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*Full Length Research Paper*

# Screening of autochthonous *Lactobacillus* species from Algerian raw goats' milk for the production of bacteriocin-like compounds against *Staphylococcus aureus*

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**Lactic acid bacteria play a key role in maintaining the balance of normal gastro-intestinal micro flora. Food contamination by *Staphylococcus aureus* is a major problem for consumer's health in Algeria, especially during the summer period. The use of bacterial interactions is a new way to limit the pathogenic germs growth. Detection of antimicrobial substances produced by lactic acid bacteria against the undesirable germs is the objective of this work. Microbiological and biochemical methods were used to identify lactic acid bacteria having an antimicrobial activity. The 2 isolates of lactic acid bacteria obtained from raw goats' milk in western Algeria's areas were identified: *Lactobacillus rhamnosus*, *Lactobacillus plantarum*. The interactions study revealed that three lactobacilli species: *L. plantarum* (58) and *L. plantarum* (68) are able to inhibit *S. aureus'* growth. In mixed culture after 24 h, *L. plantarum* reduces the growth of *S. aureus* by 1.6 log and this latter bacteria was not found after 72 h. The various tests used reveal the proteinic nature of the substance which was responsible for the growth inhibition of *S. aureus*. The ecological adaptation and growth characteristics of cultures of *L. plantarum* in food products will determine their effectiveness as bio-control agent in dairy foods.**

**Key words:** Raw goats' milk, lactic acid bacteria, *Lactobacillus plantarum*, interaction, bacteriocin, *Staphylococcus*, mixed culture.

## INTRODUCTION

Control of both pathogenic and spoilage microbe in a variety of foods is important to guarantee food quality and safety. Recently, bio-preservation has become a topic of interest (Kabuki et al., 2007; Keihei et al., 2011). This technique is used as an alternative to chemical additives for increasing self-life storage and enhancing safety of food by using natural micro flora and their antimicrobial products (Stiles, 1996). Lactic acid bacteria are believed to be safe because they have been long established as the normal flora in fermented food; thus, they have great potential for use in bio-preservation. The preserving effects of lactic acid bacteria are due to the production of antimicrobial agents such as organic acids, hydrogen peroxide and bacteriocin or related substances (Desmazeaud et al., 1996; Cocolin et al., 2007).

Bacteriocins are proteinaceous compounds that mainly inhibit closely related species (Klaenhammer, 1993). Some bacteriocins have been shown to possess the ability to inhibit the actions of unrelated genera such as Clostridia, Listeria, entero-pathogenic bacteria and Gram-negative bacteria. For these reasons bacteriocins are promising candidates for bio-preservation of food (Cleveland, 2001). Several *Lactobacillus* strains are important dairy culture starter and used for the

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manufacture of fermented food (Fitzsimmons et al., 1999; Badis et al., 2004; Pringsulaka et al., 2011b).

The discovery of bacteriocins gave a new way for food development in better hygienic quality (Fitzsimmons et al., 1999, Badis et al., 2004; Pringsulaka et al., 2011b). In recent years, there have been many reports on bacteriocins that are produced by lactic acid bacteria. However, most reports deal with bacteriocins that are produced by various lactococci, pediococci, leuconostoc, enterococci and lactobacilli (Ennahar et al., 2000; Mc Auliffe et al., 2001; Moll et al., 1991). The search for new strains of lactic acid bacteria that produce antimicrobial substances is a universal objective for the creation of new cultures starter with a high biosafety for fermented food. The inhibition of pathogenic bacteria such as *Staphylococcus aureus* by lactic micro flora was announced by Heikkila et al. (2003). The technological characterization of the lactic acid bacteria leads to the development of well defined bacterial strains with specific characteristics. The latter gradually replace the non-definite, non-defined mixtures of starters traditionally used in dairy industry (Crow et al., 1993; Papagianni et al., 2009). In order to avoid the side effects of chemical preservatives, these last years, the interest for the use of the bacteriocins or strains of lactic acid bacteria for applications as bio-preservatives caused many research tasks (Schillinger and Lücke, 1989; Budde et al., 2003; Jacobsen et al., 2003; Vermeiren et al., 2004; Cocolin et al., 2007; Alvarez-Martin et al., 2008; Pringsulaka et al., 2011). Several bacteriocin producing lactic acid bacteria offer potential applications in food preservation. The use of bacteriocins in the food industry can help to reduce the addition of chemical preservatives as well as the intensity of heat treatments, resulting in foods which are more naturally preserved and richer in organoleptic and nutritional properties (Galvez et al., 2007).

The aim of this work is the isolation of lactic acid bacteria that produce antimicrobial substances belonging to bacteriocin type able to inhibit the bacteria which causes food poisoning.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

The species of *Lactobacillus* were obtained from the collection of the laboratory of applied microbiology, Department of biology, Faculty of Science, university of Oran, Algeria. The three pathogenic bacteria responsible for food toxic-infections (*S. aureus* ATCC 25923) were obtained from the collection of Medical Analysis Laboratory of the University- Hospital centre of Oran.

