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Antimicrobial Activity of *Lactobacillus* Species Isolated from Algerian Raw Goat's Milk Against *Staphylococcus aureus*

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Abstract: 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 08 isolates of lactic acid bacteria obtained from raw goats' milk in western Algeria's areas were identified. The dominant species belonging to the *Lactobacillus* genera are: *Lb. rhamnosus*, *Lb. plantarum*, *Lb. casei*, *Lb. paracasei* subsp. *paracasei*, *Lb. acidophilus*, *Lb. delbrueckii* subsp *lactis*, *Lb. fermentum*, *Lactobacillus paraplantarum* and *Lactobacillus sakei* subsp. *sakei*. The interactions study revealed that three lactobacilli species: Lb. p*lantarum (58)*, *Lb. paracasei* subsp. *paracasei* (*55)* and *Lb. rhamnosus* (68) are able to inhibit *Staphylococcus aureus'* growth. In mixed culture after 24 h, *Lb. plantarum* reduces the growth of *Staphylococcus aureus* by 1.6 log and this latter bacteria was not found after 72h. The various tests used reveal the proteinic nature of the substance which was responsible of the growth inhibition of *Staphylococcus aureus*. The ecological adaptation and growth characteristics of cultures of *Lb. plantarum* in food product will determine their effectiveness as biocontrol 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, biopreservation has become a topic of interest [1]. This technique is used as an alternative to chemical additives for increasing self-life storage and enhancing safety of food by using natural microflora and their antimicrobial products [2]. 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 biopreservation. 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 [3,4].

Bacteriocins are proteinaceous compounds that mainly inhibit closely related species [5]. Some bacteriocins have been shown to possess the ability to inhibit the actions of unrelated genera such as Clostridia, Listeria, enteropathogenic bacteria and gram-negative bacteria. For these reasons bacteriocins are promising candidates for biopreservation of food [6]. Several *Lactobacillus* strains are an important dairy culture starter and used for the manufacture of fermented food [7,8].

The discovery of bacteriocins gave a new way for food development in better hygienic quality [7,8]. 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 [9-11]. 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 microflora was announced by Heikkila and Saris [12]. The technological characterization of the lactic acid bacteria leads to the development of well defined bacterial strains with specific characters. The latter gradually replace the nondefinite mixtures starters traditionally used in dairy industry [13].

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In order to avoid the side effect of chemical preservatives, these last years, the interest of the use of the bacteriocins or strains of lactic acid bacteria for applications as bio-preservative caused many research tasks [4,14-18]. Several lactic acid bacteria bacteriocins offer potential applications in food preservation and 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 [19].

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 of food toxi-infections (*Staphylococcus aureus* ATCC 25923*, Escherichia coli* ATCC 25921 and *Bacillus sp*) 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 during 48h [20]. The selective enumeration of *Staphylococcus aureus* is carried out on Chapman medium at 37°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 of liquid, solid (1.5% agar p/v) or the 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 during 20 min [21]. 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*. [8].

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 48h at 30°C. Blood agar plates were examined for sing of hemolysis. This test was performed in the hospital laboratory [22].

Antimicrobial Activity Detection and Assay: The production of antimicrobial substance by *Lactobacillus sp*. was detected by deferred antagonism as described by [23]. 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 during 24 h at 30°C, the bacterial lawns were examined for zones of inhibition surrounding 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 [24].

Bacteriocin-like activity was assayed by the agar-well diffusion method of Tagg and McGiven [25]. 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 *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus rhamnosus* 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 *Lactobacillus plantarum* three pathogenic bacteria *Staphylococcus aureus* ATCC 25923, *Bacillus sp.* and *Escherichia coli* ATCC 25921 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 *Lactobacillus plantarum.* After incubation at 30°C during 18 h, culture supernatants were obtained by centrifugation at 8000 t/min during 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 [1,24,26].

After the treatment, the reaction mixture was heated during 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 [24].

