PRODUCTION AND CARACTERIZATION OF BACTERIOCIN OF *Lactobacillus plantarum* F12 WITH INHIBITORY ACTIVITY AGAINST *Listeria monocytogenes*

Mohamed Sifour[1, 2]; Idoui Tayeb[1]; Houria Ouled Haddar[1,2]; Heba Namous[1]; Salima Aissaoui[1]

[1]Department of Cell and Molecular Biology, Faculty of Sciences, University of Jijel, Algeria
[2]Laboratory of Molecular Toxicology, Faculty of Sciences, University of Jijel, Algeria

e-mail: sifourm@yahoo.fr

Abstract: Thirty five lactic acid bacterial isolates from different origins (fermented Milk, chicken, olive oil, butter and newborn feces) were tested for their ability to produce bacteriocins against 12 indicator strains. These isolates presented a broad inhibitory spectrum against many indicator strains such as Methycilin resistant *Staphyococcus aureus*, *Escherichia coli*, *Salmonella* sp., *Listeria monocytogenes*… Only six isolates produced antimicrobial activity in the neutralized cell-free supernatant treated with catalase against indicator strains. The bacteriocin produced by *Lactobacillus plantarum* F12 was characterized and showed sensitivity to proteolytic hydrolysis (trypsine, chymotrypsine and pronase) and resistance to α-amylase and lipase. The activity of bacteriocin remained constant after heating at 100°C for 30 min and no change in activity was recorded after 4 h at pH 6.0. Bacteriocin production is dependent on biomass concentration; it's started at the beginning of the log phase of the bacterial growth till reached its maximum level at the stationary phase at 37°C as the optimum temperature of production. Due to the inhibitory effect of this bacteriocin on *L. monocytogenes*, it can be used to prevent food spoilage by this pathogen.

Keywords: antimicrobial activity, bacteriocin, Lactic acid bacteria, *Lactobacillus plantarum*, *Listeria monocytogenes*

INTRODUCTION

Lactic acid bacteria (LAB) are microorganisms widely used in food industry in a variety of fermentations such as the development of meat products, vegetables and many dairy products including fermented milk, cheese, yogurt and butter (Dortu & Thonart, 2009; Makhlouf, 2006). LAB produce organic acids such as lactic, acetic acid and hydrogen peroxide which possess antimicrobial activity against several pathogenic and spoilage microorganisms (Benabbou, 2009). LAB represent a major class which produces bacteriocins that become a current subject for several researches. These bacteriocins are now being explored for their potential utility in human and animal health applications, food biopreservation and agricultural uses (Parada *et al*., 2007; Todorov *et al*., 2011b).

Bacteriocins differ from most therapeutic antibiotics in being proteinaceous agents that are rapidly digested by proteases in the human digestive tract. They are ribosomally synthesized peptides, and this fact creates the possibility of improving their characteristics to enhance their activity and spectra of action (Parada *et al*., 2007). In additions, it has been shown that some strains of LAB possess interesting health-promoting properties; one of the characteristics of these properties is the potential to combat gastrointestinal pathogenic bacteria such as *Helicobacter pylori*, *Escherichia coli* and *Salmonella*. The antimicrobial spectrum frequently includes spoilage organisms and food-borne pathogens such as *Listeria monocytogenes* and *Staphylococcus.* The activity against Gram-negative bacteria such as *E. coli* and *Salmonella* has been shown, but usually only when the integrity of the outer membrane has been compromised, for example after osmotic shock or low pH treatment, in the presence of a detergent or a chelating agent, or after pulsed an electric field or high-pressure treatment. An experimental focus on bacteriocin production by probiotics LAB strains has indicated that this potential might play a considerable role during in vivo interactions occurring in the human gastrointestinal tract, for instance towards *H. pylori* (De Vuyst & Leroy, 2007; Osmanagaoclu & Beyatli, 2002;).

Several authors have recommended the use of bacteriocins combined with other preservation methods to create a series of hurdles during the manufacturing process to reduce food spoilage by microorganisms. In fact, it has been proven that application of chemical preservatives, physical treatments (heat), or new mild non-thermal physical methods (pulsed electric field, HHP, vacuum, or modified atmosphere packaging), which increase the permeability of cell membranes, positively affects the activity of many bacteriocins. Notably, combined treatments of bacteriocins with selected hurdles affecting outer-membrane permeability increase the effectiveness of some LAB bacteriocins against Gram-negative cells, which are generally resistant (Ananou *et al.*, 2007; Galvez *et al.*, 2007; Dortu & Thonart, 2009).