The species of lactobacilli were cultivated in liquid or solid MRS with pH 5.4 then incubated at 30°C for 48 h (de Man et al., 1960). The selective enumeration of *S. aureus* is carried out on Chapman medium at 30°C. The other bacteria, *Escherichia coli* and *Bacillus* sp. were grown on Muller-Hinton medium and incubated at 37°C. The media used during this work were either liquid, solid (1.5% agar p/v) or soft agar medium (0.7% agar). The skimmed milk medium (11% p/v) was sterilized at 110°C for 10 min and all other media were sterilized at 121°C for 20 min (Kihal et al., 2006). The isolated *Lactobacillus* strains were selected as bacteriocin producers because of their broad antimicrobial activity, and subjected to phenotypic identification. Cell morphology and Gram-staining reaction were examined by light microscopy. Test for catalase activity and fermentation of different sugars were also tested as described by Badis et al. (2004).

### Hemolytic activity

For testing hemolytic activity, fresh cultures of isolated strains were streaked on blood agar, containing 5% (w/v) sheep blood, and incubated for 48 h at 30°C. Blood agar plates were examined for signs of hemolysis. This test was performed in the hospital laboratory (Ghrairi et al., 2008).

### Antimicrobial activity detection and assay

The production of antimicrobial substance by *Lactobacillus* sp. was detected by the deferred antagonism assay as described by (Barefoot et al., 1983). An overnight culture of *Lactobacillus plantarum* was spotted onto the surface of an MRS plate and incubated for 16 h at 30°C to allow colonies to develop. Approximately $10^7$ CFU/ml of indicator strain were inoculated into 10 ml of MRS agar and poured over the plate on which the producer was grown. After anaerobic incubation for 24 h at 30°C, the bacterial lawns were examined for zones of inhibition surrounding the producer colonies. Inhibition was recorded as positive if the width of the clear zone around the colonies of the producer was 2 mm or larger (Tahara and Kanatani, 1996). Bacteriocin-like activity was assayed by the agar-well diffusion method of Tagg and McGiven (1971). Portions (100 µl) of serial twofold dilutions of culture filtrates of *Lactobacillus plantarum* strain were placed into each 0.5 cm diameter well of a plate, which was inoculated with approximately $10^6$ CFU/ml of a log-phase culture of *L. plantarum* after a 24 h incubation at 30°C, plates were examined for inhibition on the indicator lawn. For the activity spectrum determining of bacteriocin-like produced by *L. plantarum* pathogenic bacteria *S. aureus* ATCC 25923 were used.

### Preparation of crude bacteriocin, sensitivity to enzymes, ph and heat treatment

MRS broth was used for the preparation of crude bacteriocin from the culture of *L. plantarum*. After incubation at 30°C for 18 h, culture supernatants were obtained by centrifugation at 8000 rpm /min for 10 min at 4°C. Crude bacteriocin was concentrated by precipitation with 5% TCA and stored at 4°C. As for the sensitivity to enzymes, pH and heat treatment, the neutralized MRS culture supernatant was tested for susceptibility to proteolytic enzymes. Samples were incubated at 30°C during 1 h in the presence of trypsin, alfa-chymotrypsine and catalase (Jamuna et al., 2005; Kabuki et al., 2007; Tahara and Kanatani, 1996).

After the treatment, the reaction mixture was heated for 10 min in boiling water (100°C) for enzyme inactivation and then bacteriocin activity was measured. To determine the pH stability of bacteriocin, pH values of the culture supernatants were adjusted within the range of 5 to 7 by HCl or NaOH, and each sample was held for 1 h at 30°C. After incubation, the pH of each sample solution was adjusted to 6.5 by adding HCl or NaOH and the bacteriocin activity was measured as described above. To examine thermal stability of bacteriocin, crude bacteriocin was treated at the various temperatures mentioned above. After the treatment, the samples were rapidly cooled and the bacteriocin activity was assayed (Tahara and Kanatani, 1996).

**Assay for antimicrobial activity**

The search for possible production of inhibiting substances by the isolated bacteria was carried out according to two methods; Direct method of Tagg and Mc Given (1971), Fleming et al. (1975) and Tahara and Kanatani (1996), consists of cultivating the two strains in the same medium with double-layer. The inhibitory activity spectrum was obtained using the agar spot test (Casalta et al., 1995) against the other strains. For this, 5 µl aliquots from an overnight culture of *Lactobacillus* sp. strain grown in MRS broth being spotted onto buffered MRS agar plates (1.5% agar) and incubated for 24 h were used. Subsequently the plates were then overlaid with 6 ml of soft agar medium (Muller Hinton) seeded with actively growing cells of the test organisms (or pathogenic strain: *Staphylococcus aureus*, after solidification and then incubation at 30°C. The antimicrobial activity of *Lactobacillus* sp and the sensitivity of the pathogenic strain in question were evaluated by checking for clear zones around the spots (Tahara and Kanatani, 1996; Kaban and Kaya 2006; Guessas et al., 2006).

The indirect method of Barefoot et al. (1983) and Schillinger and Lücke (1989), makes it possible to put in contact the supernatant of lactic acid bacteria strains that produce antimicrobial substances with the test strains. Producing strains of inhibiting substances were cultivated in liquid medium MRS and incubated for 18 h. After growth, the culture was centrifugalized at 8000 rpm/min during 10 min and the supernatant was stored at 4°C. The supernatant fluid was filtered through a syringe filter with a pore size of 0.22 µm (Millipore Corporation, Bedford, USA) and adjusted to pH 6.0 with sterilized NaOH (2 M), so as to rule out inhibition through the production of organic acids. This supernatant was placed into the wells and the medium was inoculated by the test strains. These wells will receive 100 µl of tested supernatant strains and then incubated for 24 h. Inhibition of growth was determined by an area of inhibition surrounding each agar well (Miteva et al., 1998; Guessas, 2007).

