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Antibacterial activity of lactic acid bacteria isolated from raw goat's milk against spoilage and pathogenic bacteria

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ABSTRACT

The antibacterial activity of partially purified bacteriocin produced by lactic acid bacteria isolated from raw goat's milk was assessed and characterized. From thirty isolated strains, the produced bacteriocin by Pediococcus acidilacticci (BL20)and Streptococcus thermophilus (BL16) has manifested a maximum antibacterial activity against the investigated Bacillus cereus and Staphylococcus aureus ATCC 25932. The investigation of the produced bacteriocin by Pediococcus acidilacticci (BL20) indicated their stability after heat treatment, whereas, the activity of the produced bacteriocin by Streptococcus thermophilus (BL16) was decreased after the heating and completely inhibited at 121°C. The maximum antibacterial activity of the both produced bacteriocins was obtained by pH range of 4-7 and affected by the adding of trypsine. The produced bacteriocins by the selected producing bacteria were observed in the early logarithmic growth phase. Furthermore, the investigated produced bacteriocins on the bacterial growth indicated two mechanisms effect, which has manifested a bacteriostatic by Staphylococcus aureus ATCC 25932 and bactericidial by Pediococcus acidilacticci BL 20.The technological properties and the stability of bacteriocins may lead for application as biopreservative agents to control pathogens and spoiling bacteria in different food products.

Keywords: *Streptococcus thermophilus*, *Pediococcus acidilacticci*, *Staphylococcus aureus* atcc 25932, Heat Resistance, Bacteriocins, Bio-Preservative Agents.

INTRODUCTION

Lactic acid bacteria(LAB)are found in a plethora of niches, including plant material, fermented dairy, vegetable and meat products, and sour dough breads. Foods fermented by LAB are rendered more adapted for longer preservation and have improved textures, flavors and tastes [1]. The primary antibacterial effect exerted by LAB is the production of lactic acid and reduction of pH [2]. In addition, LAB produce various antimicrobial compounds, which can be classified as low-molecular-mass(LMM) compounds such as hydrogen peroxide (H_2O_2),carbon dioxide (CO_2), diacetyl (2,3-butanedione),uncharacterized compounds, and high molecular-mass (HMM) compounds like bacteriocins [3-6]. All of which can antagonize the growth of some spoilage and pathogenic bacteria foods[7].
Among HMM compounds, bacteriocins have attracted a great interest in food industry due to their Application al potentiality in food preservation. Bacteriocins ribosomally synthesized were extracellular released bioactive peptides or peptide complexes (usually30-60 aminoacids), which induced a bactericidal or bacteriostatic effect on other (usually closely related) species [8]. In all cases, the producing cell exhibits specific immunity to the action of its own bacteriocin. They are generally considered to act at the cytoplasmic membrane and dissipate the proton motive

force through the formation of pores in the phospholipids bilayer [9]. Nisin was the best defined and the only purified bacteriocin produced by LAB that has been approved for use in food products[10]. The traced aim of the present work was the isolation and the screening of a large range of lactic acid bacteria from raw milk taken from cows and goats, which manifested antagonist activity against pathogenic strains such as *B. cereus, St. aureus ATCC* 25932, *E. coli* and *Ps. aeruginosa* ATCC 27853. Furthermore, the study of bacterial growth of *St. aureus* ATCC 25932, which was responsible nosocomial infections in hospitals, in the absence and in the presence of the produced bacteriocins by *S.thermophilus (BL16)* and *P.acidilactici (BL20)* indicated a considerable biomass reduction accompanied with unbalanced growth.

MATERIALS AND METHODS

Milk sample collection

Thirteen raw goat's milk samples were collected directly from dairy farms of Saida and Sidi Bel Abbes (Algeria). For the sample collection, the udder of goat was washed twice with sterilized water and disinfected with 70% ethanol. Furthermore, a volume of 100 ml of milk from each goat was collected aseptically in sterile bottles. The samples were transported immediately under refrigeration (4°C) to the laboratory for further analysis.

