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Partial characterization of bacteriocin-like substance produced by probiotic *Lactobacillus plantarum* F12 isolated from Algerian children faeces

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The strain, *Lactobacillus plantarum* F12 with probiotic traits was isolated from Algerian healthy children faeces and identified by 16S rDNA sequencing. In this study, the antimicrobial activity and physicochemical properties of bacteriocin-like substance (BLS) produced by this strain were determined. Also, the bacteriocinogenic genes of plantaricin A, plantaricin J and plantaricin K were screened in this strain. The BLS inhibited a range of pathogenic and spoilage bacteria including *Escherichia coli, Salmonella infantis, Salmonella typhimurium*, *Shigella sonnei, Staphylococcus aureus, Listeria monocytogenes, Listeria innocua, Bacillus subtilis* and some lactobacilli sp. The BLS was proteinaceous since it was inactivated by the proteolytic enzymes (trypsin, proteinase K and pronase) but not by α-amylase and lipase. It was heat stable at different temperatures (40 - 121°C) for 30 min and retained its activity at a wide range of pH values (2 to 10). Its activity was totally preserved at -80°C for 120 days and at -20°C for 60 days. The amplification of genetic determinants of plnA, plnJ and plnK has shown the presence of these genes in *L. plantarum* F12. The ability of the BLS from *L. plantarum* F12 to inhibit several pathogenic/spoilage bacteria and its characterization demonstrated its interest as a natural food preservative, in addition to its probiotic potential in prevention and treatment of infectious diseases.

Key words: *Lactobacillus plantarum*, plantaricin J/K, plantaricin A, bacteriocin-like substance, characterization.

INTRODUCTION

Lactic acid bacteria (LAB) have an important role in fermented food production because of their beneficial influence on nutritional, organic and shelf-life properties (Leroy and De Vuyst, 2004; Savadogo et al., 2006; Gillor et al., 2008). Many of LAB produce an array of antimicrobial substances such as organic acids, hydrogen peroxide, diacetyl, antifungal substances, reuterin and bacteriocins (De Vuyst and Leroy, 2007; Reis et al., 2012).

Lactobacilli belong to LAB group, are ubiquitous and found in gastrointestinal tract in healthy human beings (Holzapfel et al., 1998; Hsieh et al., 2008). Many species of *Lactobacillus* are known to produce bacteriocins which can inhibit the growth of other bacteria including spoilage and pathogen organisms (De Vuyst and Leroy, 2007; Todorov, 2008; Martinez et al., 2013). Lactobacilli have received a considerable attention for their great potential as natural preservatives in food materials (Savadogo et al., 2006; Diep et al., 2009; Lavilla et al., 2013) and they have also been investigated with regard to their probiotic traits (Kiliç et al., 2013; Bergillos-Meca et al., 2015). The

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probiotics are defined as “live microorganisms which when administered in adequate amounts confer health benefits for the host” (FAO/WHO, 2002). Bacteriocins produced by probiotic strains might play a role during *in vivo* interactions occurring in the human gastrointestinal tract, hence contributing to gut health (De Vuyst and Leroy, 2007; Corr et al., 2009). Actually, there is intriguing potential on the use of bacteriocigenic probiotics as pharmabiotics and/or novel alternatives to existing antibiotics (Gillor et al., 2008; Dobson et al., 2012; Arthur et al., 2014).

Bacteriocins are ribosomally-synthesized peptides or proteins with antimicrobial activity, produced by different groups of bacteria (Klaenhammer, 1988; Gàlvez et al., 2007). Bacteriocins from LAB are of low molecular weight antimicrobial peptides**.** They constitute a large and heterogeneous group of bacteriocins and varied in their peptide size, post-translation modifications, chemical stability and mechanism of action (Klaenhammer 1993; Drider et al., 2006). The LAB bacteriocins have been classified into three classes according to their biochemical and genetic properties: lanthionine (class 1), small, heat-stable, non lanthionine peptides (class II) and large heat-labile proteins (class III). The class II is divided into four subclasses. The subclass IIb called two peptide complexes represents the bacteriocins in which antimicrobial activity depends on the complementary action of the two different peptides (Heng et al., 2007)**.** The genes encoding the two different peptides are also genetically closely associated, being encoded in the same operon such as plantaricin J and K (Diep et al., 1996, Diep et al., 2009; Nissen-Meyer et al., 2010).