The main purpose of our work was to select bacteriocinogenic strains from a group of LAB with antimicrobial activity isolated from different origins, to characterize the produced bacteriocin and to determine the antimicrobial spectrum of this bacteriocin produced by the selected isolate.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Lactic acid bacterial isolates used in this study were previously isolated and identified from different origins (Raibe " traditional fermented milk", chicken, olive oil, butter and newborn feces) **(Table 1)**. Isolates were grown at 37°C in Man Rogosa Sharpe (MRS) broth (Biokar Diagnostics, France) (g/l: 10g glucose, 10g beef extract, 5g yeast extract, 0.5g sodium acetate, 2g Bipotassic phosphate, 2g ammonium citrate, 0.2g magnesium sulfate, 0.05g manganese sulfate, 1ml Tween 80, pH 6.5). To confirm the purity of the isolates each strain was individually streaked on MRS agar plates (MRS broth + 15g agar) and single colonies were isolated and tested for antimicrobial activity. Indicator strains used for determining antimicrobial activity were grown on nutrient agar. The antimicrobial activity and bacteriocin assay were realized on Muller-Hinton agar.

Table 1: The total number of LAB used in this study and their origin

Test strains	**origin**
Lb. brevis H27 ThT	Traditionally extracted olive oil
P. acidilacticii	Commercial strain
Lb. plantarum	Olive oil
Lb. bifermentans, *Lb. plantarum* R17, *Lc .lactis sp lactis* R4, *Lb. delbrueckii sp lactis* R4, *Lb. curvatus* R12	Raibe "traditional fermented Milk"
Lc. lactis sp pac B7, *Lb. Curvatus* BJ, *Lb. delbrueckii sp bulgaricus* B8,	Traditional butter
Lb. delbrueckii sp delbrueckii, *Lb. bifermentans*, *St. thermophilus*, *Lc. Lactis sp cremoris* B13,*Lb. delbrueckii sp delbrueckii*, *Lb .plantarum*, *Lb. delbrueckii sp delbrueckii*, *Lc. Reffinolactis*, *Lc helveticus*, *Lb. curvatus*, *Lc .lactis sp cremoris*, *St. salivaricus sp thermophilus*, *Lc. Cremoris*, *Lc. lactis sp diacetylactis*	Butter
Lb. cremoris NNN105	Goat butter
Lb .fermentum G8, *Lb. fermentum* G12, *Lb. plantarum* G13	Chicken gizzard
Lb. plantarum F12, *Lb. curvatus* G6, *Lb. casei ssp tolerans* G4, *Lactobacillus sp.* B5, *Lb. gasseri*, *Lb. plantarum*	Newborn feces

Antimicrobial activity assay

The isolated strains were grown in MRS broth (pH 6.5) inoculated with 1% of an overnight culture and incubated at 37°C for 18-24 h. After incubation, cells were removed from the growth medium by centrifugation (6000×g for 20 min, 4°C).The cell-free supernatant was sterilized by filtering through a 0.22 µm Millipore filter. The antimicrobial spectrum of the bacteriocin from LAB was determined using the well diffusion method (WDM) (Tagg and Mc-Given, 1971). The indicator bacteria were cultured on nutrient agar for 24 h at 37°C, and used to prepare cell suspensions in 9 ml normal saline. Twenty ml of Muller Hinton agar cooled to 45°C was mixed with 110µl of the indicator strain suspension, pooled in a Petri dish and incubated aerobically for 2 to 4h at 37°C. Six mm wells were made and filled with 100 µl of the supernatants. Plates were incubated at 37°C for 24h. Inhibition zones were determined by measuring the diameter of the clear zones around the well.