Isolation and identification of lactic acid bacteria

The bacteriocin producing strains were isolated from milk, by the dilution of a volume of 10 ml samples of milk in 90 ml saline solution, and the suitable serial dilutions were plated on the MRS Agar (Fluka, France) [11], incubated at 30°C for 48 hours The typical colonies were isolated and purified. The short-time conservation of the pure isolates has been achieved on a solid culture medium; the obtained culture was maintained at 4°C and renewed every month.

The identification of isolates bacteriocin producing strains used in two steps was performed according to the methods of Badis *et al.*[12]. The first step was based on the coloration of Gram, the presence of the catalase and the production of spores of isolates strains, whereas, the second step was focused on the study of the morphological analysis such as macroscopic and microscopic observation and the type of fermentation. The determination of the physiological and biochemical criteria of the selected producing bacteriocin strains was based on the study of the bacterial growth on the culture medium MRS, containing the following concentration of the Nacl (2, 3, 4 and 6.5%), at pH range (4.2, 6.5, 7, 8), incubated at several temperatures (37, 40, 45, 50°C) for 5 days. Furthermore, the gas production from glucose in MRS culture medium has been achieved according the described method by Thomas [13], whereas, the acetone production from glucose was done by using the Voges-Proskauer test [14]. The profile of sugar fermentation in the API-50 CH was carried out according to the manufacturer's instructions (bioMerieux, Marcy l'Etoile, France).The used bacteria as indicator microorganisms for the selection bacteriocin producing strains and the antibacterial activities were described in Table 1.

Table1 : Bacterial strains used as indicator microorganisms for bacteriocin screening

Microorganisms	Media	optimal growth temperature.
Gram positive bacteria		
Bacillus cereus		30 °C ,18-24 hours
Staphylococcus aureus ATCC 25932	Nutrient agar	30 °C ,18-24 hours
Gram négative bacteria		
Escherichia coli		30 °C ,18-24 hours
Pseudomonas aeruginosa ATCC 27853		30 °C ,18-24 hours

Screening of isolates for antibacterial activity

The antagonistic activity of the bacterial isolates against all investigated pathogenic bacteria was determined by well diffusion method [15]. For the assay of the antagonistic activity of the selected producing bacteriocin strains, the bacterial biomass of the overnight cultures on MRS culture medium was harvested by centrifugation at 15000 g, for 10 minutes at 4°C. The effect of lactic acid, the pH as antimicrobial compounds, has been reduced by the adjustment of pH-value of the supernatants of the culture medium to 6.0 with of a solution of 1 N NaOH, filtered across cellulose acetate filter (0.2 µm). For this purpose, a volume of 100 µl of each overnight grown culture in a nutrient broth was inoculated, spread on the solid Muller-Hinton culture medium with the help of spreader. A sterile cork borer of diameter 3.0 mm was used to bore wells in center of inoculated solid culture medium of Muller-Hinton. Subsequently, a volume 100 µl of culture supernatant of the selected producing bacteriocin strains was introduced in well. After that, plates were kept at 4°C for 2 hours and then incubated at 37°C for 24 hours. The antagonistic activity was determined by measuring the diameter of the formed zone of inhibition around the wells. The bacterial isolate showing the widest zone of inhibition against the target microorganism was selected for further studies.

Partial purification of bacteriocin

The selected producing bacteriocin strains with a highest antagonistic activity was inoculated overnight in the MRS culture medium, incubated at 37°C for 24 hours and harvested by centrifugation at 5000 g for 10 minutes at 4°C. The recuperated supernatant was used for the partial purification of bacteriocin. Furthermore, the produced bacteriocin in the supernatant culture medium was extracted according the described methods by Joshi [15]**.**

For this purpose, different concentrations of ammonium sulfate were added to the crude extract. After that, the supernatant was stirred on a magnetic stirrer and kept at 4 °C overnight under agitation. The obtained precipitates were collected by centrifugation at 10000 g for 10 min and resuspended in a solution of 20 mmol sodium phosphate buffer, adjusted at pH-value of 6.0.The effect of the obtained precipitates on the antibacterial activity was recorded by comparison with the crude bacteriocin [15].