Several *Lactobacillus plantarum* strains have been isolated from fermented foods and numerous bacteriocins have been characterized for their use as natural food preservatives (Daeschel et al., 1990; Todorov, 2009; Gong et al., 2010; Lavilla et al., 2013). However, to the best of the authors’ knowledge, there are few reports on bacteriocins produced by *L. plantarum* from human origin. Recently, the bacteriocins of human lactobacilli strains isolated as probiotics have gained a great momentum, due to their both potential use as biological preservatives and therapeutic antibiotics (Gillor and Ghazaryan 2007; Cotter et al., 2013; Das and Goyal, 2014).

The term “bacteriocin-like substance” is applied to antagonistic substances which are not completely defined or do not fit the typical criteria of bacteriocins. The BLS have been reported to inhibit a wide range of both Gram-positive and negative bacteria as well as fungi (McGroarty, 1993). In the present study, the characterization of physicochemical properties of BLS from *L. plantarum* F12 and the screening of the plantaricin A, J and K genes in this strain were reported. *L. plantarum* F12 was previously, isolated from faeces of Algerian healthy children and screened for its probiotic traits (Bahri et al., 2014).

MATERIALS AND METHODS

Bacterial strains and growth conditions

L. plantarum F12 strain was isolated from faeces of Algerian children for its probiotic traits and identified by phenotypic and genotypic methods (Bahri et al., 2014). The strain was cultured in Man Rogosa Sharp (MRS) medium at 37°C and stored at -80ºC and left in MRS broth, supplemented with 40% (v/v) glycerol.

The following bacteria were used to detect the BLS inhibitory activity: *Staphylococcus aureus* ATCC 29222, *Salmonella typhimurium*, *Salmonella infantis*, *Escherichia coli* ATCC 25122*, Shigella dysenteriae, Shigella sonnei, Escherichia coli*, *Enterococcus faecalis, Pseudomonas aeruginosa, Listeria monocytogenes, Listeria innocua, Bacillus cereus, Staphylococcus aureus, Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus curvatus, Lactobacillus acidophilus, Lactobacillus sake* and *Streptococcus thermophilus*. The strains *L. plantarum* LMG6804 and *Streptococcus thermophilus* were studied to detect the plantaricins genes. All strains were obtained from the culture collection CWBI of the Bio-Industries Unit (Gembloux AgroBiotech, University of Liege, Belgium). Bacterial strains were stored at -80°C in culture broth with glycerol (40%). Before experimental use, *L. plantarum* F12 strain and the indictor bacteria were subcultured twice and incubation was carried out at 37°C for 48 h.

Bacteriocin-like substance assay

Preparation of culture supernatant

To prepare *L. plantarum* F12 supernatant, the strain was grown overnight in the MRS broth at 37°C. After that, 10% of the subculture obtained was seeded in 1000 ml MRS broth and incubated for 72 h at 37°C. Then, cells were separated by centrifugation (10.000 rpm for 20 min, at 4°C). The cell free supernatant was adjusted to pH 6.5 with 5 N NaOH to exclude the acidity effect due to the formation of organic acids. The neutralized supernatant was treated with catalase from Sigma (5 mg/ml) to eliminate the effect of hydrogen peroxide (H_2O_2) and then sterilized by filtrating through cellulose acetate filter (0.2 µm pore size) (Daba et al., 1991).

