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APPLICATION OF BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA ISOLATED FROM SALTED DRIED CAMEL MEAT (EL KADID) IN THE PRESERVATION OF GOAT CHEESE AGAINST *Staphylococcus aureus*

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ABSTRACT

El kadid is a traditional North African food obtained by natural dehydration of One-humped camels (*Camelus dromedarius*) meat still produced through traditional method in Algeria. This traditional food matrix constitutes a rich ecological niche for the screening of bacteriocin-producing strains and lactic acid bacteria which contributes to the safety and preservation of foods. Fifteen lactic acid bacterial isolates from *El kadid* were tested for antibacterial activity and biopreservative effectiveness against *Staphylococcus aureus.* Only four strains exhibited bacteriocin activity against the two indicator bacteria, *Listeria monocytogenes* ATCC 4379G and *Staphylococcus aureus* ATCC 25923, in the neutralized cell-free supematant treated with catalase and proteolytic enzymes. Strain LB6, identified as *Lactobacillus plantarum* and characterized by the highest antimicrobial activity, was selected for further trials. Bacteriocin rate peaked during the stationary phase. The ideal temperature and pH for its formation were 37°C and 6.5, respectively. The bacteriocin was stable at high temperatures (30 min at 100°C) and active following incubation at pH levels ranging from 2 to 12. This strain showed high activity in fresh goat cheese (11–15 mm inhibition zone) having the ability to prevent the development of *S. aureus*, also reducing the inoculum level of pathogen by about 1 log cycle. These results revealed that this strain could be a promising biopreservative starter for the dairy industry. To the author 's knowledge, this is the first report of L. plantarum being isolated in salted dried camel meat.

Keywords: *Bacteriocin*; *Lactobacillus plantarum*; *El Kadid*; cheese; *Staphylococcus aureus*

INTRODUCTION

The search for new technological approaches to maintain the nutritional values and organoleptic characteristics that can ensure food safety is a key issue for food industrie. Food preservation methods, both traditional and modern, are usually focused on drastic treatment modalities (pasteurization/sterilization). which can occasionally have a negative impact on the flavour of the food. In addition, the employment of chemical preservatives or environmental conditions is not acceptable to a certain proportion of consumers looking for foods labelled as natural (without chemicals or foreign molecules) or for products with health benefits **(Bali Panesar & Bera, 2011; Dal Bello et *al*., 2012)**. Within this context, controlled bioconservation is one of the most promising strategies, with an increase in reported industrial utilizations.

A bioprotective culture is a microbial population added to food with the goal of limiting or inhibiting the growth of food - borne pathogens. **(Beshkova & Frengova, 2012; Tabanelli et *al*., 2014)**, with a minimal texture effect and organoleptic properties of the food. The antimicrobial effect of bioprotective cultures can be obtained through their ability to colonize food environments, thus competing with undesirable microorganisms due to faster metabolism, or by producing antimicrobial compounds including bacteriocins, which confer an ecological advantage to the bioprotective culture. Lactic acid bacteria (LAB) strains are usually the greatest appropriate candidates to be used as bioprotective cultures. In fact, LABs are commonly considered as "food grade" organisms and in some cases, they have interesting characteristics for this purpose, such as low organoleptic impact, ability to lower pH as well as the secretion of antimicrobial compounds like organic acids, H_2O_2, aromatic substances, fatty acids and a wide range of bacteriocins **(Beshkova & Frengova, 2012)**.

Bacteriocins are an important group of peptides and proteins differentiated according to their dimensions, microbial focus, Tolerance and mechanism of action **(O'Connor et *al*., 2015)**. They can always be classified based on structural, physicochemical, and genomic characteristics **(Heng et *al*., 2007)**. Bacteriocins vary considerably from the most common therapeutic antibiotics because proteases in the human gastrointestinal tract digest it easily. Gram-positive and Gram-negative bacteria both release peptides produced by their ribosomes into the extracellular medium. This fact provides the opportunity to improve their antibacterial activity and their spectrum of action **(Sabo et *al*., 2004)**.