**Determination of the spectrum activity**

The direct confrontation method of Shillinger and Lücke (1989) and Sookkhee et al. (2001) was used for the determination of the spectrum activity of antimicrobial substances strains production. The selected producing species belong to *L. plantarum* (58, 68) while the test species belong to *S. aureus.*

**Characterization of the nature of the inhibiting agent**

The antimicrobial activity of *L. plantarum* can be caused by several factors such as acidity, hydrogen peroxide, phages and bacteriocins. To determine whether the inhibitory substances produced by the bacteria were proteinaceous and thus could be designated as bacteriocins, sensitivity to proteolytic enzymes (trypsin and $\alpha$ - chymotrypsin) was assessed in assays as described by Vaughan et al. (1992, 1994). Blank experiments were also performed using also no enzyme or inactivated enzyme. Gram positive *S. aureus* was used as the indicator strain in the experiments (Ghrairi et al., 2008).

**Kinetics of growth and acidification**

Associative growth of *S. aureus* and the bacteriocin producer strain *L. plantarum* (58) was performed in sterilized skim milk at 30°C. The acidity evaluation of the pure strain was carried out by tritration and pH metrical measurement (Campos et al., 2006). Each strain was inoculated in 10 ml of sterile skim milk (10% p/v). The pre-culture of *L. plantarum* was prepared by incubation at 30°C until coagulation. An amount of 3% of pre-culture was inoculated in 100 ml of skim milk which was immediately homogenized. 10 ml of the mixed culture was distributed in sterile tubes. The growth kinetics and acidification were carried out simultaneously within the regular lengths of time intervals.

The pathogenic strain of *S. aureus* (ATCC.25923) was used as test which was inoculated in skim milk at 2% which gives approximately $10^3$ CFU/ml, in pure and mixed culture with *L. plantarum*. The antimicrobial effect of the latter strain against *S. aureus* was measured by microbiological techniques as described by Kaban and Kaya (2006). Serial decimal dilutions were prepared in sterile physiological saline water and 0.1 ml samples of appropriate dilutions were spread in duplicate on selective agar plates. The number of *S. aureus* and *L. plantarum* was determined in Chapman and MRS agar media respectively (Ananou et al., 2007).

**Measurement of acid production**

A deduction of 10 ml of the culture was transferred in a conical flask of 100 ml and 5 drops of phenolphthalein indicator (2 mg/ml in ethanol 60°C) were added. The acidity was neutralized by NaOH 1/9N until the appearance of a persistent pink color. The volume of the titrating solution was measured in order to indicate the production of acidity which was estimated in dornic degrees (Kihal et al., 1996; Moulay et al., 2006).

**Growth kinetics measurement in pure and mixed culture in milk**

The enumeration of *Staphylococcus aureus* was carried out on Chapman medium. Only plates that contained 30 to 300 colonies were taken into account. The enumeration in mixed culture was done by sowing 0.1 ml of serial dilutions in two selective acidified MRS media for *Lactobacillus* and Chapman for *S. aureus* (Guessas et al., 2006; Kaban and Kaya, 2006; Otero and Macias, 2006).

**Effect of the crude supernatant of *L. plantarum***

The crude supernatant of *L. plantarum* (58) was tested against the growth of *S. aureus* in milk. The crude supernatant was obtained by the centrifugation of a culture of 18h in MRS pH 6.8 with 8000 r.p.m/min for 10 min. Protein concentration of the culture supernatant was determined by the micromethod of Bradford (1976) using bovine serum albumin fraction as standard. The supernatant was heated at 100°C. The residual activity of the crude supernatant was immediately tested after freezing and neutralization by NaOH. *S. aureus* was tested for determining the minimal inhibiting concentrations of this crude supernatant. After incubation with various dilutions, the growth was estimated by reading the optical density at 670 nm (Tahara et al., 1996; Blay et al., 2007; Pal and Ramana, 2010).

## RESULTS AND DISCUSSION

A number of 128 strains of *Lactobacillus* were isolated from raw goat's milk. The strains which produce antimicrobial substances were detected by confrontation on solid culture medium. From 128 isolates, only two strains showed an inhibiting activity. These latter strains were identified to species level by microbiological and biochemical methods, as described by Stiles et al. (1997)

**Table 1.** First identification characters of lactobacilli isolates from raw goat's milk.

| Characters | 58 | 68 |
|---|---|---|
| Form | rode | rode |
| Gram | + | + |
| Catalase | - | - |
| Fermentation ribose | + | + |
| $CO_2$ from glucose | - | - |
| $CO_2$ from gluconate | + | + |
| Arginine Hydrolysis | - | - |
| Growth at 15/45 | +/+ | +/+ |
| Group | ***Lactobacillus*** Group II, facultative heterofermenter (Streptobacteria) | ***Lactobacillus*** Group II, facultative heterofermenter (Streptobacteria) |

Klein et al. (1998) and Carr et al. (2002).