Determination of the bacteriocin-like substance inhibitory spectrum

Bacteriocin-like substance activity of neutralized cell free supernatant, treated with catalase (NFSC) was tested by agar well diffusion assay (WDA) as described by Barefoot and Klaenhammer, (1983). Aliquots of 60 µl from NFSC were placed in wells (6 mm) cut in molten agar previously seeded with indicator strains. After 4 h at 4°C, the plates were incubated without aeration, at 30°C for LAB indicator and aerobically, at 37°C, for the other indictor strains for 18 to 24 h. Inhibition of growth was determined by the presence of an inhibition zone surrounding each agar well.

Determination of the bacteriocin-like substance titre

E. faecalis was used to determine the BLS titre. Two fold serial dilutions of NFSC were prepared. The titres of the BLS were quantified by the method described above. The antimicrobial activity of bacteriocin-like substance, expressed in activity units (AU) per milliliter, was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition of the indicator strain (Todorov and Dicks, 2005).

Table 1. PCR primers and conditions used for detection of bacteriocin gene fragments.

Targeted genes	Primers*	Sense	Expected size (bp)	Annealing temperature (°C)	Reference
Plantaricin (A)	5'GTACAGTACTAATGGGAG 3' 5'CTTACGCCAATCTATTATACG 3'	Forward Reverse	450	53	Remiger et al. (1996)
Plantaricin (J)	5'TAACGACGGATTGCTCTG 3' 5'AATCAAGGAATTATCACATTAGTC 3'	Forward Reverse	475	51	Rojo-Bezares et al. (2007)
Plantaricin (K)	5'AATCGCAGTGACTTCCAGAAC 3' 5'AGAGCAATCCGTCGTTAATAAATG 3'	Forward Reverse	469	53,7	Rojo-Bezares et al. (2007)

*Synthetic primers sequences were collected from Europeen Molecular Biology Laboratory (EMBL) and have been supplied by SIGMA-PROLIGO.

Characterization of the bacteriocin-like substance

Sensitivity to heat

To determine the effect of temperature on the BLS, the method of Barefoot and Klaenhammer, (1983) was performed with slight modifications. 1 ml of the NFSC 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. The residual activity of the BLS against *E. faecalis* was assayed by the WDA method.

pH sensitivity

The sensitivity of the BLS to pH variations was estimated by adjusting the pH of NFSC samples (5 ml) to pH 2, 4, 5, 6, 7, 8, 9, 10, 11 and 12 with 1 M HCl and 1 M NaOH. The samples were incubated for 4 h at room temperature. Then, they were neutralized to pH 6.5 to remove the effect of pH. The remaining bacteriocin-like substance activity was assayed against *E. faecalis* by the method described above.

Storage stability

The NFSC was stored at -80, -20, 4 and 37°C at different time intervals for four months. Samples were taken from the stored material to determine the BLS activity (Ten Brink et al., 1994) using *E. faecalis* by the WDA method.

Enzyme treatments

The sensitivity of the BLS to various enzymes was tested with the following enzymes: trypsin (Fluka), α-amylase (Fluka), lipase (Sigma), proteinase K (Sigma), pronase (Merck). The enzymes were added to the NFSC at a final concentration of 1.0 mg/ml. Samples were incubated for 1 h, 30 min at 37°C (Jack et al., 1996). The residual activity of BLS was performed as previously mentioned.

Detecting genes of plantaricin A and plantaricin J/K by polymerase chain reaction

The presence of plantaricin gene (A) and plantaricin cluster genes J/K were screened in the bacteriocin producer *L. plantarum* F12. The Wizard® genomic DNA purification kit (Promega, Madison, USA) was used to isolate the total DNA from the liquid culture of bacteria. To amplify and sequence plantaricin genes, the polymerase chain reaction (PCR) was carried out using the total bacterial DNA as a template. The PCR was prepared using specific primers and conditions (Table 1). After amplification, the PCR products were separated by electrophoresis in 1% (w/v) agarose gels, visualized by ethidium bromide staining then purified with the Microcon YM-100 kit (Bedford, MA, USA) according to the manufacturer's instructions. Universal primers and BigDye Terminator v3.0 kit were used for the sequencing of amplicons and the nucleotide sequences analysis was performed using the Vector NTI (Version 8) software package (BD Biosciences, San Jose, USA). *S. thermophilus* DNA and *L. plantarum* LMG6804 DNA were used as a negative and a positive control, respectively**.**