At lower concentrations, LAB's bacteriocins kill bacteria compared to antimicrobial peptide from eukaryotes, presumably due to their interference on target cells that have a specific binding site **(Cotter et al., 2005; Drider et *al*., 2006)** Numerous bacteriocins, like the Nisin produced by *Lactococcus lactis*, characterized by a spectrum of action. On the other hand, most bacteriocin are associated with a limited spectrum of action directed against a very few bacterial species. The food industry faces a major challenge in developing LAB strains that produce bacteriocins that are effective against the most common foodborne pathogens. **(Beshkova & Frengova, 2012)**. Strains of *Lactobacillus plantarum* species may be of special importance as this bacterium is one of the widely studied LABs and, due to its versatility, can be isolated from various foods of plant and animal origin **(Ricciardi et *al*., 2012**). Members of this bacterium are known to produce a wide variety of bacteriocins, mainly plantaricins, but also pediocins. Pathogen detection in fermented and non-fermented foods, as well as in medicine, has been studied using *L. plantarum* strains **(Sabo et *al*., 2014).**

The most common bacterial pathogen implicated in a wide range of human and animal diseases, such as foodborne toxi-mediated diseases, is *Staphylococcus aureus. S. aureus* can be a major cause of the intramammary infection in cows that produce milk; consequently, if animals' milk with *Staphylococcal mastitis* is used for consumption or make cheese, the risk of food contamination is high, particularly if unpasteurized milk is used **(Muñoza et *a*l., 2007)**.

The goal of our research was to find and isolate bacteriocin-producing LAB from *El kadid. El kadid* is a traditional North African food obtained by natural dehydration of the camel meat *(Camelus dromedarius*) produced under artisanal conditions in Algeria **(Benkerroum, 2013)**. This traditional fermented food is potential source of LAB that could be used in many other types of foods as biopreservative remains largely unknown. Regrettably, there is no information on the LAB isolated from dried salted camel meat produced in Algeria, known as El kadid. The present research was carried out to study the antimicrobial activities of LAB strains by their antagonistic activities against spoilage bacteria. *L. plantarum* have been used to test their activity against *S. aureus* which is a dangerous bacterium in cheese.The production of bacteriocins has been studied in the same

species. The competent strains were used in a biopreservation test of fresh cheese against the proliferation of *S. aureus*.

MATERIALS AND METHODS

Microorganism and growth conditions

Fifteen cultures of LAB isolated from dried and salted camel meat (El Kadid) on MRS agar (Fluka, Almaty, Kazakhstan). The preparation of El Kadid is done following the method described by **Bouchefra et al. (2019)**. In brief, samples of camel meat (thigh portions) were gathered from various animals in the region of Oued Souf in Algeria. After slaughtering, the muscles of thigh were rinsed under running water. Then, they were cut into strips of thickness and size (about 1 cm wide and 10-20 cm long). After that, other ingredients such as salt (6.0g salt/100g of meat), pepper and olive oil were added to the meat strips in a bowl and left overnight to macerate 4 °C. Afterwards, we proceed with the drying phase. For a period of about 10 days and between 9 am and 15 pm, the meat strips were left to dry on ropes under the sun, under a temperature of 30 °C and humidity percentage of 67%. In order to obtain a uniform drying product, the stripes were regularly turned on all sides. The drying phase ends when the meat turned brown and reaches the right fibrous appearance. Lastly, the meat strips were cut into pieces of 2-3 cm long and placed inside a sealed jar. After that, the jar was stored at room temperature. The LAB was undergoing preliminary identification by microscopic examination, Gram stain, catalase and acid production. The cultures have been kept at -80°C in MRS broth (Fluka) with 50% (v/v) added glycerol. The antimicrobial activity was evaluated using indicator strains: *Escherichia coli* ATCC 27853, *Listeria monocytogenes* ATCC 4379G, *Pseudomonas aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *Bacillus cereus* ATCC 10876 and were grown in nutrient broth (Fluka).

Screening and partial characterisation of bacteriocin activity

The LAB isolates were cultured in MRS broth (pH 6.5) at 30°C for 24h and centrifuged at 4500g for 22min at 4°C. Fractions of free supernatant from cells (CFS) were collected and filtred via a 0.22 µm Millipore filter (Millipore, Bellarica, MA, USA) and subjected to further characterization.
First, the pH was corrected to 6.0-6.5 to remove any organic acids' antimicrobial reaction with NaOH (1 mol/l). To exclude the inhibitory activity of H_2O_2, the supernatants were additionally incubated with catalase (1mg/ml) (Sigma-Aldrich, Taufkirchen, Germany). The antimicrobial spectrum of the CFS fractions was estimated using the agar-well diffusion test developed by Tagg and McGiven (1971). Muller Hinton agar plates (Sigma-Aldrich, Taufkirchen, Germany) were inoculated with 100 µl of each indicator strain: *Escerichia coli* ATCC 27853, *Listeria monocytogenes* ATCC 4379G, *Pseudomonas aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *Bacillus cereus* ATCC 10876 A total of 100 µl of potential CFS containing bacteriocin was inoculated into five mm wells. The plates were first incubated for 24 h at 37°C after a 4h diffusion time at 4°C, and then the inhibition zones were examined. MRS broth without microorganisms was employed as a negative control. All of the tests were carried out twice. The protein origin of the generated antimicrobial substances was tested using LAB cultures that showed positive results for antimicrobial activity. Proteolytic enzyme sensitivity was evaluated by incubation of CFS with trypsin (Sigma-Aldrich from bovine pancreas 1,000-2,000 UI/mg, Taufkirchen, Germany), α-chymotrypsin (Sigma-Aldrich ≥40UI/mg, Taufkirchen, Germany) and α-amylase (Sigma-Aldrich from porcine pancreas ≥1000UI/mg, Taufkirchen, Germany), with a final concentration of 1 mol/litre for each enzyme. No inhibition halo following proteinase treatment of CFS reflects the proteinaceous nature of the antimicrobial agents **(Ribeiro et *al*., 2014)**.