## Characterization of the isolates

The strains retained gave small colonies of approximately 1 mm of diameter, lenticular with a white or milky color, smooth surface and a regular circular circumference were observed on solid medium. The microscopic examination revealed that the tested strains were Gram positive, with a cellular rod form associated in pairs or in chains (Table 1). Table 2 adds to the characteristics for a better identification of the species used in this study.

These data guide us to classify the isolated bacteria to the genus level according to their cellular morphologies and their association mode and the type of gram staining (Joffin and Leyral, 1996). On the basis of microbiological (Table 1), physiological and biochemical (Table 2) analysis results, the establishment of the percentage of reliability of each strain in comparison with references (rephrase) (Stiles et al., 1998; Klein et al., 1998; Carr et al., 2002).

## Hemolytic activity

In eight strains of *Lactobacillus* isolated from the raw goat's milk and used in this work no hemolytic activity was observed. However, absence of hemolytic activity should be a selection criterion for (bacteriocin-producing) starter strain for dairy use; absence of hemolytic activity in *Lactobacillus* indicates that these bacteria are non-virulent (De Vuyst et al., 2003).

## Detection of inhibitory activity

All of the eight *Lactobacillus* species retained were tested for their antimicrobial activity (Table 3, Figure 1). The zones of inhibition in the spot in the lawn method were more easily visualized. Our results show that all isolated strains (*L. plantarum*, *Lactobacillus rhamnosus*) have inhibitory effect against *S. aureus*; the most inhibiting species was *L. plantarum* (58).

The inhibiting activity of *Lactobacillus* sp can have two factors; the first is the production of lactic or acetic acid; indeed, the *Lactobacillus* are known for a great resistance to acid pH (until a pH close to 3.5) contrarily to the other genera of lactic acid bacteria (Wong, and Chen, 1988; Podolak et al., 1996; Wilson et al., 2005); whereas the second comes from the production of another substance and probably of the bacteriocins (Larsen et al., 1993; Oyetayo et al., 2003; Avila et al., 2005; Cocolin et al., 2007).

## Determination of the antimicrobial compounds

Our results of the inhibition study show that several strains can give an inhibition zone on solid medium. All tests of the determination of the antimicrobial compounds show that our eight *Lactobacillus* strains cannot regenerate $H_2O_2$. The effect of acidity and phage lyses were also studied and no effect was observed. Juilliard et al. (1987) reported that the production of $H_2O_2$ occurred in aerobic conditions.

The phages can be the origin of bacterial growth inhibitions (McGrath et al., 2002; Lewis et al., 1991). The test of the phage appeared negative for the strains of *Lactobacillus*.

The synergism effect of the antibacterial action cannot exclude the effect of acids (Deegan et al., 2006), hydrogen peroxide (Piard and Desmazeaud, 1991), diacetyl (Condon, 1987) or bacteriocin-like substances (Alexandre et al., 2002) by the strains of *Lactobacillus*.

**Table 2.** Physiological and biochemical characters of *Lactobacillus* strains having an antimicrobial activity isolated from fresh raw goat's milk.

| **Carbon hydrates** | **58** | **68** | ***Lactobacillus plantarum* ATCC 14917** |
|---|---|---|---|
| Blanc | - | - | - |
| Glycérol | - | - | - |
| Erythritol | - | - | - |
| D- Arabinose | - | - | - |
| L- Arabinose | + | + | + |
| D- Ribose | + | + | + |
| D- Xylose | - | - | - |
| L- Xylose | - | - | - |
| D- Adonitol | - | - | - |
| MDX | - | - | - |
| D- Galactose | + | + | + |
| D- Glucose | + | + | + |
| D- Fructose | + | + | + |
| D- Mannose | + | + | + |
| L- Sorbose | - | - | - |
| L- Rhamnose | - | - | - |
| Dulcitol | - | - | - |
| Inositol | - | - | - |
| D- Mannitol | + | + | + |
| D- Sorbitol | + | + | + |
| MDM | + | - | + |
| MDG | - | - | - |
| NAG | + | + | + |
| Amydaline | + | + | + |
| Arbutine | + | + | + |
| ESC | + | + | + |
| Salicine | + | + | + |
| D- Celiobiose | + | + | + |
| D- Maltose | + | + | + |
| D- Lactose 1 | + | + | V |
| D- Melibiose | + | + | + |
| D-Saccharose | + | + | + |
| D- Trehalose | + | + | + |
| Inuline | - | - | - |
| D- Mélézitose | + | + | + |
| D- Raffinose | + | + | + |
| Amidon | - | - | - |
| Glycogène | - | - | - |
| Xylitol | - | - | - |
| Gentiobiose | + | + | + |
| D-Turanose | + | + | + |
| D- Lyxose | - | - | - |
| D- Tagatose | - | - | - |
| D- Fucose | - | - | - |
| L-Fucose | - | - | - |
| D- Arabitol | - | - | - |
| L-Arabitol | - | - | - |
| GNT | + | + | + |
| 2KG | - | - | - |
| 5KG | - | - | - |
| Identified as | *Lactobacillus plantarum* | *Lactobacillus plantarum* | *Lactobacillus plantarum* |

MDX: Méthyl-βD-Xylopyranosid, MDM: Méthyl-αD-Mannopyranosid, MDG: Méthyl-αD-Glucopyranosid, NAG: N-Acétylglucosamin, ESC: Esculine citrate iron, 2KG: Potassium 2-cétogluconat, 5KG: Potassium 5-cétogluconat, GNT: Potassium Gluconat. 1 origine bovine.

