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Isolation and Identification of Lactic Acid Bacteria from Algerian Goat's Milk and Their Major Technological Traits

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Abstract : Bacteriocins produced by lactic acid bacteria offer potential as tools for ensuring food safety and quality. The current research aimed to identify and characterize a bacteriocin-like substance produced by *Pidiococcus pentocaseus* sp. isolated from Algerian goat's milk. The production of this antimicrobial during the stationary growth phase, destruction of antimicrobial activity by proteolytic enzymes indicated that the antimicrobial is a bacteriocin. The bacteriocin proved to be active against gram positive bacteria like isolates of *Staphylococcus aureus*, and was heat labile as the antimicrobial activity was destroyed by heating after 120°C for 15 min. Bacteriocin activity was stable after three months of storage at 4°C and -20°C, for 75 min of exposure to UV light and at pH values between 2.0 and 6.0. This bacteriocin was also found to lyse sensitive cells. Results revealed that an increase in bacteriocins activity of the strain against *Staphylococcus aureus* on increasing the concentration of NaCl and KCl up to 5% and in the presence of spices 5%. Results presented here support the idea that the bacteriocin may propose some industrial advantage that renders it as a good natural food biopreservative candidate.
Keywords : *Pidiococcus pentocaseus* sp., *Staphylococcus aureus*, bacteriocin-like substance, physicochemical characterization, food ingredients.

1. Introduction

Bacteriocins are generally recognized as "natural" compounds able to influence the safety and quality of foods (1). They are highly specific antibacterial proteins produced by strains of bacteria active mainly against some other strains of the same or related species (2). The bacteriocins produced by LAB are potent biopreservative agents and the applications of these in food are currently the subject of extensive research.Some studies of characterization of bacteriocins show that these molecules can be active under certain ranges of temperature and pH. Sensibility to proteolytic enzymes evidences the proteinaceous characteristic of bacteriocins (3). Thermal processing is used extensively within the food manufacturing process and can have adverse effects on the bio-active capability of a bacteriocin, potentially rendering it less effective. The chemical and physical properties of a food, e.g. pH, and fat content, can also have a significant role in the suitability of a particular bacteriocin. Some food ingredients might interfere with the bacteriocin activity. An other study indicated that the use of lactocin 705 to control *L. monocytogenes* was less effective in the presence of curing ingredients such as sodium chloride, sodium nitrite, ascorbic acid, alginate and sodium lactate (4, 5, 6). Regarding the application of bacteriocin-producing starters trains in food fermentation, the major problem is related to the in situ antimicrobial efficacy which can be negatively influenced by various factors, such as binding of the bacteriocins to food components (fat or protein particles) and food additives (e.g. triglyceride oils), inactivation by proteasesor other inhibitors, changes in solubility and charge, changes in the cell envelope of the target bacteria (7, 8, 9).The search for new bacteriocins with wider spectrum of activity and compatibility

with different food system is being studied by some investigators. The aim of the current study was to select bacteriocin producing LAB from such products in order to use these proteinaceous inhibitors to improve the microbial quality and safety of foods. The effect of some environmental parameters on the level of bacteriocin activity is evaluated for the purpose to obtaining better and stable bacteriocin activity.

2. Materials and methods

2.1. Culture and media

Pc. pentosaceus sp. was isolated in the Applied Microbiology Research Laboratory, Oran University and was maintained on MRS medium (10, 11). The pathogenic culture *L. ivanovii* was isolated from different clinical specimens (Oran Hospital, Algeria) and it found to be the most suitable indicator for the quantification of antimicrobial effects of the bacteriocin investigated in both agar and broth system. All the raw material for isolation were procured from the local market and stored at 4°C until the time of use. The reagents used in the study were of analytical grade and procured from Merck, Germany.

2.2. Morphological and biochemical Identification of bacterial strains

The isolate was then subjected for morphological and biochemical characterization using conventional methods and the isolates were identified up to species level (12).