LAB identification by 16S Rrna gene sequencing

The genomic DNA has been obtained from the pure culture adopting the Instagene matrix (Bio-Rad Laboratories,Italy) as instructed by the producer. The 16S rRNA gene has a 600-bp fragment. was amplified with LpigF / LpigR primers (5'-TACGGGGGAGGCAGCAGTAG-3 'and 5'-CATGGTGTGTGTGTGTGTGACGGGGCGGGGGT-3'; Eurofins MWG Operon, Germany). The PCR amplification has just been carried out using the Fisher Scientific Taq DNA polymerase kit (Italy). The respective chemical reagent mixes consist of 5x buffer, 2mM $MgCl_2$, 1.25 U Taq polymerase ,50 mM of dNTPs, each primer is 0.5 mM and the model DNA is 0.5µl, for a total volume of 50µl. On a T3000 thermal cycler (Biometra, Germany), replication was performed with an initial denaturation at 94°C for 5 min, followed by 35 cycles (94°C for 2 min, 56°C for 1 min, and 72°C for 2 min), and a final extension at 72°C for 5 min. Electrophoresis was used to isolate the PCR compounds from a 1.5% (w/v) agarose gel (Lonza, Italy) colored with ethidium bromide (0.5 mg/ml).The amplicon was eluted from its agarose gel and then further purified using the QIAquick PCR purification kit (Quiagen, USA). The same primers were used for the amplification and sequencing at BMR (Padova, Italy). Identity of the sequence with the known LAB was assessed using BLASTn (http://blast.ncbi.nlm.nih.gov/).

Quantitative monitoring of bacteriocin production

A volume of 1 ml of an 18-h culture was drawn and inoculated with 100 ml of MRS broth, then incubated for 48 h at 37°C.Periodically, the samples were monitored for bacterial growth at 660 nm optical density (OD660), bacteriocin activity against *S. aureus* and pH changes in the culture (**Sifour *et al.*, 2012**). Bacteriocin activity levels were calculated in arbitrary units (AU/ml) **(Rajaram et *al*., 2010)**.

Optimisation of bacteriocin production

To find out the temperature is best for producing bacteriocin., a 0.5 ml overnight culture has been inoculated into 50 ml of MRS broth. Incubation temperatures were 30, 37, and 40°C, respectively. Samples were taken after 24 h. To be able to identify the impact of initial pH on bacteriocin production, the MRS broth's pH was controlled at 3.5, 6.5, 7.5, and 9.5 before inoculation, and supernatant samples were collected after 24 h.

Effect of pH and temperature on the stability of bacteriocins

By varying the pH of CFS, the effect of pH on bacteriocin stability was investigated. (Obtained as reported in screening and partial characteriation of bacteriocin activity section) to 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 with NaOH or HCl (1mol/ml). After leaving the CFS to stand at 30 °C for 1 h, the bacteriocin activity of the remaining bacteriocin was tested against *S. aureus* 25923. In addition, the thermal tolerance of bacteriocin was further characterized by heating the CFS at 40, 60, 80 and 100°C for 10, 20, 30 and 60 min. Heat-treated CFS was then investigated for residual bacteriocin activity **(Sifour et *al*., 2012).**