**Table 3.** Interactions between 8 selected *Lactobacillus* strains, on solid medium and the diameter of inhibition zones was measured (mm).

| Code Strains | 58 | 55 | 68 |
|---|---|---|---|
| 58 | 0 | 7 | 5 |
| 55 | 8 | 3 | 5 |
| 68 | 6 | 3 | 4 |
| 2 | 6 | 4 | 2 |
| 13 | 3 | 3 | 5 |
| 52 | 4 | 7 | 2 |
| 31 | 7 | 4 | 3 |
| 54 | 5 | 4 | 5 |

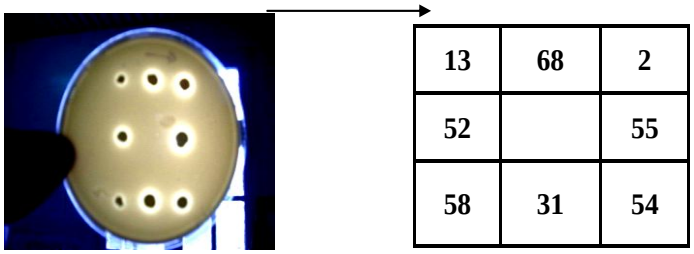


**Figure 1.** Interactions between the isolates of the *Lactobacillus* species with the test one *Lactobacillus plantarum* (Strain 58) on solid medium.

**Table 4.** The action of the proteolytic enzymes and the heat treatment on the antimicrobial activity of the crude supernatants of *Lactobacillus* strains towards the growth of *Staphylococcus aureus*.

| Strains | α-chymotrypsin | Trypsin | Heath treatment 100°C |
|---|---|---|---|
| 58 | - | - | + |
| 68 | - | - | - |

**Table 5.** Interactions and inhibition spectrum of *Lactobacillus* strains towards food spoilage bacteria and food-born pathogen *Staphylococcus aureus.*

| Strains | *Staphylococcus aureus* | | |
|---|---|---|---|
| | MNT | MT | % |
| 58 | 28 | 20 | 28.5 |
| 68 | 19 | 14 | 26.3 |
| Average | 21.87 ± 4.85 | 15 ± 2.26 | 31.41 |

MNT: medium not buffered, MT: buffered medium.

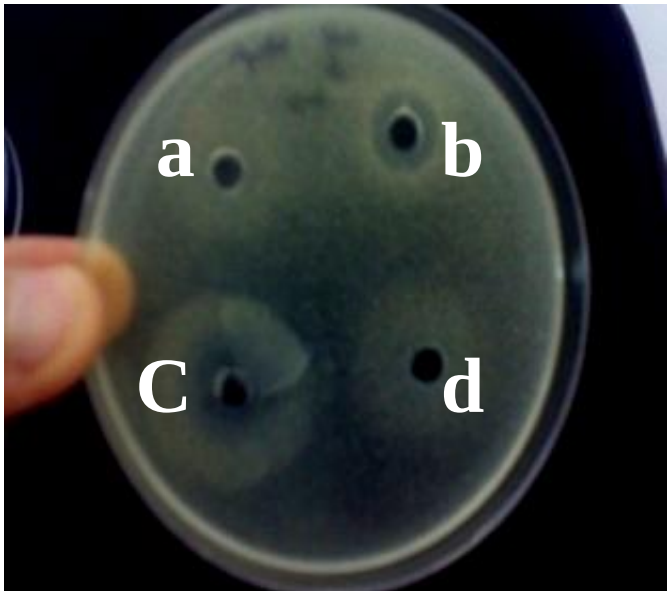


**Figure 2.** The action of the proteolytic enzymes and the heat treatment on the antimicrobial activity (inhibition zones) of crude supernatant towards the growth of *Staphylococcus aureus*. (a: α-chymotrypsin, b: crude supernatants, c: crude supernatants with heat treatment at 100°C and d: Trypsin).

## The effect of the proteolytic enzymes on the inhibiting substance

All bacteriocins compounds are of protein nature (Callewaert and Vuyst, 2000; Aslim et al., 2005). To identify this nature of the antimicrobial substances, the action of proteolytic enzymes (trypsin and α-chymotrypsine) was tested and the results were noted in Table 4 and 5 which gives the effect of enzyme and heat treatment on the antimicrobial activity of the crude supernatant. The actions of α-chymotrypsin (Figure 2) reduced totally the antimicrobial activity of the *Lactobacillus* strain. Whereas, only one *Lactobacillus* strain (58) could resist to the heat treatment at 100°C.

Several bacteriocin of *Lactobacillus* have a large antimicrobial spectrum for gram positif and gram negative bacteria, for example the plantaricin C19 produced by *L. plantarum* (Nettles et al., 1993). The antimicrobial substances produced by lactic acid bacteria strains isolated from the goat's milk were investigated by Klaenhammer (1988). Our results indicated the case of the inhibiting substances produced by *L. plantarum* (58) strain. During the characterization of the bacteriocin, important variations in the spectra of activity are noted. It is also noted that the sensitivity of a strain depends on the genera, the species and even on the subspecies (Kalchayanand et al., 1992). The activity of strain supernatant was lost after the treatment with proteolytic enzyme indicating that active component secreted extracellularly was proteinaceous in nature and confirming that growth inhibition of sensitive strain of *Lactobacillus* was caused by bacteriocin (Ghrairi et al., 2008).