Assay for Antimicrobial Activity: The search for possible production of inhibiting substances by the isolated bacteria is carried out according to two methods:

Direct method of Tagg and Mc Given [25], Fleming *et al*. [25] and Tahara and Kanatani [27], consists in cultivating the two strains in the same medium with double-layer. The inhibitory activity spectrum was obtained using the agar spot test [28] 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 are used. Subsequently the plates were then overlaid with 6 ml of softagar medium (Muller Hinton) seeded with actively growing cells of the test organisms (or pathogenic strain: *Staphylococcus aureus, Bacillus sp.* and *Escherichia coli),* 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 spots [24,29,30].

The indirect method of Barefoot *et al*. [23] and Shillinger and Lücke [14], makes it possible to put in contact the supernatant of lactic acid bacteria strains that produce antimicrobial substances with the strains test. Producing strains of inhibiting substances were cultivated in liquid medium MRS and incubated during 18 h. After growth, the culture was centrifugalized at 8000 t/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 2M NaOH, so as to rule out inhibition through the production of organic acids. This supernatant was placed into the wells and the medium was sown by the test strains. These wells will receive 100 µl of tested supernatant strains and then incubated during 24 h. Inhibition of growth was determined by an area of inhibition surrounding each agar well [31,32].

Determination of the Spectrum Activity: The direct confrontation method of Shillinger and Lücke [14] and Sookkhee *et al*. [33] was used for the determination of the spectrum activity of antimicrobial substances strains production. The selected producing species belong to the *Lactobacillus plantarum* (58) while the species tests belong to *Staphylococcus aureus*.

Characterization of the Nature of the Inhibiting Agent: The antimicrobial activity of *Lactobacillus 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 a variety of proteolytic enzymes (trypsin and α-chymotrypsin) was assessed in assays as described by Vaughan *et al*. [34] and Vaughan *et al*. [35]. Blank experiments were also performed by no enzyme and inactivated enzyme. gram positive *Staphylococcus aureus* indicator were used in the experiments [22].

Kinetics of Growth and Acidification: Associative growth of *Staphylococcus aureus* and bacteriocin producer strain, *Lactobacillus plantarum* (58), was done in sterilized skim milk at 30°C. The acidity evaluation of the pure strain is carried out by titrimetrical and pH metrical measurement [36]. Each strain is inoculated in 10 ml of sterile skim milk (10% p/v). The pre-culture of *Lactobacillus plantarum* is prepared by incubation at 30°C until coagulation. An amount of 3% of pre-culture is inoculated in 100 ml of skim milk which is immediately homogenized. 10 ml of the mixed culture is distributed in sterile tubes. The growth kinetics and acidification are carried out simultaneously within the regular lengths of time intervals.

The pathogenic strain of *Staphylococcus aureus* (ATCC.25923) is used as test which is inoculated in skim milk at 2% which gives approximately 10^3 cfu/ml, in pure and mixed culture with *Lactobacillus plantarum*. The antimicrobial effect of the latter strain against *Staphylococcus aureus* was measured by microbiological techniques as described by Kaban and Kaya [29]. 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 *Staphylococcus aureus* and *Lactobacillus plantarum* was determined in Chapman and MRS agar media respectively [37].

Measurement of Acid Production: A deduction of 10 ml of the culture is transferred in a conical flask of 100 ml and

5 drops of phenolphthalein indicator (2 mg/ml in ethanol 60°) are added. The acidity is neutralized by NaOH 1/9N until the appearance of a persistent pink color, the volume of the titrating solution was measured and to indicate the producing of acidity which was estimated in dornic degree [38,39].

j-Growth Kinetics Measurement in Pure and Mixed Culture in Milk: The enumeration of *Staphylococcus aureus* is carried out on Chapman medium. Only plates that contain between 30 to 300 colonies are taken into account. The enumeration in mixed culture is done by sowing of 0,1 ml of serial dilutions in two selective acidified MRS media for *Lactobacillus* and Chapman for *Staphylococcus aureus* [29,30,40].