Biopreservative efficiency of bacteriocin in fresh goat cheese

Preparation of fresh goat cheese

The cheese was prepared in accordance with the manufacturer's instructions of **Coelho et *al* (2014)**, with a few changes. Raw goat's milk was processed at 73°C for 20 seconds and then refrigerated. $CaCl_2$ (0.25g/l, Merck) and NaCl (11 g/l) were added to the milk. For any case, milk heated to 32°C was placed in four 0.6 l containers and separately injected with a 1% LAB culture in UHT skim milk and incubated for 48h at 30°C, yielding a cell density of approximately 10^7 CFU/ml. The control cheese was processed without LAB inoculum. *S. aureus* (1% v/v) was inoculated into goat's milk (immediately after the LAB inoculum) to achieve a cell load of approximately 10^5 CFU/ml. A control cheese with only *S. aureus* was produced. Rennet (0.25 g/l) has been applied in the milk and incubated at 30°C for 40 min. As soon as the coagulum was fairly rigid, it was partitioned into pieces and cooked at 37°C for 30 minutes. The whey has been drained and the curd was placed in sterile circular perforated containers (8 cm in diameter). The cheeses were conserved in the refrigerator (4°C).

Analysis of fresh goat cheese

LAB counts were performed at (time=0) 6, 24, 48, and 72 h of storage at 4°C. The pH of the cheese was assessed by a pH meter (HANNA HI 2210, Bucharest, Romania) according to **AOAC (1995)**.

Antimicrobial activity of cheese and evaluation of S. aureus in fresh goat product

The agar disc spread assessment was used to test cheese samples for antibacterial activity **(Ribeiro et *al*., 2014)**. Samples of cheese (5 g) were processed by centrifugation at 4800 g during 8 min. Phosphate buffer (0.5 M, pH 7.0) was used to neutralize the obtained supernatants. The filtrate was then passed through a 0.22 m membrane filter and double-placed in wells (5 mm in diameter) formed in Muller Hinton agar plates with cultures of *S. aureus* as indicator microorganism. The inhibition zone following anaerobic storage at 37°C for 24 h was calculated in mm. To count *S. aureus* in cheeses, 25 g samples were taken at various times (0, 6, and 24 h, as well as 2, 3, and 7 days) and reduced in 225 ml phosphate - buffered saline. After wards shaking, the mixture was diluted and spread on Baird Parker (Sigma-Aldrich) medium, and the plates were then incubated for 48 h at 37°C.The samples were then diluted and spread on the Baird Parker (Sigma-Aldrich) medium.

Data analysis

The data were reported as the average of three studies with standard deviation (SD). Microsoft Excel 2016 was used for data analysis. For antimicrobial activity of cheese statistical analysis was performed with GraphPad prism software (version7.0). Analysis of variance was used to compare the findings of three replications (ANOVA: Analysis of Variance). The difference between the samples was judged significant when the $P < 0.05$.

RESULTS AND DISCUSSION

Screening and partial characterisation of antimicrobial activity

Fifteen cultures were isolated from the traditional salted and dried camel meat using MRS agar; they were preliminarily classified as LAB: not spore forming, Gram-positive and catalase negative. These isolates have been evaluated for their potential to inhibit pathogens and production of bacteriocins. In the current study, only four isolates showed inhibition against the two test microorganisms, *L. monocytogenes* ATCC 4379G and *S. aureus* ATCC 25923 **(Table 1)**.

Table 1 Antimicrobial activity of the neutralized CFS of LAB isolates against Listeria monocytogenes ATCC 4379G and Staphylococcus aureus ATCC 25923.Results are the mean of three samples. The standard deviation is provided

Isolate	Target strain Inhibition diameter (mm)	
	Listeria monocytogenes ATCC4379G	*Staphylococcus aureus* ATCC 25923
LB2	16±0.10	12±00
LB6	17±0.30	18±0.10
LB8	15±0.40	16±0.20
LB5	10±0.10	14±0.20