Characterization of bacteriocin

Effect of temperature

In order to explore the effect of temperature on the extracted bactériocin, a volume of 5 ml of supernatant was introduced in different test tubes and heated at the following temperature (60, 80, 100°C)for one minute, the reaction was stopped by heating at 121°C for 20 minutes under pressure. The heat-treated bacteriocin samples were employed for antimicrobial activity assay as described earlier [16].

Effect of pH

In order to investigate the effect of pH value on the extracted bacteriocin a volume of 5 ml of supernatant was introduced in different test tubes and the pH-value was adjusted from 2 to 10, by the using 1 NaOH or 1 Hcl solution, incubated at 37°C for 90 min and the bacteriocinogenic activity was assayed against *B. cereus* and *St.aureus* as described earlier [16].

Effect of proteolytic enzyme

The effect of the proteolytic enzyme activity on the extracted bacteriocin has been investigated by the incubation of supernatant containing bacteriocin, in the presence of Trypsine at a final concentration of 1 mg/ml, incubated at 37°C for 1h. Enzyme reaction was stopped by heating at 65°C for 10 mn [17, 18] and the antimicrobial activity assay as described earlier [16].

Bacterial growth and bacteriocin production

In order to study the bacterial growth of the selected producing bacteriocin lactic acid, a volume of 10 ml of the inoculated overnight culture was introduced in tube, containing 100ml of MRS broth, incubated at 30°C. After that, samples were withdrawn from the culture and used for determination for the optical density at 660 nm, extracellular pH, and determination of the antibacterial activity [19].

Study of *Staphylococcus aureus* ATCC growth in the presence of the produced bacteriocins

In order to explore the mode of action of the produced bacteriocins by *S. thermophiles* (BL16), *P. acidilactici* (BL20), against *St. aureus* ATCC 25932, the viability of the treated cells has been investigated. The bacterial growth of *St.aureus*ATCC25932in the absence (control) and in the presence of the produced bacteriocins by *S. thermophiles* (BL16), *P.acidilactici* (BL20), has been assayed.For this purpose, *St. aureus*ATCC25932 was inoculated in the MRS culture medium with initial optical density of 0.5 at 630 nm according the protocol described by Abbouni and coworkers [26, 27].A volume of 1 ml of the recovered of the produced bacteriocins by *S. thermophiles* (BL16), *P. acidilactici*(BL20), was added 4 hours after the onset of the bacterial growth. The obtained results (Figure 5, 6)showed a considerable inhibition of the growth *St.aureus*ATCC25932, after the adding a volume 1 ml of the produced bacteriocins by *S.thermophilus*(BL16), *P. acidilactici*(BL20), to the culture medium of the above tested strain during early exponential growth phase, which was explained the absence of the phenomenon of bacterial lysis in the presence of the produced bacteriocin*S.thermophilus*(BL16) and bacterial lysis in the presence of the produced bacteriocin by *P. acidilactici*(BL20).

RESULTS AND DISCUSSION

Antibacterial activity

Based on the achieved morphological tests, thirty isolated LAB strains from Algerian dairy milk were identified as belonging to six groups : (1)white, round or lenticular colonies; cocci, diplococcic and in chain cells; thermophilics and homofermentative (Presumptive *S. thermophilus*); (2)white round or lenticular colonies; cocci, diplococcic and in chains; mesophilics and homofermentative (presumptive lactococci); (3) small white colonies with maroon convex center; rolled up or filamentous long rods, alone or in chains; homofermentative (presumptive thermophilic lactobacilli); (4) small round or lenticular white colonies, small rods in chains; homo or heterofermentative and arginine-positive (presumptive mesophilic lactobacilli); (5) transparent colonies; very small round cocci and in oval

chains; mesophilic; heterofermentative (presumptive leuconostocs) and (6)smooth, round, grayish or whitish colonies; cocci in tetrads and homofermentative (presumptive *Pediococci*).