Effect of the Crude Supernatant of Lactobacillus Plantarum: The crude supernatant of *Lactobacillus plantarum* (58) is tested against the growth of *Staphylococcus aureus* in milk. The crude supernatant is obtained by the centrifugation of a culture of 18h in MRS pH 6.8 with 8000 t/min during 10 min. Proteins concentration of the culture supernatant were determined by the micromethod of Bradford [41] using bovine serum albumin fraction as standard. The supernatant is heated at 100°C. The residual activity of the crude supernatant is immediately tested after freezing and neutralization by NaOH. *Staphylococcus aureus* is tested for determining the minimal inhibiting concentrations of this crude supernatant. After incubation with various dilutions, the growth is estimated by reading the optical density at 670 nm [24,42].

RESULTS AND DISCUSSION

A number of 64 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 64 isolates, 11 strains only showed an inhibiting activity. These latter strains were identified to species level by microbiological and biochemical methods, as described by Stiles *et al*. [43]; Klein *et al*. [44] and Carr *et al.* [45].

Characterization of the Isolates: The strains retained give small colonies of approximately 1mm of diameter, lenticular with a white or milky color, smooth surface and a regular circular circumference were observed on solid medium. The microscopic examination reveals that the tested strains were gram positive, with a cellular rod form associated in pairs or in chains (Table 1).

Table 2 and 3 add 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 strain [46]. 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 [43-45] allows to determine the nearest species (Table 3).

Hemolytic Activity: In 08 strains of *Lactobacillus* isolated from the raw goat's milk and used in this work no hemolysis activity was observed. However, absence of haemolytic 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 none virulent [47].

Detection of Inhibitory Activity: All of the 08 *Lactobacillus* species retained were tested for their antimicrobial activity (Table 4, Fig. 1). The zones of inhibition in the spot in the lawn method were more easily visualized. Our results show that all isolated strains (*Lactobacillus plantarum* (01)*, Lactobacillus rhamnosus* (03)*, Lactobacillus acidophilus* (01), *Lactobacillus sakei* subsp. *sakei* (01), *Lactobacillus casei* (01) and *Lactobacillus paracasei* subsp. *paracasei* (01)) have inhibitory effect against *Staphylococcus aureus,* the most inhibiting species was *Lactobacillus plantarum* (58).

The inhibiting activity of *Lactobacillus sp* can have two; 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 [48-50]; whereas the second

Table 1: Morphological characteristics of the lactobacilli isolated from raw goat's milk

Strain's code	Cell's forme	Association type
2	Rod	In diplobacille and chain
7	Short rod	In diplobacille and chain
13	Fine and long rod	In chain and palissade
22	Short rod	In diplobacille and chain
31	Rod	In diplobacille and chain
52	Short rod	In diplobacille and chain
54	Short rod	Diplobacilles
55	Rod	Diplobacilles
55*	Rod	Isolated and in diplobacille
58	Short rod	In diplobacille and chain
68	Short rod	In diplobacille and chain

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

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Lactobacillus paracasei subsp. *paracasei*	55	+	-	-	+/+	-	+	-	+	+	-	-	-	+	+	-	+	+	+	+	-	+	+	+
Lactobacillus paraplantarum	55*	+	-	-	-/	-	+	-	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+
Lactobacillus acidophilus	2	+	-	-	-/+	-	+	-	+	+	+	+	+	-	+	-	+	+	-	+	+	-	+	+
Lactobacillus delbruekii subsp. *lactis*	22	+	-	-	-/+	-	+	+	+	+	+	-	+	-	+	-	+	+	-	+	-	-	+	+
Lactobacillus sakei subsp. *sakei*	31	+	-	-	+	-	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	-	+	+
Lactobacillus rhamnosus	54	+	-	-	+/+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+
Lactobacillus rhamnosus	52	+	-	-	+/+	-	+	+	+	+	+	-	+	+	+	-	+	+	+	+	-	+	+	+
Lactobacillus fermentum	7	+	-	+	-/+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+
Lactobacillus rhamnosus	68	+	-	-	+/+	-	+	+	+	+	+	-	+	+	+	-	+	+	+	+	-	+	+	+
Lactobacillus plantarum	58	+	-	+	+	-	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Lactobacillus casei	13	+	-	-	+	-	+	+	+	+	-	-	+	+	+	-	+	+	+	+	-	+	+	+