The diameters of the inhibition zones were ranging from 10 to 18 mm**.** No activity was observed against *P. aeruginosa* ATCC 27853, *E. coli* ATCC 27853 and *B. cereus* ATCC 10876 (data not shown). The highest diameter (18 mm) was obtained with the CFS of the strain LB6 against *S. aureus* ATCC 25923, whereas the lowest diameter (10 mm) was obtained with the CFS of the strain LB5 against *L. monocytogenes* ATCC 4379G. These CFSs were ineffective to inhibit the growth of test strains when trypsin, α-chymotrypsin, and amylase were added, but inhibition was observed when they were neutralized and treated by catalase. This indicated that the inhibitory substance was probably a bacteriocin. These results were consistent with those of **Nieto-Lozano et *al.* (2002)**, who reported that *L. plantarum* had an inhibitory activity towards *S. aureus* and not towards *P. aeruginosa*, *E. coli* and *Salmonella* spp. In addition, several studies have confirmed that strains of *L. plantarum* may have antibacterial activity against some *Listeria* isolates **(Nieto-Lozano et *al*., 2002)**. In addition, **Ammor et *al.* (2005)** and **Albano et *al*. (2007)** reported that *L. plantarum* may inhibit the growth of S. *aureus*, *L. monocytogenes* (Gram-positive bacteria) more than *Salmonella arizonae*, *E. coli* and *P. aeruginosa* (Gram-negative bacteria). Subclass IIa has been assigned to a number of bacteriocins produced by *L. plantarum* **(Drider et *al*., 2006)**, in particular plantaricin 423, which was discovered to stop *L. monocytogenes* from multiplying in salami **(Dicks et al., 2004).** Bacteriocins can be expressed by a gene situated in a plasmid (plantaricin 423) or in the chromosome (plantaricin ST31**) (Todorov et *al*., 1999; Powell et al.,2007; Todorov, 2009).** Various bacteriocins from *L. plantarum* have potent antimicrobial action against a variety of pathogens**.** Indeed, the plantaricin produced by *L. plantarum* ZJ008 demonstrated broad-spectrum inhibitory activity and was particularly active against *Staphylococcus* species **(Zhu et al., 2014). Lim and Dong-Soon (2009)** noted that *S. aureus* was most sensitive to the occurrence of *L. plantarum* KC 21, showing approximately 70% inhibition. Because of its antimicrobial capabilities, strain LB6 was selected for additional studies, while *S. aureus*, which was the most sensitive to the examined bacteria, was used below as an indicator strain.

Monitoring of bacteriocin production during *Lactobacillus plantarum* LB6 growth

The strain LB6 was recognised by partial 16S rRNA sequencing, and it was assigned as *L. plantarum* (99–100% similarity values). GenBank accession number MT891322. *L. plantarum* LB6 ability to produce bacteriocin in a liquid medium was investigated; results are shown in **Figure 1**, which shows cell growth monitoring, pH decreases of the culture and production of bacteriocin in the supernatant during 48 h incubation in MRS broth. Bacteriocin activity towards *S. aureus* ATCC 25923 increased progressively with bacterial proliferation, reaching its maximum production level (2500 AU/ml) after 24 h of incubation (when the cells have already attained the stationary phase). Following this peak, bacteriocin activity decreased in time. The pH level of the culture medium increased from 6.73 to 4.27 during the same period. Maximum activity of a bacteriocin of *L. plantarum* ST8KF **(Powell et *al*., 2007)** was also reported at the beginning of the stationary growth phase. The decline in the activity of the bacteriocin during the successive stages may be related to the bacteriocin degradation through extracellular proteolytic enzymes. Comparable declines were also reported for bacteriocins from *Enterococcus faecium* ST311LD **(Todorov & Dicks, 2005a)**, *Pediococcus acidilactici* NRRL B5627 **(Anastasiadou et *al*., 2008)** and *E. mundtii* ST4SA **(Todorov & Dicks, 2009).**

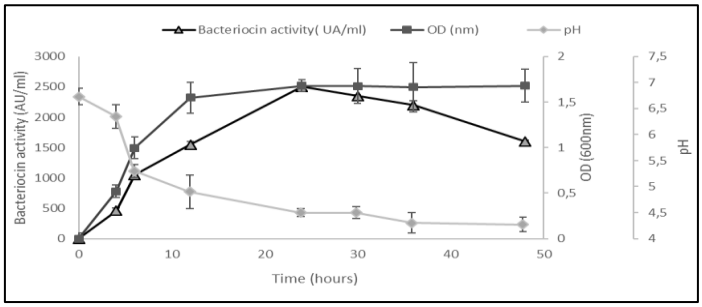

Figure 1 Monitoring of bacteriocin production in arbitrary unity per ml (AU/ml), pH and growth of *Lactobacillus plantarum*
LB6 in MRS broth at 37°C during 48h of incubation. The results are the means ± standard deviation of three replicates

Optimization of bacteriocin production

To learn more about how growing conditions affect bacteriocin production, *L. plantarum* was grown in varying pH levels and temperatures, and the bacteriocin activity was tested against *S. aureus* ATCC 25923. Regarding the effect of the initial pH (**Figure 2a**), the maximum activity was observed at pH 6.5 (1650 AU/ml). A slightly lower value was noted at pH 7.5, while a marked reduction was evident at pH 9.5.