2.3. Antimicrobial Activity Detection and Assay

The production of antimicrobial substance was detected using the spot agar method. An overnight culture of the *Pc. pentosaceus* sp. strain was spotted onto the surface of an MRS (Merck, Germany) plate. Spotted plates were overlaid with a second layer of MRS Agar. The plates were incubated at 37°C for 48h and then overlaid with active growing sensitive cells of *S. aureus* (10^8 CFU/mL), imbedded in a thin layer (7mL) of soft MRS (MRS with 7.5% (w/v) agar). After anaerobic incubation during 24h at 30°C, the bacterial lawn was 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 2mm or larger (13).

2.4. Bacteriocin-like substance production and extraction

Antimicrobial substance production was carried out in 250 mL Erlenmeyer flasks each containing 100 mL medium MRS (maltose (2%); yeast extract (0.05%); meat extract (0.1%) and with 2% inoculum at 37°C, pH 7.5 and 100 rpm for 18 hrs. For extraction of t, the fermentation broth was centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was filtered through pre-sterilized 0.22 µm filters (Merck, Germany) and pH was neutralized using 0.1N NaOH (14). This crude bacteriocin was further used for estimating the extension of shelf life of food products.

2.5. Characterization of bacteriocin-like substance

In all tests, the filter sterilized CFS subjected to the different parameters was tested for their antimicrobial activity against *S. aureus* using the ODM (Optical Density Measure) method at 600 nm (15).Untreated bacteriocin-containing CFSs of the producer strain were inoculated with the same indicator strain served as control. Selected enzymes including trypsin, pepsin, and papain were dissolved in 40 mM Tris-HCl (pH 8.2), 0.002 M HCl (pH 7), and 0.05 M sodium phosphate (pH 7.0) respectivelyto a final concentration of 0.1 mg/mL. Other enzymes such as lipase and α- amylase were dissolved in 0.1 M potassium phosphate (pH 6.0), and 0.1 M potassium phosphate (pH 7.0) respectively to a final concentration of 0.1 mg/mL. Equal aliquots of filter sterilized CFS of test strain and each enzyme solution were mixed, incubated at 37°C for each enzyme for 2 hours and heated in boiling water for 5 min to inactivate the enzymes. These sample mixtures and the control (CFSs without enzyme treatment) were inoculated with the indicator strain as previously mentioned and tested for antimicrobial activity by the ODM (16).

2.6. Detection of bacteriocin activity during growth (growth kinetics)

MRSm broth was inoculated with 2% (v/v) of an overnight pre-culture of the test strain (*Pc. pentosaceus* sp.) and incubated at 37°C, where changes in $O.D_{600}$was recorded every 2 h. The growth kinetics experiment (17) was performed with a minor modification. The indicator strain (10^8 CFU/mL) was grown at 37°C in MRS broth in the presence of the test strain CFS. Optical density measurements were recorded every 2

h for 12 h. The bacteriocin activity was expressed by the percentage of growth reduction to the indicator strain and determined from the ratio between the optical densities of the treated cultures and untreated ones (the indicator strain without the CFS). This ODM method was used in all the antimicrobial assays.

2.7. Physical and biochemical characterization of bacteriocin-like substance

2.7.1. The influence of heat, pH, and UV on bacteriocin activity

The thermal stability of crude bacteriocin preparations was assessed by exposing the CFSs to different temperatures (18) ranging from 0°C to 121°C (0°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 90°C, 100°C and (121°C and 15 Lbs) for 15 minutes before being tested for their antimicrobial activity. The effect of pH on the activity of bacteriocin was tested by adjusting CFSs from 2 to 12 (at increment of one pH unit) with sterile 1 N NaOH or 1N HCl (19) after 1hour of incubation at room temperature (25°C), the samples were tested for antimicrobial activity by the ODM. Similarly, sterile petri dishes containing aliquots of 10 mL crude bacteriocin preparations were exposed to the UV irradiation (Philips bulb, wavelength 340 nm, 220-240 V, 50 Hz,) situated at a distance of 30 cm (20, 21). Times of exposure to UV light ranged from 15 to 75 min. After each time interval, bacteriocin activity was estimated to UV light by ODM as previously stated together with unexposed bacteriocin-containing CFSs that served as the experimental controls.