Statistical analysis

The data were calculated with mean values, and standard deviations (mean±SD) were determined from triplicate trials. Statistical significance of the results was evaluated by analysis of variance (ANOVA). Statistical significance was attributed to $P<0.05$.

RESULTS

Determination of bacteriocin-like substance inhibitory spectrum

The BLS of *L. plantarum* F12 displayed a large spectrum of antimicrobial activity. It inhibited 16 of the 19 indicator strains. All pathogenic bacteria tested were inhibited by the BLS except *P. aeruginosa.* The spoilage and pathogenic bacteria *L. monocytogenes*, *L. innocua* and *B. cereus* were strongly inhibited by the BLS. However, there was no bacteriocigenic activity against the LAB *L. rhamnosus* and *L. sake* (Table 2).

Determination of the bacteriocin-like substance titre

The BLS titre from *L. plantarum* F12 was performed by WDA method against *E. faecalis.* It showed an antimicrobial activity of 51000 AU/ml.

Table 2. Inhibitory spectrum of neutralized cell free supernatant, treated with catalase of *L. plantarum* F12.

Indicator organisms	Sensitivity
Escherichia coli ATCC 25122	+++
Escherichia coli	++
Salmonella infantis	+++
Salmonella typhimurium	+++
Shigella dysenteriae	+
Shigella sonnei	++
Enterococcus faecalis	+++
Pseudomonas aeruginosa	-
Staphylococcus aureus ATCC 29222	+++
Staphylococcus aureus	++
Listeria monocytogenes	+++
Listeria innocua	+++
Bacillus cereus	+++
Lactobacillus rhamnosus	-
lactobacillus plantarum	+
Lactobacillus curvatus	++
Lactobacillus acidophilus	++
Lactobacillus sake	-
Streptococcus thermophilus	++

- No inhibition zone; +, 5 mm < zone < 10 mm; ++, 10 mm < zone < 15 mm; +++, zone >15 mm. Each data point is the average of repeated measurements from 03 independently replicated experiments, n = 3. P<0.05.

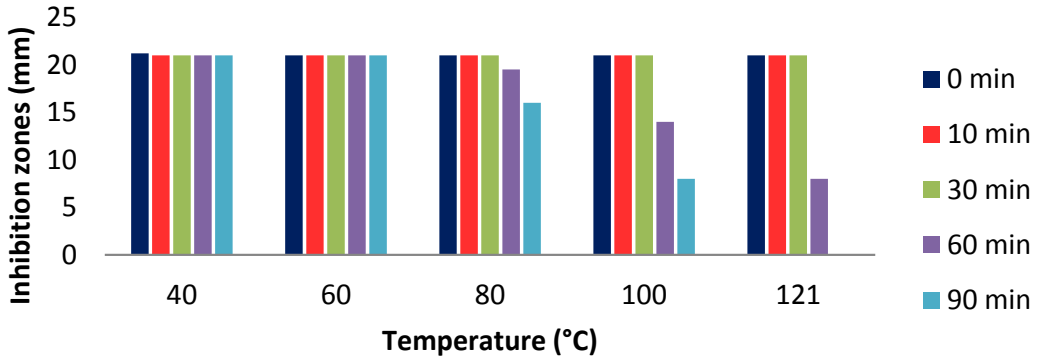

Figure 1. Effect of temperature variation on bacteriocin-like substance from L. plantarum F12. Each data point is the average of repeated measurements from 03 independently replicated experiments, n = 3. P<0.05.