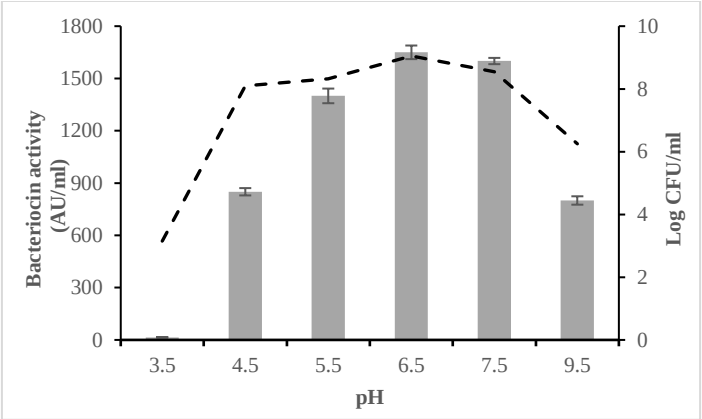

Figure 2a Effect of initial culture medium pH on bacteriocin production (AU/ml) and on viable cells (log CFU/ml) of *Lactobacillus plantarum* LB6
The bacteriocin activity decreased with pH decrease: at pH 4.5, it was halved with respect to the optimum, while at pH 3.5 it was not detectable. This behaviour was related to the extent of growth. In fact, the maximum growth level (9.05 log CFU/ml) was obtained at pH 6.5, while pH 9.5 and 3.5 were characterized by a low cell number (6.25 and 3.15 log CFU/ml). Regarding the effect of the LB6 growth temperature (**Figure 2b**), the higher bacteriocin activity was recorded in the CFS in which *L. plantarum* LB6 was grown at 37°C (1545 AU/ml), while this value decreased in the samples incubated either at 30 and 40°C (1018 AU/ml and 956 AU/ml, respectively), even if all of the samples reached the stationary phase.

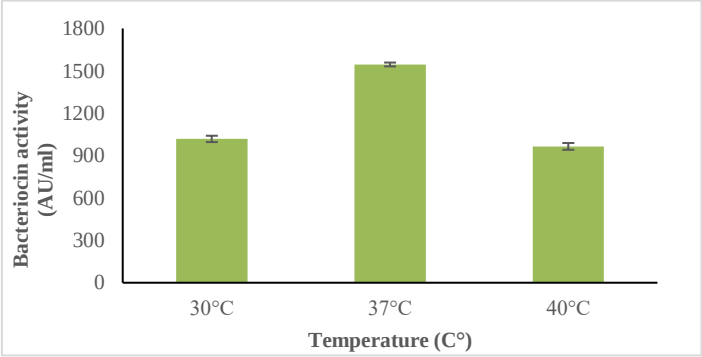

Figure 2b Effect of growth temperature (30, 37, 40°C) on bacteriocin production by *L. plantarum* LB6 in MRS medium

Concerning pH's influence on bacteriocin synthesis, identical results were shown for other LAB bacteriocins. For instance, bacteriocin ST16Pa was induced in MRS broth at pH levels ranging from 6.0 to 6.5. At pH 4.5, this strain grew much slower, which is consistent with lower bacteriocin production. This indicates the important role for the initial pH of the MRS broth for bacteriocin production **(Todorov et *al*., 2011)**. Comparable results were also found for other bacteriocins generated by *L. plantarum* strains **(Daeschel et al.,1990; Kelly et al.,1996; Todorov et *al*., 2000;**

Todorov & Dicks, 2005b; Todorov et *al*., 2010). The current work reveals that for optimum *L. plantarum in vitro* growth and bacteriocin synthesis, an initial pH of at least 6.0 is required. The optimal production and growth temperature of *L. plantarum* LB6 are associated with each other, as has been observed for other bacteriocins, like lactocin A, enterocin 1146, lactocin S and nisin Z **(Todorov & Dicks, 2004).** Low molecular weight bacteriocins are very thermostable, unlike those with higher molecular weight which are very sensitive to heat treatments **(Alves et *al*.,2006).** bacteriocins are generally stable in acidic and neutral media, on the other hand they lose their stability in basic media **(Kostinek et *al*.,2007).**

Effect of pH and temperature on the stability of LB6 bacteriocin

The stability of bacteriocin was investigated at various pH levels and temperatures. For this purpose, the CFS obtained after growth under optimal conditions (37°C and initial pH of 6.5) was adjusted to different pH values, the results of the residual activity are reported in **Figure 3a**. As it can be observed, pH has a substantial impact on the antibacterial activity of L. plantarum LB6 bacteriocin.; it was higher in the pH range of 6 to 8, extensive at pH 6.5, excluding the function of organic acids in the inhibition of *S. aureus* growth.