1= Strains code, 2 = Gram, 3 = Catalase, 4 = Arginine, 5 = Growth 15/45, 6 = Production Of gaz, 7 = Acétones, 8 = Esculine, 9 = Galactose, 10 = Fructose, 11 = Arabinose, 12 = Raffinose, 13 = Mannose, 14 = Mannitol, 15 = Maltose, 16 = Xylose, 17 = Cellobiose, 18 = Sucrose, 19 = Ribose, 20 = Saccharose, 21 = Mélibiose, 22 = Sorbitol, 23 = Glucose, 24 = Lactose

Table 3: Pre-identification of the isolates and related species of the lactobacilli using phenotypic characteristics

Strains code	Species of *Lactobacillus (Lb.)*	Parentage of authenticity
2	*Lb. acidophilus*	90%
7	*Lb. fermentum*	75%
13	*Lb. casei*	50%
22	*Lb. delbruekii* subsp. lactis	64%
31	*Lb. sakei* subsp. *sakei*	90%
52	*Lb. rhamnosus*	67%
54	*Lb. rhamnosus*	67%
55	*Lb. paracasei* subsp. *paracasei*	64%
55*	*Lb. paraplantarum*	82%
58	*Lb. plantarum*	55%
68	*Lb. rhamnosus*	73%

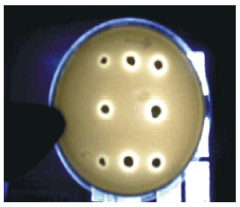
comes from the production of another substance and probably of the bacteriocins [51-53].

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 (08) *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*. [54] reported that the production of H_2O_2 has occurred in aerobic conditions.

The phages can be the origin of bacterial growth inhibitions [55-56]. The test of the phage appeared negative for the strains of *Lactobacillus*.

The synergism effect of the antibacterial action can not exclude the effect of acids [57], hydrogen peroxide [58], diacetyl [59] or bacteriocin-like substances [60] by the strains of *Lactobacillus*.

The Effect of the Proteolytic Enzymes on the Inhibiting Substance: All bacteriocins compounds are of protein nature [61,62]. 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 5 which give the effect of enzyme and heat treatment on the antimicrobial activity of the crude supernatant.



13	68	2
52		55
58	31	54

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

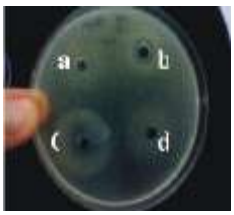


Fig. 2: The action of the proteolytic enzymes and the heat treatment on the antimicrobial activity (inhibition zones) of crude supernatant towards the growth of *Staphylococcus aureus*. (a: α-chymotrypsin, b: crude supernatants, c: crude supernatants with heat treatment at 100°C and d: Trypsin)

The actions of α-chymotrypsin reduce totally the antimicrobial activity of the *Lactobacillus* strain. Whereas, the trypsin enzyme cannot decrease the antimicrobial activity of two *Lactobacillus* strains 52 and 31. Only one *Lactobacillus* strain (58) can resist to the heat treatment at 100°C.

Several bacteriocins of *Lactobacillus* have a large antimicrobial spectrum for gram positive and gram negative bacteria, for example the plantaricin C19 produced by *Lb. plantarum* [63]. The antimicrobial substances produced by lactic acid bacteria strains isolated from the goat's milk were investigated by Klaenhammer [64]. Our results indicated the case of the inhibiting substances produced by *Lb. plantarum* (58)

Table 4: 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

Table 5: 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	-	-	-
55	-	-	-
52	-	+	-
54	-	-	-
31	-	+	-
2	-	-	-
13	-	-	-

strain. During the characterization of the bacteriocins, 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 [65]. The activity of strain supernatant was lost after the treatment with proteolytic enzyme indicating that active component secreted extracellulary was proteinaceous in nature and confirming that growth inhibition of sensitive strain of *Lactobacillus* was caused by bacteriocin [22].