Characterization of the bacteriocin-like substance

The BLS was heat stable at different temperatures (40 - 121°C) for 30 min. After this time, there was a decline in its activity as observed with prolonged treatment. However, there was no activity after 90 min at 121°C as shown in Figure 1.

The BLS activity was stable at pH values ranging from 2 to 8. Its activity started to decrease at pH 9 and it became completely inactive at Ph 12 (Figure 2). Regarding the effect of storage (Figure 3), the BLS maintained its total activity at -80°C for 120 days and at -20°C for 60 days. However, a decrease on its stability was observed at 4°C for 30 to 120 days and at 37°C for 30 days. After 60 days of storage, the BLS was completely inactivated at 37 °C.

The treatment with trypsin, proteinase k and pronase led to a total loss of activity of the BLS. However, α-amylase and lipase do not affect the BLS activity (Figure 4).

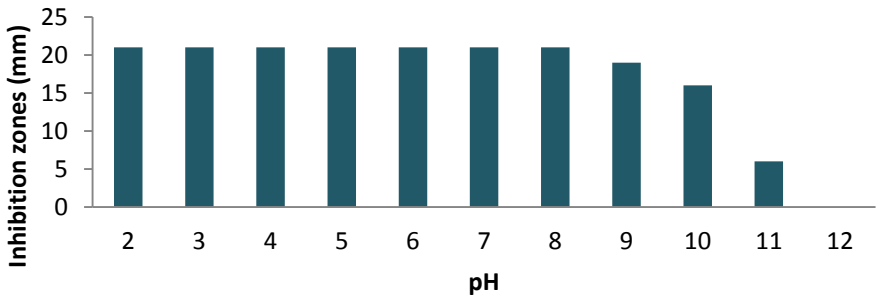

Figure 2. Effect of pH variation on the activity of bacteriocin-like substance from *L. plantarum* F12. Each data point is the average of repeated measurements from 3 independently replicated experiments, n = 3. P<0.05.

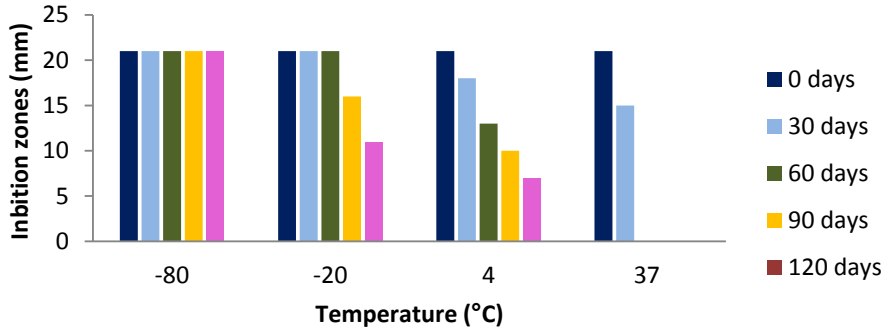

Figure 3. Effect of storage on the activity of bacteriocin-like substance of *L. plantarum* F12. Each data point is the average of repeated measurements from 3 independently replicated experiments, n = 3. P<0.05.