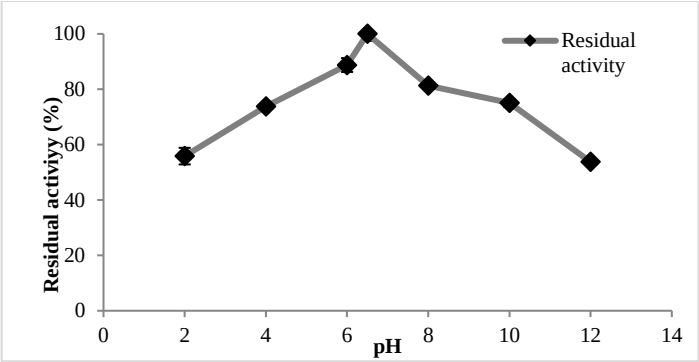

Figure 3a Residual activity of *L. plantarum* LB6 bacteriocin at different pH values On the other hand, the effect of temperature (40,60,80 and100°C) was also investigated, the results reported that bacteriocin produced by *L. plantarum* LB6 is heat stable (**Figure 3b**). Actually, residual activity of the produced bacteriocin stayed unchanged for 30 min after heating at 80 or 100°C, and then declined after 60 min of heating. At lower temperatures, no significant difference was observed.

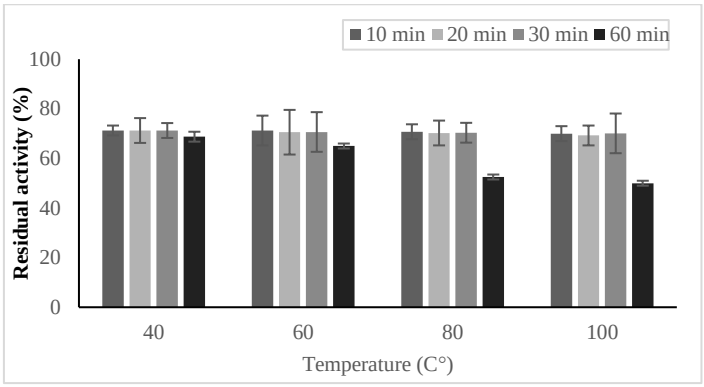

Figure 3b Residual activity of *L. plantarum* LB6 bacteriocin at different temperatures

The antimicrobial activity of *L. plantarum* was maintained in the pH range of 2.0 to 12.0 and at all thermal treatments applied, at least within the first 30 min. The bacteriocin of *L. plantarum* LB6 showed high thermal and pH stability, these features render it appropriate for application in heat-processed foods, similar to acidocin M46 **(Ten Brink et *al*.,1994).** These findings are in line with what has previously been published by **Enan et *al*. (1996**). Who showed that JLA-9 plantaricin remained stable after 3h of incubation at pH range between 2.0 and 7.0, although its activity was greatly decreased at pH 10. The activity of bacteriocin ST16Pa produced by *L. plantarum* was unaffected with a pH in the range of 2.0 to 12.0 and stayed unchanged at 100°C for 2 hours **(Todorov et *al*., 2011)**. Loss of antimicrobial activity at extreme pHs could be attributed to protein instability or protein aggregation **(De Vuyst et *al*., 1996**).

Biopreservative efficiency of bacteriocin in fresh goat cheese

Based on the obtained *in vitro* results, further experiments were performed to assess the biopreservative efficiency of *L. plantarum* LB6 in a real food. For this purpose, fresh goat cheese was prepared with addition of the bacteriocinogenic strain LB6 as described by **Coelho et *al.* (2014)**. Cheese samples were collected to measure pH, LAB growth counts, and bacteriocin activity against *S. aureus* and the result are reported in **Table 2.** The pH values were initially 6.5 in both samples and the pH decreased to 5.9 in cheese inoculated with *L. plantarum* LB6 after 72 h. This was probably due to the multiplication of *L. plantarum* LB6 that, starting from a level of 6.77 log CFU/g, attained a number of about 8 log UFC/g. Conversely, the pH of the control cheese remained stable during the storage time. The results regarding the bacteriocin activity in cheeses made with the *L. plantarum* LB6 culture, reported in the same table, showed clear inhibition zones, 12 mm (at time 0) and 11 mm after 72h of storage at 4°C.