Screening for bacteriocin producing strains

The cultures of LAB that showed antimicrobial activity against indicator bacteria based on the well diffusion assay were tested for their potential to produce bacteriocins. The assay of bacteriocin was carried out as follow; the cell-free supernatants of LAB were adjusted to pH 6.0-6.5 using NaOH 5N to exclude the antimicrobial effect of organic acids. Inhibitory activity of hydrogen peroxide was eliminated by the addition of catalase at a final concentration of 1mg/ml. The catalase-treated samples were incubated for 2h at 37°C, after incubation the treated and neutralized cell-free supernatants were then tested for antagonistic activity against indicator bacteria by the WDM as described above (Ghalfi et al., 2006). Bacteriocin activity was expressed in arbitrary units (AU/ml). One AU was defined as the reciprocal of the highest level of dilution resulting in a clear zone of growth inhibition (Rajaram *et al*., 2010). Zone of 1 mm and above was considered as inhibition.

Characterization of bacteriocin

The bacteriocin samples were characterized with respect to thermal and pH stability, and susceptibility to denaturation by enzymes. The effect of temperature on the bacteriocin was tested by heating the cell-free supernatants to 40, 60, 80 and 100°C during 60 min. Aliquots of each treatment were taken after: 0, 15, 30 and 60 min. 100µl of each heat-treated sample were used for the well diffusion method, the residual activity was determined using methycilin-resistant *Staphylococcus aureus* (MRSA) as indicator organism. The effect of pH on the bacteriocin was determined by adjusting the cell-free supernatant between pH 2.0 and 12.0 with sterile 1N

HCl or 5N NaOH. The adjusted supernatants were incubated for 4 h at room temperature, 100µl of each sample were tested by the WDM using MRSA as indicator organism and the residual activity was determined.

The supernatants were treated with the following enzymes at a final concentration of 1mg/ml: lipase (Sigma), trypsine (Sigma), α-chymotrypsin (Merck), pronase E (Merck), α- amylase (Fluka). 5µl of the enzyme solution were added to 100µl of the cell-free supernatant. Controls consisted of only cell-free supernatant and tris-HCl buffer. Both the samples and the controls were incubated at 37ºC for 2 hours and heated in boiling water for 5 min to inactivate the enzymes. The remaining bacteriocin activity was determined by the WDM described above using the MRSA as indicator organism. All enzymes were used at a final concentration of 1mg/ml and maintained in tris-hydrochloric buffer (pH 8.0).

Monitoring of bacteriocin production

One ml of an 18h-old culture was used to inoculate 100 ml of MRS broth and incubated at 37°C for 48 hours. Samples were taken after time interval and examined for bacterial growth (OD 660nm), changes in culture pH, and antimicrobial activity against MRSA. The WDM was used and the activity expressed as AU/ml as described previously.

Effect of temperature on bacteriocin production

To determine the optimum temperature for bacteriocin production, we used 100ml Erlenmeyer flask. In each flask, 50ml of MRS broth was inoculated with 0.5ml of an overnight culture. The Erlenmeyer flasks were incubated at different temperatures: 30, 37 and 40°C. Samples were collected after 24h and examined for bacteriocin production as described earlier.

RESULTS AND DISCUSSION

Screening for bacteriocin producing isolates

Thirty five LAB isolated from different origins were examined for displaying bacteriocin activity against a set of 12 indicator strains. These strains presented a broad inhibitory spectrum since they were able to inhibit many of the indicator strains tested such as *E. coli* ATCC29522, *K. oxytoca*, *K. pneumoniae*, *Proteus mirabilis*, *Salmonella* sp. *S. aureus* ATCC29523, *P. aeruginosa*, *E. coli* ATCC25922, MRSA, *B. subtilis*, *E. coli* ATCC28484, *L. monocytogenes* and the pathogenic *Klebsiella* 111. The inhibitory effect, which was observed by the formation of clear and distinct zones around the wells, may be due to the production of several antimicrobial compounds like organic acids, hydrogen peroxide or bacteriocins (Labioui *et al*., 2005).