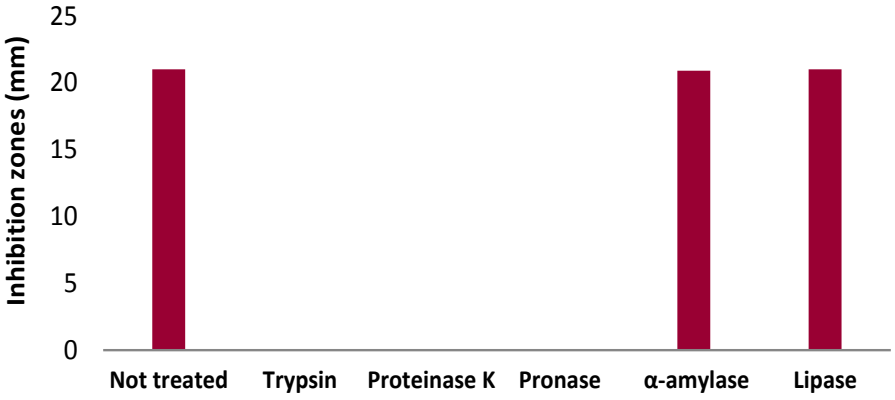

Figure 4. Effect of enzyme treatments on the activity of bacteriocin-like substance of *L. plantarum* F12. Each data point is the average of repeated measurements from 3 independently replicated experiments, n = 3. P<0.05.

Detecting genes of plantaricin A and plantaricin J/K by PCR

Results of PCR amplification of genomic DNA, performed in order to detect the genes of bacteriocins from *L. plantarum* F12, are shown in Figure 5. It was found that the genes plnA, plnJ and plnK, observed in the wells 1, 2 and 3 respectively, were presented in this strain.

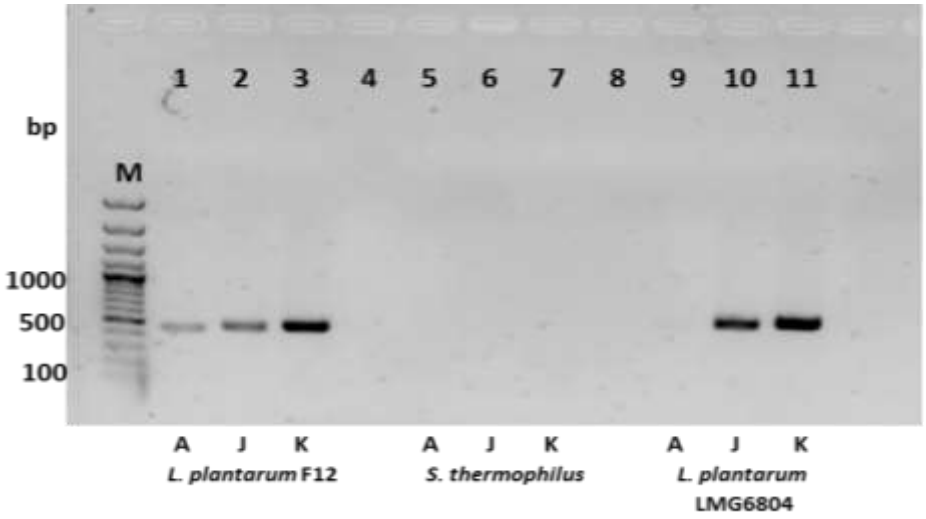

Figure 5. Agarose gel showing results from the genomic DNA PCR amplification from *L. plantarum* F12 using specific primer pairs for plnA; plnJ and plnK observed in the wells 1, 2 and 3 respectively. *S. thermophilus* DNA and *L. plantarum* LMG6804 DNA were used as a negative and a positive control, respectively.

DISCUSSION

In this study, preliminary characterizations were carried out by using NFSC prepared from probiotic *L. plantarum* F12. The NFSC was active against several indictor bacteria that will be discussed sooner. The antimicrobial activity of NFSC was not due to acidity or hydrogen peroxide. So, the activity was neither lost after neutralizing of pH value nor after treatment with catalase. In otherwise, the total loss of the antagonistic effect of NFSC with the all proteolytic enzymes (trypsin, proteinase K and pronase) indicates its proteinaceous nature. The other enzymes tested (α-amylase and lipase) did not cause any inactivation suggesting the lack of lipid or carbohydrate moiety in the NFSC. According to those results, the antimicrobial compound(s) contained in NFSC of *L. plantarum* F12 could be considered as bacteriocins or BLS. Since this (these) compound(s) have not yet been characterized on amino acids and nucleotides sequences, they will be referred to as BLS (McGroarty, 1993; Corsseti et al., 2004).