2.7.2. The influence of NaCl, KCl, and spices on bacteriocin activity

Different concentrations of inorganic salts such as NaCl and KCl (0, 0.5, 1, 3 and 5 % w/v) were examined for their inhibitory effect on bacteriocin preparations (2). Equal aliquots of both filters sterilized CFSs of each test strain and each salt solution were mixed, inoculated with the indicator strains as previously mentioned. Spices used (green pepper, red pepper, garlic, and pimon) as local food additives were also studied for their possibility of influencing the effectiveness of bacteriocin activity (22). Each spice was dissolved in 10 mL of sterile warm distilled water, vortexed for 5 min, followed by centrifugation, filter-sterilization, and mixed for 2 h with the CFSs to get a final concentration of 0.5, 1, and 3% (v/v). Salts and spices-treated preparations were assayed for antimicrobial activity as previously described.

2.8. Stability during storage

The method devised by (21) was used to study the stability of bacteriocin preparations during different storage conditions. The crude bacteriocin was stored at –20ºC and +4ºC for different interval of time (15, 30, 45, 60, and 90 days). Samples were taken from the stored material to determine the bacteriocin activity as previously mentioned. Bacteriocin-containing CFSs of producer strain that was not subjected to storage, and inoculated with the same indicator strain served as control.

2.9. Efficacy of protective lactic acid bacteria in growth control of S. aureus on the goat's milk

Commercial goat's milk was heat treated at 90°C for 15 min and was inoculated with overnight-grown culture of *Pc. pentocaseus* sp. at 2 mL/100 mL, *S. aureus* at 10^8 CFU/mL was also simultaneously added to the milk. Fermentation was carried out at 37°C for 12 h and the change in the number of viable cells of *S. aureus* was measured at 2 h intervals. To determine the number of the indicator strain, a 1 mL aliquot of the sample was withdrawn and immediately chilled on ice, diluted and plated onto (NA) agar. The plates were incubated at 37°C for 48 h and the colonies developed were counted (23).

Statistical analysis

Data were expressed as mean ± standard deviation. Statistical significance was determined using one-way analysis of variance on the replicates, where a p-value of ≤ 0.05 was considered significant.

3. Results

The selection of isolate strain was based on the display of high bacteriocin activity and their potential of inhibiting the growth of the food-borne pathogen *S. aureus* using the agar spot method. The average diameter of the inhibition zone ranged from 1-14 mm in size (24).

The isolate obtained in this study was considered LAB based on their positive Gram reaction, non-motility, absence of catalase activity, spore formation, and the rod or coccal shape (data not shown), while their

carbohydrate fermentation profiles, as determined with API50 CH test strips, showed a high similarity (Table. 1). Identifications made by the API database indicated that the isolate was a strain of *Pc. pentosaceus* sp.

The antimicrobial substance produced by *Pc. pentosaceus* sp. was inactivated to the tested enzymes and displayed a higher growth reduction potential against *S. aureus* (Table. 2). However, since the activity of the filtrate was not completely inhibited, it is possible that the bacteriocin may also be bound toother molecule/s like a lipid or a carbohydrate moiety. These data clearly show that the antimicrobial substance was of a proteinaceous nature.

Table 2: Effect of enzymes treatment (1 mg/mL) on bacteriocin activity against *S. aureus*. Results are expressed as % of means values of growth reduction (n= 3) ± standard deviations

Enzymes	Enzyme concentration 1 mg/mL
Papain	15±0.2
Pepsin	45±0.2
Trypsin	80±0.5
α- amylase	40±0.3
Lipase	62±0.2

During growth the peak of bacteriocin production occurred in the late logarithmic phase and reached its maximum during the stationary phase (Do. 0.2 growth reduction of the indicator strain) after 12 h at 37°C. Incubation of *S. aureus* (10^8 bacteria per mL) in the presence of bacteriocin (1 µg mL^{-1}) at 37°C resulted in 90% killing, indicating that the bacteriocin has a bactericidal effect (Fig. 1).