The activity of the inhibitory agent was tested under conditions which eliminate the possible effect of organic acids by adjusting the pH of the cells-free supernatant to 6.0 and of hydrogen peroxide by catalase treatment. Six of 35 strains (*Lb.plantarum* F12, *Lb.curvatus* G6, *Lb. gasseri*, *Lb. plantarum*, *Lb. casei* ssp *tolerans* G4, and *Lactobacillus* sp. B5) produced antimicrobial activity in the neutralized cell-free supernatant against four indicator strains (MRSA, *Bacillus subtilis*, *L. monocytogenes* and pathogenic *Klebsiella* 111). When the cell-free supernatant was treated with catalase (1mg/ml) the six strains confirmed their activity only against three indicator strains (MRSA, *L. monocytogenes* and *B. subtilis*). The diameters of inhibition zones of the indicator strains by the cell-free supernatant neutralized and treated with catalase are ranging from 14 to 20 mm**.** The highest diameter (20mm) was obtained with the cell-free supernatant of *Lb. plantarum* F12 and *Lb. curvatus* on *B. subtilis*, whereas the lowest diameter was obtained with the cell free supernatant of *Lactobacillus* sp. B5 against MRSA.

The fact that, the cell-free supernatants (neutralized and treated with catalase) inhibited the growth of the indicator strains gives evidence that the antimicrobial activity is due to the production of bacteriocins (Tatsadjieu *et al.,* 2009). Gram-positive indicator bacteria are much more sensitive to bacteriocin of our LAB strain than Gram-negative indicator bacteria. These results indicated that our LAB had an inhibitory spectrum towards closely related Gram-positive bacteria. The resistance of Gram-negative bacteria is attributed to the particular nature of their cell membrane; the mechanism of action described for bacteriocin involved a phenomenon of adsorption. Ivanova *et al*. (2000) found that, the bacteriocin produced by *Lactococcus lactis* subsp. *lactis* B14 inhibited only wide range of strains from the group of closely related LAB. The known bacteriocins does not still act on the sorts taxonomic close, for example, nisin has an inhibitory effect against a wide variety of Gram-positive food-borne pathogens and spoilage microorganisms and can also act on several Gram-negative bacteria when the integrity of their outer membranes is disrupted (Savadogo *et al.,*2004). The isolate *Lb. plantarum* F12 was selected for further studies. *L. monocytogenes* has become one of the most significant food borne pathogens. In food industry, the control of this pathogen remains a challenge because of its widespread occurrence and its ability to survive and persist even in hostile environment (Hartmann *et al*., 2011). For this reason we tested the ability of bacteriocins produced by *Lb. plantarum* F12 to inhibit this bacterium. Hartman *et al*. (2011) observed that the cell-free supernatant produced by eight antagonistic bacteria strains were able to inhibit *L. monocytogenes* in different food matrices. In another study, Singh and Prakash, (2009) found that, several LAB strains isolated from cottage cheese are capable of inhibiting pathogenic microorganisms in the food environment and display crucial antimicrobial properties with respect to food preservation and safety. They can also be used more specifically to inhibit certain high–risk bacteria like *L. monocytogenes* in food. Application of bacteriocins may help reduce the use of chemical preservatives and /or

the intensity of heat and other physical treatments, satisfying the demands of consumers for foods that are fresh tasting, ready to eat, and lightly preserved.

Characterization of bacteriocin

The effect of heating, pH and enzymes were studied in this work by using MRSA as indicator strain. Based on the results showed in **Fig. 1**, the inhibitory compounds produced by the tested isolate were considered to be heat stable. The activity of bacteriocin produced by *Lb. plantarum* F12 remained constant after heating at 100°C for 30 min followed by subsequent decline after 60 min. Similar results were recorded for a number of bacteriocins produced by *Lactobacillus* spp. and *Lactococcus* spp.. In addition, lacticin NK24 produced by *Lc. lactis* NK24, lost only 87.5% of its activity after 30 min at 100°C and was completely inactivated after 15min at 121°C (Todorov *et al*., 2011b).On the other hand, Sarika *et al*. (2010) observed that, the bacteriocin GP1 produced by *Lb. rhamnosus* had a remarkable stability over heat treatment even at the autoclaving temperature for 20 min. Heat stability of *Lb. plantarum* F12 at 100°C is important if the bacteriocin is used as a food preservative, because many procedures of food preparation involve a heating step.