The thirty isolates were screened for their antagonistic activity against pathogenic bacteria such as *B. cereus, St.aureus* ATCC 25932, *E.coli* and *Ps. aeruginosa* ATCC 27853by the using well diffusion method. Eight isolates has manifested an excellent antagonistic activity against the tested Gram positive (Figure 1). Furthermore, the isolates did not show any antagonistic activity against the investigated Gram negative bacteria such as *E. coli* and *Ps. aeruginosa 27853*. Similar results have been obtained by *S. thermophilus T2*, isolated from dairy product of Algerian Raib [12].Subsequently, all isolates manifested antagonistic activity with diameter of inhibition zone greater than 8 mm were used for further studies such bacterial growth at different temperatures, pH values, salt condition, gas production from glucose and sugar fermentation profile. The obtained results from the previous screening showed that *P.acidilacticci (BL20)* and *S.thermophilus* (BL16) (Table 2) has manifested an excellent antagonistic activity against *B.cereus* CIP 6624, *B.subtilis* ATCC 6633, *E. coli* CIP 35218. The both isolated, selected strains were used for further investigation.

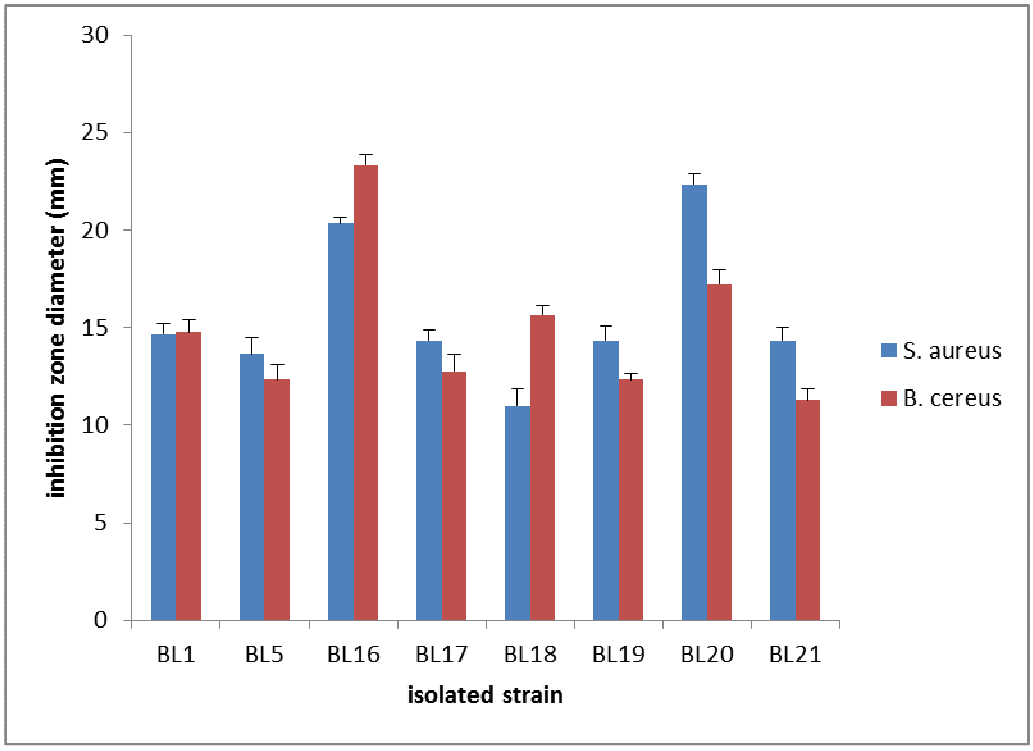

Figure 1: The obtained diameter of zone inhibition by *St. aureus ATCC 25932* and *B. cereus* in the presence the isolated, selected antagonistic lactic acid bacteria

Partial purification of bacteriocin

The extracted bacteriocin of *P. acidilacticci* (BL20) and *S.thermophilus* (BL16) from the supernatant of the used MRS culture medium was partial purified with ammonium sulphate. The obtained results have indicated that the antagonistic activity bacteria against pathogenic bacteria has been considerably augmented. The highest antagonistic activity of *P.acidilacticci* (BL20) and *S.thermophilus* (BL16) was obtained by the precipitation of the bacteriocin with (30–40%) of ammonium sulphate saturation (Figure 2)**,** where the diameter of inhibition was increased from12 to 23 mm. Joshi and co-workers has reported that the produced bacteriocin by *Lactobacillus*, isolated from carrot fermentation, was precipitated from cell free supernatant by the using (20- 30 %) ammonium sulphate saturation [15].