### Inhibitory effect of *Lactobacillus* against *S. aureus*

The inhibitory spectrum of culture supernatants of eight *Lactobacillus* strains was assayed by the agar well diffusion method. The inhibitory activity was directly evaluated against food spoilage bacteria and food-born pathogen including three strains (*S. aureus, Escherichia coli* and *Bacillus* sp.) is shown in (Table 5).

The results obtained in the interaction between the most powerful *Lactobacillus* shows an inhibition of 8 strains (Figure 3). A strong inhibition of *S. aureus* growth was obtained by *L. plantarum* (58). This inhibition was due to an inhibiting substance like-bacteriocin. All used tests in this study confirm the nature of this substance as shown by Tagg et al. (1971); Todorov and Dicks (2005). *Lactobacillus* strains 52, 54, 31, 2 and 13 expressed an inhibition growth of *S. aureus*, Bacillus sp. and *E. coli* with various diameters but lower than those obtained by strains 58, 55 and 68.

*L plantarum* strains (58) gave an inhibition diameter of 20 mm for *S aureus*. This strain (58) was retained as producing bacteriocin for carrying out this study. Generally the median values recorded by measuring the diameter of the inhibition zones, show that the Gram positive bacteria (*S. aureus*, 15±2.26 mm ) were sensitive to the inhibiting substances produced by the lactobacilli compared to the Gram negative bacterium (*E. coli,* 8.62 ±1.84 mm). Todorov and Dicks (2005) observed that a high level of bacteriocin was produced when the cells were grown in the presence of $K_2HPO_4$. Little is known about the influence of potassium ions on the production of bacteriocins. In the case of bacteriocin produced by *L. plantarum,* the level of $K_2HPO_4$ is needed to increase bacteriocin production (Todorov et al., 2005). The growth conditions used in this study were similar to those cited above.

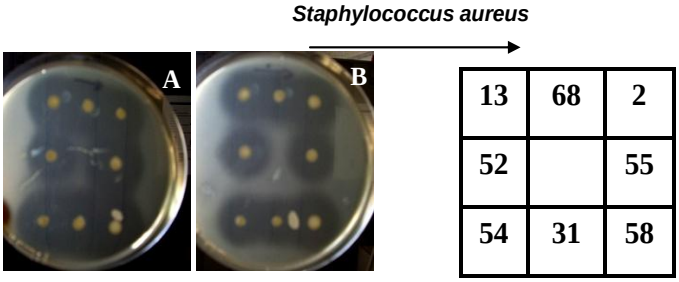


**Figure 3.** The inhibiting activity of *Lactobacillus* towards *Staphylococcus aureus* by the appearance of the clear zones around the colonies in none buffered (A) and buffered medium (B).

### Growth kinetics and acidification

#### *Evolution of pH and acidity in mixed culture in skim milk*

The technological aptitude of the lactic acid bacteria is often based on the study of the acidifying capacity. All the species of *Lactobacillus* retained showed a high acidifying activity, which exceeded that produced by *S. aureus* which was 36.3°D in 24 h (Figure 4). *L. plantarum* (58) produced the highest level of lactic acid 50°D in 24 h. Other species of *Lactobacillus*, *L. plantarum* (68) produced 48°D and 43.5°D respectively in 24 h.

In mixed culture of *L. plantarum* and *S. aureus*, a high production of lactic acid (69.4°D) was observed. Whereas, the production of lactic acid decrease in mixed culture with *Lb. plantarum* (68) (59.4°D) was noted.

Kinetics of pH evolution (Figure 4) showed that *L. plantarum* decreased the pH up to 4.32 in 24 h whereas the pH of *L. plantarum* (68) was 4.76. The final pH of the culture of *S. aureus* was 5.13. In the mixed cultures the pH reached 4.57 in *L. plantarum* (68) and 4.6 in *L. plantarum* (58).

Lactic acid production in the culture of *L. plantarum* and *Lacyobacillus brevis* studied by Kask et al. (1999) and Katina et al. (2002) was 50°D. These results are in agreement with our strains and in particular with *L. plantarum*. The latter produces a quantity of lactic acid higher than 40°D and almost reaches a maximum value of 100°D (Katina et al., 2002).

The most important quantity of lactic acid production was observed in *L. plantarum* (52°D) in pure culture after 72 h. Whereas, in mixed culture with *S. aureus* the species of *Lactobacillus* produced a higher quantity of acid, which was 71°D in *L. plantarum*, followed by *L. plantarum* (68) with 64°D. In synthetic medium saturated with glucose, Callewaert and De Vuyst (2000) reported that *Lactobacillus reuteri* produces an acidity of 400°D in 24 h.

The results of the present study argue in favour of controlling *S. aureus* at the beginning of the dairy industry process when pH and temperature conditions may favour enterotoxin production. Our results suggest that the growth in mixed culture of *L. plantarum* and *S. aureus* can take into account their interactions.