As shown in **Fig. 2**, the antimicrobial activity of *Lb. plantarum* F12 is significantly influenced by pH. In this respect, it was observed that the residual activities were significantly higher in the range of pH 6.0 to pH 10.0 then those at pH 2.0, 4.0 and 12.0; with a maximum activity at pH 6.0, suggesting that compounds other than acids inhibited growth of MRSA. These observations are in agreement with those reported by Ogunbanwo *et al*. (2003) who showed that *Lc. brevis* excreted other compounds such as bacteriocins that inhibited the growth of pathogens. According to these results, we can say that the antimicrobial activity of *Lb. plantarum* F12 presents stability in the range of pH from 2.0 to 12.0. This property has been considered highly useful for their application as food preservative.

The effect of various enzymes on the inhibitory agent was studied. Complete inactivation or significant reduction in activity was observed after treatment of the cell-free supernatant with chymotrypsine, trypsine and pronase which confirmed the proteinaceous nature of the active agent. The other enzymes tested in our study (amylase and lipase) did not cause inactivation. This confirmed that carbohydrate and lipid moieties if existing were not required for the inhibitory activity. Similar results were recorded by Todorov *et al*. (2004) for bacteriocins produced by *Lc. plantarum* ST13BR whereas Ivanova *et al*. (2000) observed that trypsine, chymotrypsine and rennin had no effect on bacteriocin produced by *Lc. lactis* subsp. *lactis* b14 isolated from boza Bulgarian Traditional cereal beverage.

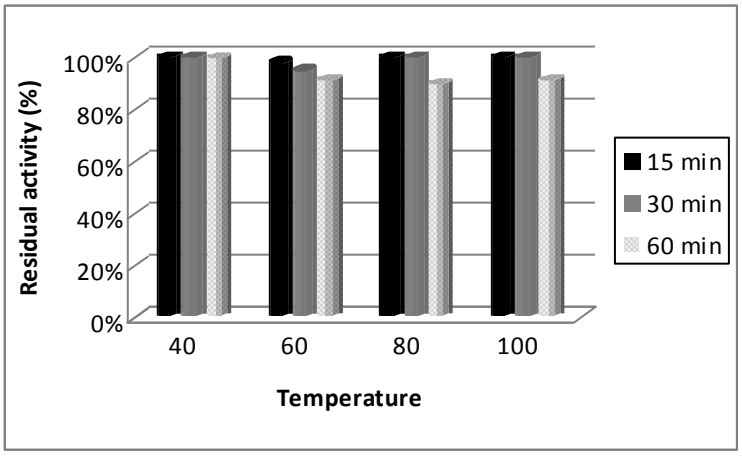

Fig. 1: Effect of temperature on bacteriocin activity produced *Lb. plantarum* F12.

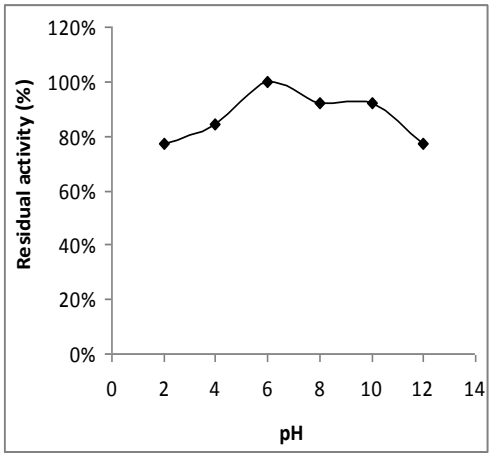

Fig. 2: Effect of pH on bacteriocin activity produced by *Lb. plantarum* F12.

Monitoring of bacteriocin production

Bacteriocin production was monitored during 48 hours of growth in MRS broth. **Fig. 3** illustrates the growth, pH and the level of *Lb. plantarum* F12 bacteriocin production through 48 hours of incubation. Data showed that bacteriocin production started at the beginning of the log phase of the bacterial growth (after 4 h), and increased gradually with bacterial growth till it reached its maximal level (2416 AU/ml) after 30 hours of incubation (in stationary phase). After 32 h of incubation a decrease in bacteriocin production was observed (2166 UA/ml). During the same period of growth the pH of the medium decreased from 6.5 to 4.5. The growth of *Lb. plantarum* F12 increased gradually and reached its optimum after 32 h and remained more or less constant during the following 16 hours. Several studies have shown that bacteriocin production is dependent on biomass concentration. Todorov and Dicks, (2005) reported that optimal levels of plantaricin ST194BZ, produced by *Lb. plantarum* ST194BZ, were obtained in growth media that supported high biomass production, such as MRS. A similar bacteriocin production profile was reported for bacteriocin ST311LD produced by *E. faecium* ST311LD isolated from fermented olives, in which maximal bacteriocin production was reported after 20 hours growth in MRS broth, followed by a decrease in activity in the following 5 hours. The decrease in activity of bacteriocins produced by *Lb. plantarum* F12 at the end of the monitored period could be explained by the degradation of the bacteriocin by extracellular proteolysis enzymes, similar decreases have also been observed for bacteriocins produced by *Enterococcus faeciu*m ST311LD (Todorov &Dicks, 2005), *Enterococcus mundtii* ST4SA and *Pediococcus acidilacticii* NRRL B5627 (Todorov *et al*., 2011a).