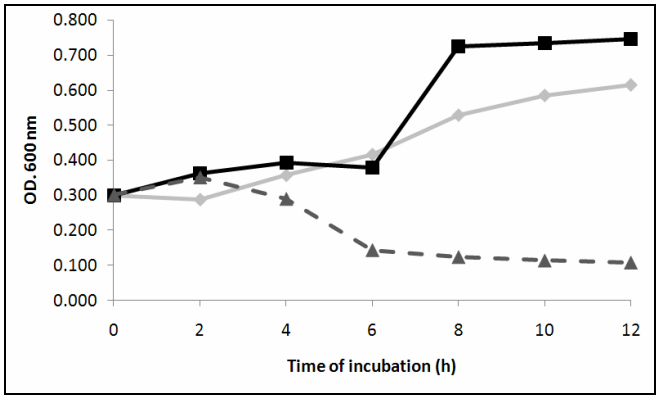

Figure 1: Growth kinetics of *Pc. pentosaceus* sp., Growth kinetics of *S. aureus*. *S. aureus* (10^8 CFU/mL) were grown at 37°C in MRS broth for 12 h in the presence of the test strain CFS.

The effects of heat and pH were determined. Bacteriocin of *Pc. pentosaceus* sp. (Table. 3) was considered to be extremely heat stable as antibacterial activity was not altered by heat treatment after 15 min at 121°C. The pH stability was studied in the range of pH 2–12. It was observed that this bacteriocin was active at pH values from 2 to 12. pH adjustment to 6.5 was chosen to eliminate the possible effect of organic acids.

Table 3: Effect of heat and pH on bacteriocin activity produced by *Pc. pentosaceus* sp. against the indicator strain *S. aureus*. Results are expressed as % of means values of growth reduction (n= 3) ± standard deviations

Temperature °C	**30**	**60**	**90**	**120**
Bacteriocin of *Pc. pentosaceus* sp.	40± 0.2	56± 0.2	73± 0.3	80± 0.2
pH	**3**	**6**	**9**	**12**
Bacteriocin of *Pc. pentosaceus* sp.	50± 0.5	50± 0.1	64± 0.2	52± 0.3

The influence of UV light on bacteriocin activity was studied. It was observed that bacteriocin produced by *Pc. pentosaceus* sp. was completely destroyed after 75 minutes exposure to UV light.

Table 4: Effect of UV light on bacteriocin activity produced by *Pc. pentosaceus* sp. against the indicator strain *S. aureus*. Results are expressed as % of means values of growth reduction (n= 3) ± standard deviations

Time of exposure (min)	Bacteriocin of *Pc. pentosaceus* sp.
15	4.60 ± 0.2
30	4.96 ± 0.2
45	4.64 ± 0.4
60	4.64 ± 0.3
75	4.24 ± 0.2

Table 1: Morphological, physiological and biochemical properties of LAB isolate (*Pc. pentosaceus* sp.)

Tests		Isolate/ *Pc. pentosaceus* sp.
Morphology		**White colonies 1-2 mm**
Growth at temperature °C	10	-
	15	nd
	30	+
	37	+
	45	+
Growth at pH	3.5	+
	4.5	+
	5.5	+
	6.5	+
	7.5	+
	8.5	+
	9.5	+
Growth at NaCl %	4	+
	6.5	+
	10	nd
	15	-
CO_2 from glucose		-
ADH		+
Thermoresistance at 60°C/ 30min at 45°C		+
Fermentation Type		Ho
Methylene blue (BM 1%) at 42°C		+
Citrate		+
Fructose		+
Mannane		+
Maltose		+
Trehalose		+
Manose		+
Xylitol		+
Melibiose		+
Palmitine		+
Raffinose		+
Xylose		+
Methyl-D-glucose		+
Inositol		-
Sorbitol		+
N- Acetylglucosamine		+
Mannitol		+
Galactose		+

Legend: Growth (+), no growth (-), homofermentation (Ho).