### Growth evolution in mixed culture in milk medium

#### *Enumeration of S. aureus in pure and mixed culture with Lactobacillus sp.*

The initial enumeration of the 3 *Lactobacillus* strains *L. plantarum* (58), and *L. plantarum* (68) was 3.19 log and 3.9 log respectively. After 24 h of incubation, the number of *L. plantarum* was 8.19 and 5.07 log CFU/ml for *S. aureus* in pure culture. The number of the latter bacteria increased by 1.85 log CFU/ml after 24 h of incubation. In mixed culture after 24 h of incubation, a reduction of 1.6 log CFU/ml in the number of *S. aureus* was observed. This reduction proved the inhibiting effect of *L. plantarum* (Figure 5). A decrease of inhibition effect was observed

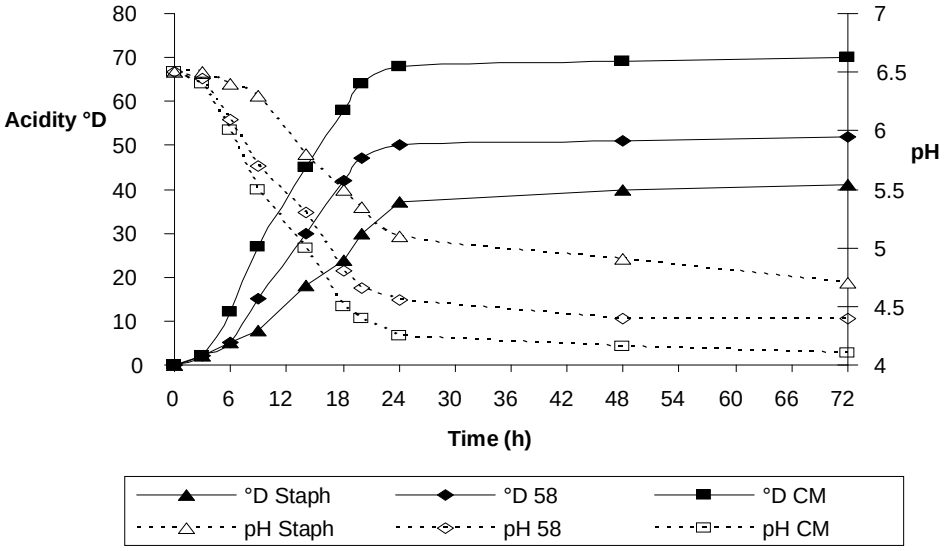


**Figure 4.** Acidity (full symbol) and pH (empty symbol) evolution in pure culture and mixed culture of *Lactobacillus plantarum* (58) and *Staphylococcus aureus* in milk.

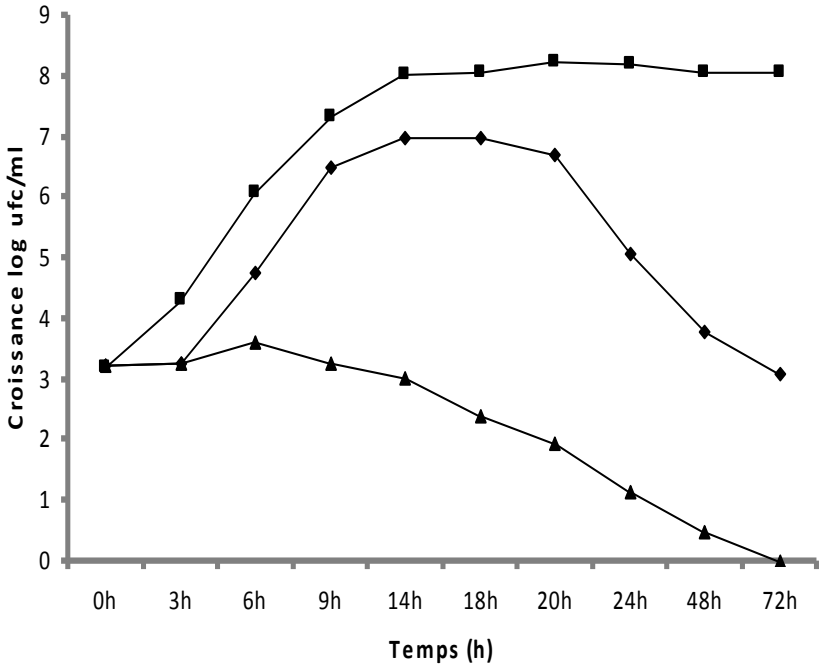


**Figure 5.** Kinetics of growth of *Lb. plantarum* (■) and *Staphylococcus aureus* (♦) in pure culture and *Staphylococcus aureus* (▲) in mixed culture in milk.

for the other strains of *Lactobacillus* sp. towards *S. aureus* (results not showed).