Characterization of bacteriocin

Effect of temperature

The obtained results showed that the antagonistic activity of the produced bacteriocin by *S.thermophilus* (BL16) against *Staphylococcus aureus* ATCC25932 was stable after heat treatment at a temperature of 60°C for 10 minutes (Figure 3),whereas, the activity has decreased after the treatment at a temperature of 80°C for 10min. Furthermore, the antagonistic activity has decreased drastically after heating at 100°C and 121°C for20 minutes. The obtained results of the antagonistic activity of the produced bacteriocin are similar to the effect to thermophilin T produced by *S.thermophilus*[21].

The antagonistic activity of the produced bacteriocin by *P.acidilactici* (BL20) against *St.aureus*ATCC25932 was decreased after treatment at (60, 80,100°C) for 10 min, where the diameters of inhibition zones were 18.5±0.95mm, 15±0.45mm, 12.4±0.57mm respectively. Similar results have been reported for pediocin produced by *Pediococcus CAPEA* [22].

Table 2: Illustration of the physiological and biochemical characteristics of the isolated, selected antagonistic strains

Physiological and biochemical characteristics	Isolated strains	
	BL16	BL20
Production of gaz	+	+
Growth at : 37°C	+	+
40°C	+	+
45°C	+	+
50°C	+	+
Growth at pH : 4.2	-	+
6.5	+	+
7	NT	+
8	NT	+
Growth at different concentration of NaCl:		
2 %	+	+
3 %	+	+
4 %	-	+
6.5%	-	+
VP	-	+
NO_3	-	-
ORTP	-	-
ADH	-	+
ESC	-	-
Sugar Fermentation		
Glucose	+	+
Arabinose	+	+
Mannose	-	+
Manitol	-	-
Maltose	-	-

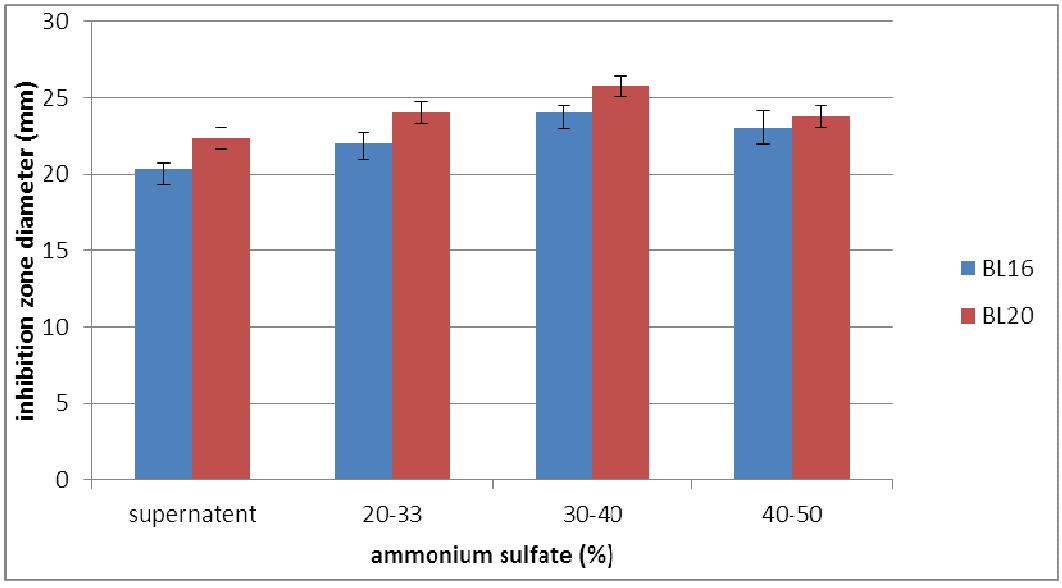

Figure 2: Display of the antagonistic activity of the produced bacteriocin by *P. acidilacticci* (BL20) and *S.thermophilus* (BL16), purified by (30–40%) ammonium sulphate saturation

