro ligol | 478 54 [21] |
|           | R2     | 5'-AATCCCTTTGCTAATCCAC-3' | Reverse | Sigma-Pro ligol | 478 54 [21] |
|           | R3     | 5'-CACCACATCTCAGGACAC-3' | Reverse | Sigma-Pro ligol | 478 54 [21] |
|           | R4     | 5'-GTGTAGCTCCGTCAGGCTCA-3' | Reverse | Sigma-Pro ligol | 478 54 [21] |

Table 3
Primer sequences used for the amplification and sequencing reactions of bacteriocin gene fragments.

| Target | PCR primer | Expected size (bp) | Annealing temperature (°C) | Reference |
|--------|------------|--------------------|---------------------------|-----------|
| PinEF  | P: 5'-GGCATAGTTAAAATTCCCCCC-3' | 428 | 53 | [19] |
|       | R: 5'-GGCATAGTTAAAATTCCCCCC-3' | | | |
| PinJ   | F: 5'-GAAWTRMMANCAATTAYMGGTGG-3' | 475 | 51 | [19] |
| PinK   | F: 5'-CTGTAGACCGTACCTAACCATC-3' | 469 | 53 | [19] |
| Sakacin P | F: 5'-GAATRMMANCAATTAYMGGTGG-3' | 120 | 51 | [20] |
| Enterocin P | F: 5'-TGAAAGAAAAATTTAAGTTCAGCTTGAT-3' | 488 | 60 | [21] |

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 × 10⁷ CFU/mL of the indicator microorganisms and further incubated at 37 °C 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 (10⁵ 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. Aliquots 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 l’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
PinEF and PinK 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
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, 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 | Strain       | Medium-incubation temperature | 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                    | ++++        |
| 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. dysenteriae     | CECT 457     | BHI, 37 °C                    | ++++        |
| L. innocua         | CECT 910     | BHI, 37 °C                    | ++++        |
| E. coli            | ATCC 25122   | BHI, 37 °C                    | ++++        |
| E. coli            | DH108        | BHI, 37 °C                    | ++++        |
| M. luteus          | ATCC 10420   | BHI, 37 °C                    | ++++        |
| E. faecalis        | EM X2        | BHI, 37 °C                    | ++++        |
| B. cereus          | ATCC 9884    | BHI, 37 °C                    | ++++        |
| L. sake            | LTH 673      | MRS, 37 °C                    | ++++        |
| L. sake            | 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                    | -           |

*: ATCC: American Type Culture collection; CECT: Colección Española de Cultivos Tipos; DSM: Deutsche Sammlung von Mikroorganismen; **: 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                        |

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).

Table 6

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

| Indicator organism | Strain       | Medium-incubation temperature | Sensitivity |
|--------------------|--------------|-------------------------------|-------------|
| L. innocua         | BUG 499      | 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. dysenteriae     | CECT 457     | BHI, 37 °C                    | ++++        |
| L. innocua         | BUG 498      | BHI, 37 °C                    | ++++        |
| C. maltaromaticum   | DSM 20722    | BHI, 37 °C                    | ++++        |
| M. luteus          | ATCC 10420   | BHI, 37 °C                    | ++++        |
| C. maltaromaticum   | DSM 20730    | BHI, 37 °C                    | ++++        |
| C. maltaromaticum   | CECT 7508    | BHI, 37 °C                    | ++++        |
| B. cereus          | ATCC 9884    | BHI, 37 °C                    | ++++        |
| E. coli            | ATCC 25122   | BHI, 37 °C                    | ++++        |
| E. coli            | DH108        | BHI, 37 °C                    | ++++        |
| E. faecalis        | EM X2        | BHI, 37 °C                    | ++++        |
| L. sake            | LTH 673      | MRS, 37 °C                    | ++++        |
| L. sake            | 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                    | -           |

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

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.
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 \textit{L. plantarum}. \textit{Kılıç et al.} isolated twenty \textit{L. plantarum} strains from the fecal samples of humans\[26\]. These strains were investigated \textit{in vitro} for their characteristics as potential new probiotic strains.

A clear identification of species, especially within the genus \textit{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 \textit{L. plantarum} species. The 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 LbM2a 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 LAB but also against other Gram-positive and Gram-negative bacteria[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]. Bovicon 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 protease inhibitors on bacteriocin of L. plantarum bacST202Ch and bacST216Ch[37]. Ultrafiltration studies showed that the antagonistic activity was detected at molecular weight comprised between 10000 and 30000 kDa, which corresponds to the molecular weight range in which the plantaricins PInE, PInF, PinJ, and PinK 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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