Inhibitory Effect of *Lactobacillus* against *Staphylococcus aureus*: The inhibitory spectrum of culture supernatants of 08 *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 03 strains (*Staphylococcus aureus, Escherichia coli* and *Bacillus* sp.) is shown in (Table 6).

The results obtained in the interaction between the most powerful *Lactobacillus* shows an inhibition of 8 strains (Fig. 3).

***Staphylococcus aureus*:** A strongly inhibition growth of *Staphylococcus aureus* was obtained by *Lb. plantarum* (58). This inhibition is 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*. [66]; Todorov and Dicks [67].

Lactobacillus strains 52, 54, 31, 2 and 13 expressed an inhibition growth of *Staphylococcus aureus,*

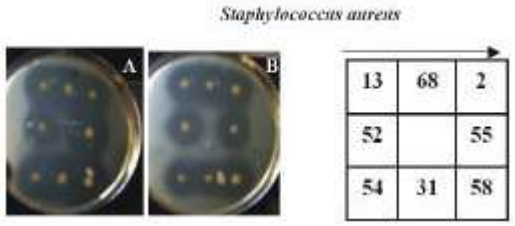


Fig. 3: The inhibiting activity of *Lactobacillus* towards *Staphylococcus aureus* by the appearance of the clear zones around the colonies in none buffered (A) and buffered medium (B)

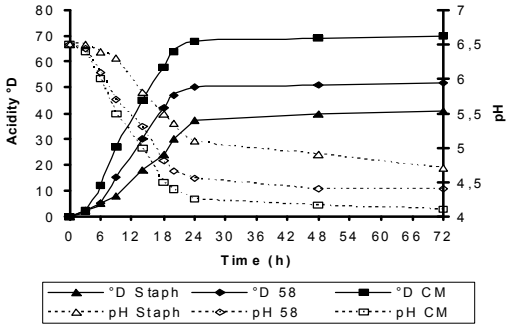


Fig. 4: Acidity (full symbol) and pH (empty symbol) evolution in pure culture and mixed culture of *Lactobacillus plantarum* (58) and *Staphylococcus aureus* in milk

Bacillus sp. and *E. coli* with various diameters but lower than those obtained by strains 58, 55 and 68.

Lactobacillus plantarum strains (58) give an inhibition diameter of 20 mm for *Staphylococcus aureus*, 11 mm for *Bacillus sp.* and 10 mm for *E. coli*. 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 (*Staphylococcus aureus,* 15±2,26 mm and *Bacillus* sp. 11,25±2,25 mm) are sensitive to the inhibiting substances produced by the lactobacilli compared to the negative gram bacteria (*E. coli*, 8,62±1,84 mm). Todorov and Dicks [67] 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 a bacteriocin produced by *Lb. plantarum*, level of K_2HPO_4 were needed to increase bacteriocin production [67]. The growth conditions used in this study are similar to that sited above.

Table 6: Interactions and inhibition spectrum of *Lactobacillus* strains towards food spoilage bacteria and food-born pathogen

	Staphylococcus aureus			*Bacillus sp*			*Escherichia coli*		
Strains	MNT	MT	%	MNT	MT	%	MNT	MT	%
58	28	20	28.5	26	11	57.7	14	10	28.5
68	19	14	26.3	26	13	50	13	10	23
55	21	15	28.5	25	13	48	11	11	0
52	15	15	0	25	14	44	11	6	36
54	25	14	44	24	10	58	16	7	56
31	25	12	52	26	12	53.8	12	7	41
2	16	15	6.25	25	10	60	12	10	16
13	26	15	42.3	23	7	70	11	8	27
Total	175	120	31.42	200	90	55	100	69	31
Average	21.87±4.85	15±2.26	31.41	25±1.06	11.25±2.25	55	12.5±1.77	8.62±1.84	31.04

MNT: medium not buffered, MT: buffered medium

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 exceeds that produced by *Staphylococcus aureus* which was 36.3°D in 24h (Fig. 4). *Lb. plantarum* (58) produced the highest level of lactic acid 50°D in 24h. The other species of *Lactobacillus*, *Lb. rhamnosus* (68) and *Lb. paracasei* subsp. *paracasei* (55) produced 48°D and 43.5°D respectively in 24h.