Effect of temperature on bacteriocin production

The effect of temperature on bacteriocin production by *Lb. plantarum* F12 was tested in Erlenmeyer flasks cultures containing sterile MRS and maintained at different temperatures (30, 37 and 40°C). **Fig. 4** shows the effect of temperature on bacteriocin production. The optimum temperature for the production of bacteriocin was 37°C, thus the bacteriocin activity at this temperature was higher than that observed at 30 and 40°C. According to these results we can say that, the optimum temperature for production and the one for growth are correlated, as observed elsewhere for lactocin A, enterocin 1146, lactocin S and nisin Z (Todorov *et al*., 2004). So, growth temperature seems to play an important role on bacteriocin production. Different results were recorded by Mataragas *et al*. (2003), as they found that the optimum temperature for the production of bacteriocins produced by *Leuconostoc mesenteroides* L124 and *Lb. curvatus* L442 was 25°C and was lower than that of growth (30°C).

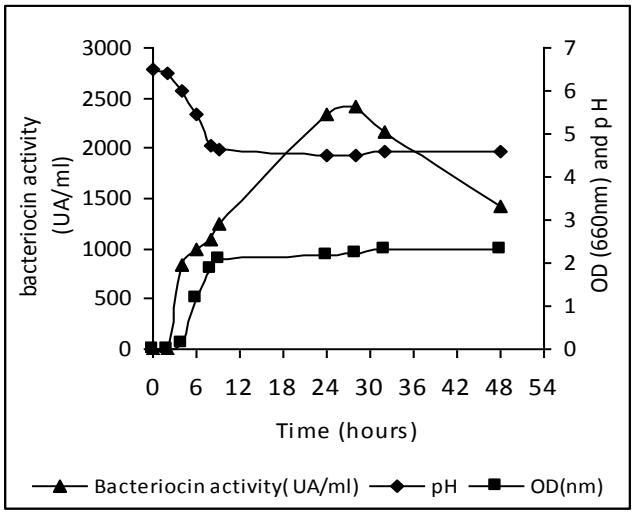

Fig. 3: Monitoring of bacteriocin production from *Lb. plantarum* (F12) in MRS medium at 37°C during 48 h.

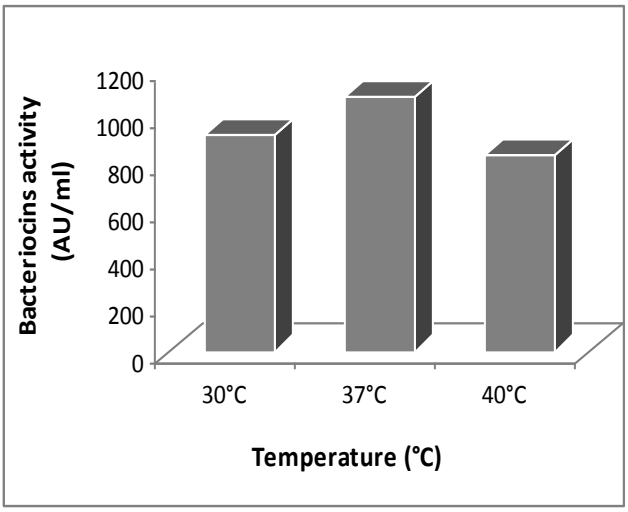

Fig. 4: Effect of temperature on bacteriocin production from *Lb. plantarum* F12 in MRS medium at 37°C.

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