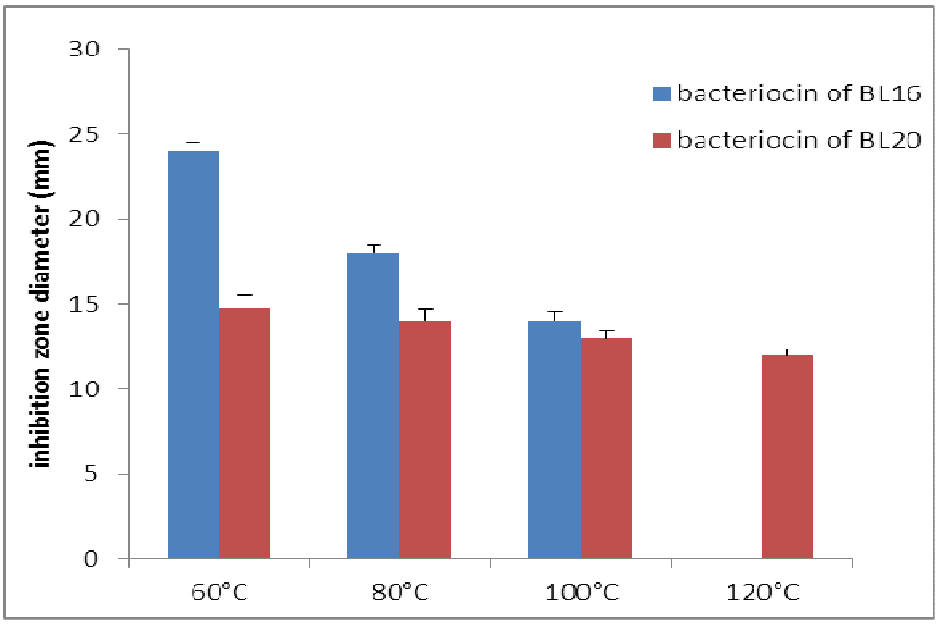

Figure 3: Effect of temperature on the antagonistic activity of the produced bacteriocins by *S.thermophilus (BL16)* and *P.acidilactici (BL20)* against *St. aureus ATCC 25932*