After 72 h of incubation, viable cell number of *L. plantarum* (58) and *L. plantarum* (68) reached 8.05 and 8log CFU/ml respectively. No growth could be detected for *S. aureus* after 72 h of incubation in the presence of *L. plantarum*. The inhibiting activity was slightly weak in the two other *Lactobacillus* species than *L. plantarum* (68). This variation of the inhibiting effect of the species of *Lactobacillus* towards *S. aureus* was also observed by Rodriguez et al. (2005). Production of multiple bacteriocins by *L. plantarum* caused an important

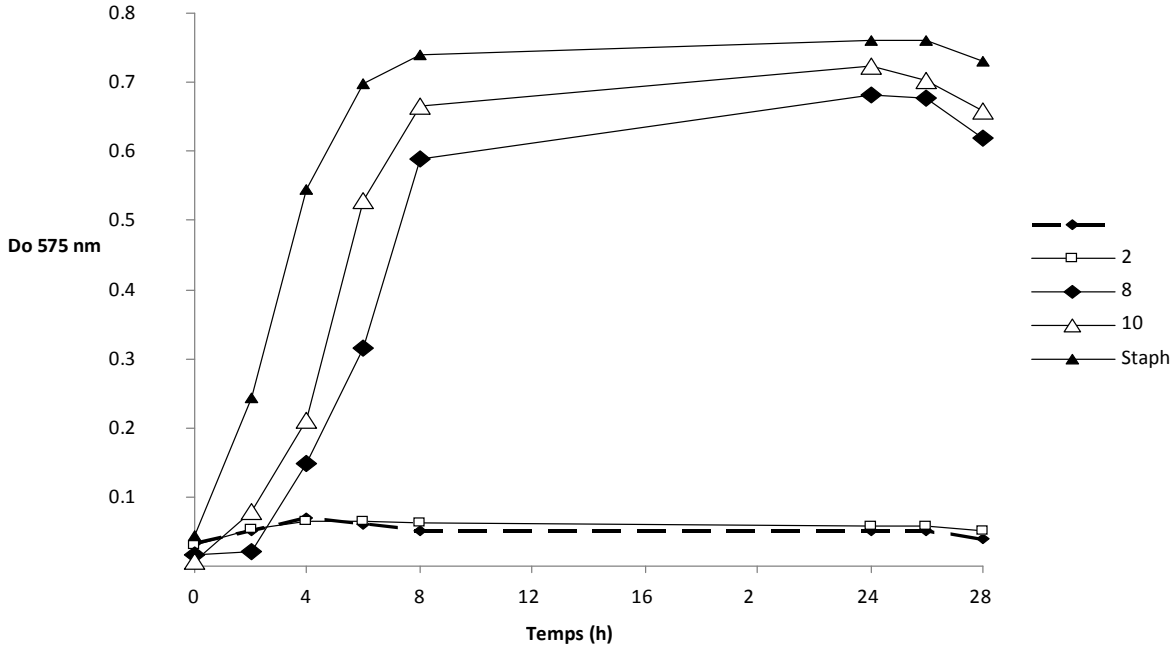


**Figure 6.** Effect of various dilutions of crude supernatant of *Lactobacillus plantarum* (58) heated with 100°C towards the growth of *Staphylococcus aureus*.

inhibition of *S aureus* (Hernandez et al., 2005). Work of Arquès et al. (2005) showed that after 72 h incubation, the number of *S. aureus* falls down to 0.46 log CFU/ml.

## Effect of crude supernatant of *L. plantarum* on the growth of *S. aureus*

The influence of *L. plantarum* (58) supernatant was tested for evaluating the growth of the test strain of *S. aureus* after 6 h of incubation (Figure 6). The results showed clearly the absence of growth of *S. aureus* in first dilutions (1/2 and 1/4) in which death rate was higher than 90% (Figure 6). Whereas, for dilutions (1/256 and 1/1024) death rates were in close proximity to 50%. After 24 h of incubation, death rate in the first three dilutions (1/2, 1/4 and 1/8) could reach 95.1, 93.4 and 92.5 respectively compared to the test strain growth. While the death rate decreased considerably in dilutions 1/256 and 1/1024 and reached easily 4 and 4.9% respectively.

In optimal growth conditions of the bacteriocin producer strain, *Lactobacillus acidophilus*, the culture supernatant contains 4.9 mg/ml of peptides (Kanatani et al., 1995). This concentration of protein in the culture supernatant was two times higher compared to our strain *L. plantarum* (58). These results are not surprising since it is well known that the culture medium and incubation conditions affect the bacteriocins production in the genus of *Lactobacillus* (Todorov et al., 2005; Babic et al., 2011).

The death rate observed was inversely proportional to the dilutions level. Inhibition was high in the first dilutions where the concentration of peptides was higher than 2.5 mg/ml. The concentrations of peptides 0.31 mg/ml (1/8) dilution produced a middle inhibiting effect near 40% of death. In the last dilution 1/1024 which represents peptides concentration of 2.4 µg/ml, the death rate in 24 h was near 4.9%. Ananou et al. (2007) reported that the addition of enterocin AS-48 had an inhibitory effect on the growth of *Staphylococcus* sp, reducing viable counts below detection limits for the highest bacteriocin concentration tested (40 µg/ml). However the bacteriocin concentration required stopping *Staphylococcus* growth during prolonged incubation was markedly higher compared to the minimal bactericidal concentration value of 15 µg/ml. It is well known that activity of bacteriocin can be influenced by the chemical composition and the physical conditions of food.

In conclusion, the lactic acid bacteria originally isolated from raw goat's milk are probably the best candidates for improving the microbiological safety of dairy product because they are well adapted to the condition of milk and should be more competitive than lactic acid bacteria from other sources. The results from the present study suggest that the bacteriocin-producing strain *L. plantarum* (58) could be used to improve the safety of traditional fermented foods of dairy origin where *Lactobacillus* commonly occurs. The selective use of this bacteriocinogenic strain may improve the microbiological quality of such

foods.

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