In mixed culture of *Lactobacillus plantarum* and *Staphylococcus aureus*, a high production of lactic acid (69.4°D) was observed. Whereas, the production of lactic acid decrease in mixed culture with *Lb. rhamnosus* (59.4°D) and *Lb. paracasei* subsp. *paracasei* (47.5°D) is noted.

Kinetic of pH evolution (Fig. 4) showed that *Lb. plantarum* decreased the pH up to 4.32 in 24h whereas the pH of *Lb. rhamnosus* and *Lb. paracasei* subsp. *paracasei* were 4.76 and 4.7 respectively. The final pH of the culture of *Staphylococcus aureus* is 5.13. In the mixed cultures the pH reaches 4.45 in *Lb. paracasei* subsp. *paracasei*, 4.57 in *Lb. rhamnosus* and 4.6 in *Lb. plantarum*.

Lactic acid production in the culture of *Lb. plantarum* and *Lb. brevis* studied by Kask *et al*. [68] and Katina *et al*. [69] was 50°D. These results are in agreement with our strains and in particular with *Lb. plantarum.* The latter produces a quantity of lactic acid higher than 40°D and almost reaches a maximum value of 100°D [69].

The most important quantity of lactic acid production was observed in *Lb. plantarum* (52°D) in pure culture after 72h. Whereas, in mixed culture with *Staphylococcus aureus* the species of *Lactobacillus* produce a higher quantity of acid, which was 71°D in *Lb. plantarum*, fallowed by *Lb. rhamnosus* and *Lb. paracasei* subsp. *paracasei* with 64°D and 62°D respectively. In synthetic medium saturated with glucose, Callewaert and De Vuyst [61] reported that *Lb. reuteri* produces an acidity of 400°D in 24h.

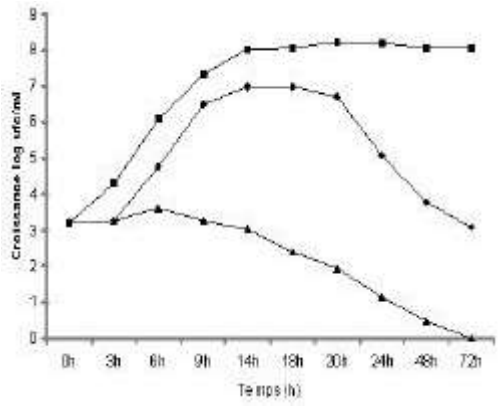


Fig. 5: Kinetics of growth of *Lb. plantarum* (■) and *Staphylococcus aureus* (◆) in pure culture and *Staphylococcus aureus* (▲) in mixed culture in milk

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 *Lb. plantarum* and *S. aureus* can take into account their interactions.

Growth Evolution in Mixed Culture in Milk Medium

Enumeration of *Staphylococcus aureus* in Pure and Mixed Culture with *Lactobacillus* sp.: The initial enumeration of the 3 *Lactobacillus* strains *Lb.plantarum*, *Lb. paracasei* subsp. *paracasei* and *Lb. rhamnosus* was 3.19 log, 3.9 log and 3.55 log respectively. After 24h of