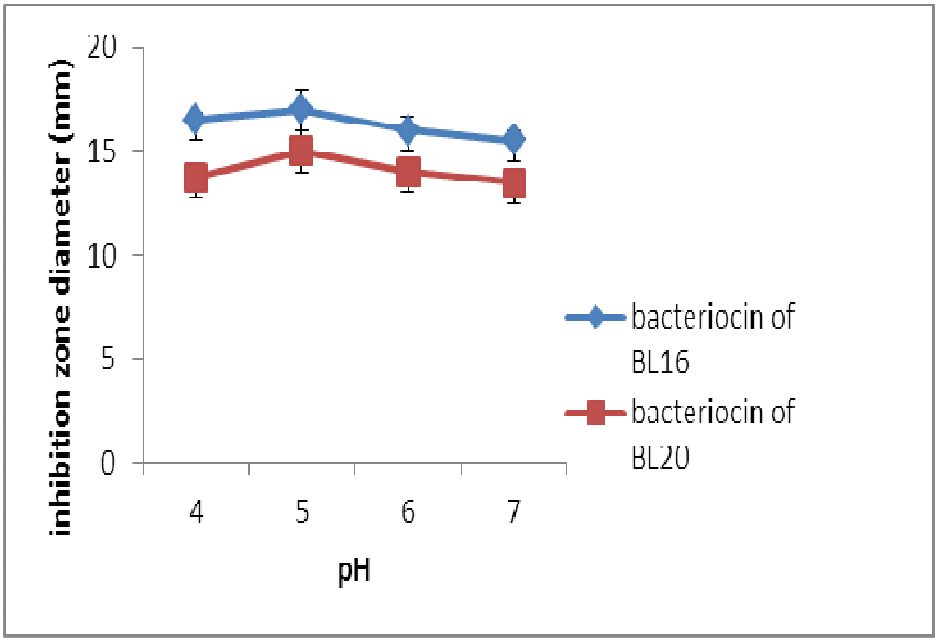

Figure 4: Effect of pH on antagonistic activity of produced bacteriocinsby *S.thermophilus*(BL16) and *P.acidilactici*(BL20) against *St.aureus ATCC25932*.

Effect of the pH value on the Antagonistic activity

The obtained results showed a considerable reduction of the antagonistic activity of the produced bacteriocin of *S.thermophilus*(BL16) against *St. aureus* ATCC25932 at pH values of 4 and 7. Furthermore, the pH optimum for antagonistic activity of the produced bacteriocin was at pH values between5 and 6(Figure 4).However, the antagonistic activity of the produced bacteriocin by *P.acidilactci* (BL20) against *St.aureus*ATCC25932 was stable at pH value between 4-7. Similar results have been reported for bacteriocin produced by *L lactis D*53**,** *Pediococcussp*[23, 24].

Effect of proteolytic enzymes

The obtained results showed a considerable loss of the antagonistic activity against *B.cereus* and *St. aureus* ATCC 25932 after treatment with trypsin. The sensitivity of the produced bacteriocin by *S.thermophilus* (BL16) trypsin

indicated their protein nature. The sensitivity of the analyzed substances to the treatment with proteolytic enzymes confirmed their protein nature.

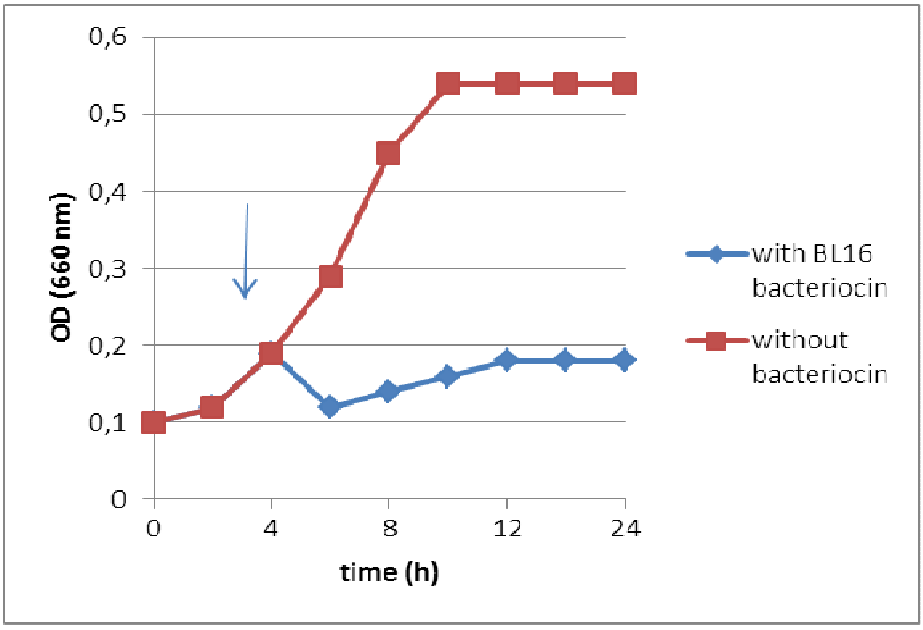

Figure 5: The mode of action of the produced bacteriocin by *S. thermophilus*(BL16) on the growth of *St. aureus* ATCC 25932