The BLS from *L. plantarum* F12 displayed a large spectrum of antimicrobial activity. It inhibited numerous indicator strains including pathogenic and spoilage bacteria such as *Escherichia coli, Salmonella infantis, Salmonella typhimurium*, *Shigella sonnei, Staphylococcus aureus, Listeria monocytogenes, Listeria innocua, Bacillus subtilis* and some lactobacilli sp. Depending on the strains, *Lactobacillus* is known to produce BLS which are active against different bacterial species (Abo-Omer, 2007; Ghanbari et al., 2013, Anacarso et al., 2014; Sabia et al., 2014). Recently, there was a greatest need of more natural and safe food products. For this purpose, the bacteriocinogenic compounds provide an alternative to reduce chemical preservatives and intensity of heat treatment in food preservation (Gàlvez et al., 2007). So, BLS of LAB may offer a wide applicability in food biocontrol because of their antimicrobial broad spectrum against both pathogens and spoilage bacteria.

The titration of the BLS of *L. plantarum* F12 showed a high antimicrobial activity (51000 AU/ml). Our result fall in the range of Diop et al. (2007), where titration value is between 10^4 and 10^5. However, this is higher than that of the study carried by Todorov in 2008 (30000 AU/ml) and Ogunbanwo et al. in 2003 (6400 AU/ml).

For its application as biopreservative, the bacteriocin must maintain its activity in thermally processed food. In general, plantaricins are high thermoresitant (Todorov and Dicks, 2005; Rojo-Bezares et al., 2007; Martinez et al., 2013). But, some of them were reported to be less heat stable such as plantaricin TF711 that retained only 70% of its activity after boiling and non activity upon autotclaving (Hernandez et al., 2005). The BLS of *L. plantarum* F12 was stable at high temperatures and autoclaving. According to Todorov (2009), bactriocins of class II are heat resistant up to temperatures 100°C,or autoclavable, through this, we can supposed our BLS belongs to class II.

About pH variations, several bacteriocins were found to be highly stable at acidic conditions and many of them are inactivated under alkaline pH values (Ten Brink et al., 1994; Hernandez et al., 2005; Todorov and Franco 2010). This is in line with our study; the BLS of *L. plantarum* F12 remained active in acidic pH range from 2 to 6, but its activity decreased at pH 9 until its full inactivation at pH 12. While, some bacteriocins remained

active in acidic, neutral and alkaline conditions such as bacteriocin ST71KS (Martinez et al., 2013).

Another criterion required for the use of bacteriocins in food industry is their stability during storage. The antimicrobial activity of the BLS from *L. plantarum* F12 was maintained in frozen state at -80°C for four months and at -20°C for two months. The bacteriocins stability at low temperature for a long duration allows their use in refrigerated and frozen foods in order to prevent the pathogenic and spoilage psycrotrophic micro-organisms growth (Hernandez et al., 2005).

According to Diep et al. (1996), plnJ and plnK genes coded respectively for plantaricine J and plantaricine K; two small cationic bacteriocin-like peptides belonging to the subclass IIb. The plnA induces transcription of genes organized in the operon of plnJKLR which encompass the gene pair of pln JK (Diep et al., 1996, 2009). *L. plantarum* F12 harbors the bacteriocin genes plnJ, plnK and plnA. The presence of these genes in *L. plantarum* F12 assumes that the BLS produced by this strain can be encoded by these genes and belongs to the subclass IIb.

Conclusion

L. plantarum F12, a probiotic strain isolated from human samples, produced a BLS which could interest the food industry as a biopreservative and medical sector as a pharmabiotic. It displays a strong antimicrobial activity against pathogens and spoilage bacteria, is stable to heat treatment and storage and active on a wide pH range. However, further studies were needed to get more characterizations of this BLS.

Conflict of interest

The authors have not declared any conflict of interest

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