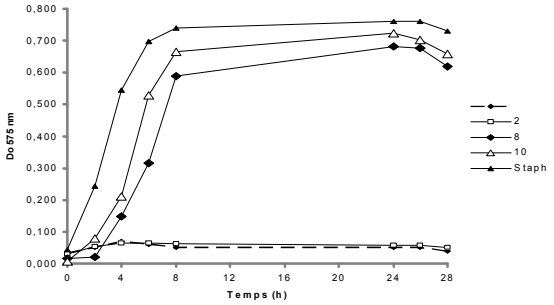


Fig. 6: Effect of various dilutions of crude supernatant of *Lactobacillus plantarum* (58) heated with 100°C towards the growth of *Staphylococcus aureus*

incubation the number of *Lb. plantarum* was 8.19 log cfu/ml and 5.07 log cfu/ml for *Staphylococcus aureus* in pure culture. The number of the latter bacteria increases by 1.85 log cfu/ml after 24h of incubation. In mixed culture after 24h of incubation, a reduction of 1.6 log cfu/ml in the number of *S. aureus*. This reduction proved the inhibiting effect of *Lb. plantarum* (Fig. 5). A decrease of inhibition effect was observed for the other strains of *Lactobacillus* sp. Towards *S. aureus* (results not showed).

After 72 H of incubation, viable cell number of *Lb.plantarum*, *Lb. rhamnosus* and *Lb. paracasei* subsp. *paracasei* reaches 8.05 log cfu/ml, 8 log cfu/ml and 8.04 log cfu/ml respectively. No growth could be detected for *Staphylococcus aureus* after 72h of incubation in the presence of *Lb. plantarum*. The inhibiting activity is slightly weak in the two other *Lactobacillus* species. *rhamnosus* and *paracasei* subsp. *paracasei*. This variation of the inhibiting effect of the species of *Lactobacillus* towards *Staphylococcus aureus* was also observed by Rodriguez *et al*. [70]. Production of multiple bacteriocins by *Lb. plantarum* causes an important inhibition of *Staphylococcus aureus* [71]. Work of Arquès *et al*. [72] showed that after 72h incubation, the number of *Staphylococcus aureus* falls down to 0.46 log cfu/ml.

Effect of Crude Supernatant of *Lb. plantarum* on the Growth of *Staphylococcus aureus*: The influence of *Lb. plantarum* (58) supernatant was tested for evaluating the growth of the test strain of *Staphylococcus aureus* after 6h of incubation (Fig. 6). The results showed clearly the absence of growth of *Staphylococcus aureus* in first dilutions (1/2 and 1/4) in which death rate is higher than 90% (Fig. 6). Whereas, for dilutions (1/256 and 1/1024) death rates are in close proximity to 50%. After 24h of incubation, death rate in the first three dilutions (1/2, 1/4 and 1/8) can reach 95.1, 93.4 and 92.5 respectively compared to the test strain growth. While the death rate decrease considerably in dilutions 1/256 and 1/1024 and reach easily 4% and 4.9% respectively.

In optimal growth conditions of the bacteriocin producer strain, *Lactoacillus acidophilus,* the culture supernatant contain 4.9 mg/ml of peptides [73]. This concentration of protein in the culture supernatant was two times higher compared to our strain *Lactobacillus plantarum* (58). These results are not surprising since it is well known that the culture medium and incubation condition affects the bacteriocins production in the genus of *Lactobacillus* [67].

The death rate observed is inversely proportional to the dilutions level. Inhibition is high in the first dilutions where the concentration of peptides is higher than (2.5 mg/ml). The concentrations of peptides 0.31 mg/ml (1/8) dilution produce a middle inhibiting effect near of 40% of death. In the last dilution 1/1024 which represents peptides concentration of (2.4 µg/ml) the death rate in 24h is near to 4.9%. Ananou *et al*. [37] reported that the addition of enterocin AS-48 has 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 *Staphylucoccus* growth during prolonged incubation were 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 and the results from the present study suggest that the bacteriocin-producing strain *Lactobacillus plantarum*(58) could be used to improve the safety of traditional fermented foods of dairy origin were *Lactobacillus* commonly occur. The selective use of this bacteriocinogenic strain may improve the microbiological quality of such foods.

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