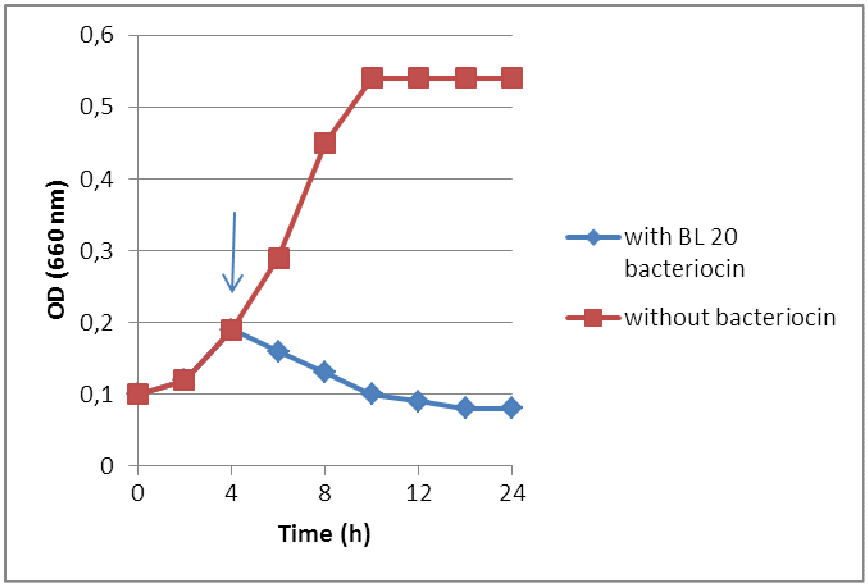

Figure 6: The mode of action of the produced bacteriocin by *P.acidilactici* (BL20) on the growth of *St. aureus* ATCC 25932

Study of *Staphylococcus aureus* ATCC growth in the presence of the produced bacteriocins

In analogy to S.*thermophilus* (BL16), a maximum antagonistic activity of *P.acidilactici* (BL20) was observed at the beginning of the stationary phase followed by a constant level as was the case with thermophilin ST-1, where the production was directly related to the growth phase, the highest concentrations were achieved in the early stationary phase [21]. The early studies on cells of *P. pentosaceus* at logarithmic and stationary phase showed that the production of pediocin is very high during the stationary phase. This physiological behavior is observed in other bacteriocins such as nisin and ACCEL pediocin [25].

In conclusion, the produced bacteriocins by *S.thermophilus*(BL16), *P.acidilactici* (BL20), has induced unbalanced growth and furthermore the arrest of the cell cycle *St.aureus*ATCC25932, in comparison with the untreated biomass (balanced growth).In Previously studies, Bhunia and co-workers [28] has reported that the mode of action of

pediocin AcH by produced from *P.acidilactici H* against *L. plantarum NCDO 955* manifested a considerable reduction the bacterial biomass after the adding of the purified bacteriocin.

Application of bactériocins in food preservation may beneficial in several aspects [29,30], to decrease the risks of food poisoning, to decrease cross contamination in food chain and to improve shelf life of foods products, food protection during temperature abuse episodes; decrease economic losses due to food spoilage. Therefore, there may also be a potential market for bacteriocins as natural substitutes for chemical preservatives and in the preservation of functional foods and nutraceuticals [31].

CONCLUSION

The obtained results in this study showed that the produced bacteriocin from *S. thermophiles* and *P. acidilactici*, isolated from Algerian raw goat's milk manifested a wide spectrum of antagonistic activity against *St. aureus ATCC 25932*.

The technological properties indicated that the produced bacteriocins possess some important potential to use as biopreservative agents to control pathogens and spoiling bacteria in food products as such or in combination with other preservation methods. Since the lactic acid fermentation is employed mostly for development of products, especially for enhancer flavor and taste of the fermented products. Furthermore, it would be of interest to find out which functional group is responsible for the antagonistic activity of the produced bacteriocin from *S. thermophiles* and *P. acidilactici* and also whether any of them is a novel compound with antagonistic activity which would make it a promising candidate for the production of new antimicrobials.

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