Table 2 Values of pH, growth counts of LAB (log CFU/g) and bacteriocin activity against *Staphylococcus aureus* ATCC 25923 presented as diameter of inhibition zones (mm) of fresh goat cheese stored at 4°C. Results are the mean of two samples. The standard deviation is reported

		Storage time (hours)				
	Samples	0	6	24	48	72
pH	Control cheese	6.5±0.05	6.5±0.14	6.6±0.28	6.6±0.26	6.7± 0.23
	Cheese + LB6	6.4±0.08	6.3±0.19	6.1±0.06	6.0±0.12	5.9 ±0.06
LAB counts (Log CFU/g)	Control cheese	4.55±0.02	4.87±0.01	5.52±0.03	5.89±0.08	6.02±0.05
	Cheese + LB6	6.77±0.04	6.84±0.08	7.06±0.04	7.11±0.04	7.97±0.05
Bacteriocin activity (mm)	Control cheese	0	0	0	0	0
	Cheese + LB6	12±0.10	14.5±0.10	15±0.10	14.5±0.90	11 ±0.80

In the last experiment, the fresh goat cheese samples were prepared with the addition of *S. aureus* (at about 5 log CFU/g) to assess the *in situ* effect of the bacteriocinogenic strain *L. plantarum* LB6. Results regarding the cell counts of *S. aureus* in fresh cheese during storage at 4°C for 7 days are reported in **Figure 4**.

Without the protective culture of *L. plantarum*, *S. aureus* grew throughout a 7-day period, reaching high concentrations (7.36 log CFU/g). In contrast, *S. aureus* counts were reduced in the fresh cheese inoculated with *L. plantarum* of about 1 log unit (cell counts of 3.85 Log CFU/g when the storage term is up). The results obtained from *in situ* bacteriocin production clearly showed the presence of an antagonistic activity against *S. aureus* in fresh cheese made with *L. plantarum* LB6 ($P < 0.05$). In a similar study, staphylococcal inhibition was lower but remained significant in cheese, with approximately 1.5-log observed inhibition **(Trmcic et *al*., 2010).** The *in-situ* production of bacteriocin may depend on physical and chemical factors and diet-related factors. However, the production of bacteriocins can also rely on parameters related to microbes, like total microbial loading and biodiversity, bacterial activities in food during storage, nutritional competition or the generation of other antagonistic compounds, and bacteria's physiological condition are all factors to consider. **(Reis et *al.*, 2012)**.

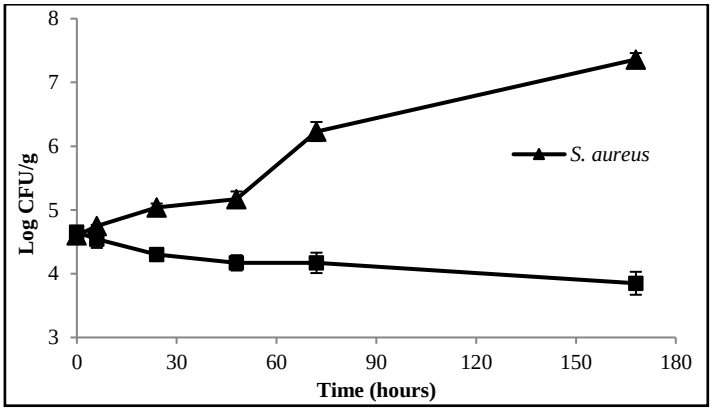

Figure 4 Biopreservation test in fresh goat cheese at 4°C by comparing the growth of *S. aureus* in cheese without the addition *of Lactobacillus plantarum* LB6 (Cheese 1) or in the presence of *Lactobacillus plantarum* LB6 (Cheese2). Results are expressed as log CFU/ml

At the beginning of storage period, no difference was made from the pH of the control cheese and the cheese inoculated with *L. plantarum* LB6, but the latter samples had already antimicrobial activity against *S. aureus*. This would suggest that the inhibitory activity implies a mechanism other than acid production. **Charlier et *al.* (2008)** reached similar conclusions, suggesting that inhibition of *S. aureus* in cheese might take place independently of acidification. **Trmcic et *al.* (2010)** have also suggested that nutrient-related phenomena are possible elements implicated in the inhibition of *S. aureus*.

CONCLUSION

During the manufacture of processed products, chemical preservatives are commonly used. However, the increasing consumer demand for natural products has made it more crucial to research alternatives strategies. Regarding this issue, the application of biopreservative cultures in food industry, aiming at inhibiting or reducing spoilage and/or pathogenic microorganisms, is increasing. In the current paper, *L. plantarum* LB6 isolated from *El Kadid* demonstrated an interesting inhibitory activity against *L. monocytogenes* and *S. aureus* and a high pH and thermal stability. Tthis activity is strongly linked to the production of a bacteriocin which will be characterized and identified in a future study. Challenge test showed that the bacteriocinogenic *L. plantarum* LB6 inhibited the growth of *S. aureus* in fresh goat cheese. These results revealed that this strain could be a promising biopreservative culture for the dairy industry.

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