Different concentrations of NaCl and KCl were selected to examine the effect of inorganic salts on the activity of the CFSs of *Pc. pentosaceus* sp. against the indicator strain used after 2 hours of exposure (Table. 4). There was an increase in bacteriocin activity on the indicator strain with the increase in the concentrations of NaCl and KCl up to 5%.

The spices used in this experiment w/v (red pepper, black pepper, garlic, and pimon) in concentrations ranging from 0.5% affected the bacteriocin activity differently. The addition of 0.5% spices solution to the bacteriocin resulted in a significant activity against the indicator strain (up to 90% reduction of growth). Storage of the active compounds at +4°C for three months and in a frozen state -20°C did not affect the antibacterial activity (Table. 5).

Table 5: Effect of inorganic salts and spices on bacteriocin activity produced by *Pc. pentosaceus* sp. against the indicator strain *S. aureus*. Results are expressed as % of means values of growth reduction (n= 3) ± standard deviations

Inorganic salts	**NaCl %**			
	0.5	**1**	**3**	**5**
Bacteriocin of *Pc. pentosaceus* sp.	84.4±0.7	84.8±1.6	95.9±1.3	94.3±3.0
Spices (0.03 mg/mL)	Red pepper		Green pepper	
Bacteriocin of *Pc. pentosaceus* sp.	80.64 ± 0.5		60.61 ± 0.5	
Inorganic salts	**KCl %**			
	0.5	**1**	**3**	**5**
Bacteriocin of *Pc. pentosaceus* sp.	83.5±1.5	92.2±0.6	91.5±1.2	64.0±2.0
Spices (0.03 mg/mL)	Garlic		Pimon	
Bacteriocin of *Pc. pentosaceus* sp.	51.96 ± 0.3		60.61 ± 0.2	

Table 6: Effect of time and temperature of storage on bacteriocin activity produced by *Pc. pentosaceus* sp. against the indicator strain *S. aureus*. Results are expressed as % of means values of growth reduction (n= 3) ± standard deviations.

Time of storage (days)	**Bacteriocin of *Pc. pentosaceus* sp.**	
	-20°C	**4°C**
15	50 ± 0.2	80 ± 0.1
30	50 ± 0.2	80 ± 0.2
45	55 ± 0.3	78 ± 0.2
60	48 ± 0.4	70 ± 0.1
90	46 ± 0.4	56 ± 0.1

Bacteriocin produced by *Pc. pentosaceus* sp. resulted in rapid inactivation of *S. aureus* from an initial population of 10^8 CFU mL^{-1} to a resistant population of 10^3-10^4 CFU mL^{-1}, when both strains were grown in association in the goat milk, the number of *S. aureus* gradually decreased and became undetectable at 12 h at 37°C. *Pc. pentosaceus* sp. appeared to be a suitable starter culture for preservation of the goat milk (Fig. 02).

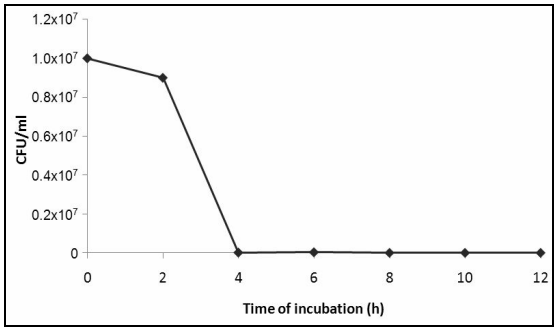

Figure 2: Reduction in population of *S. aureus* in goat milk with the addition of *Pc. pentosaceus* sp.) as starter at 37°C.

4. Discussion

The aim of the current investigation was to isolate and identify bacteriocin-producing LAB from goat milk that could be used locally for improving food biopreservation and biosafety. Choice was based on its broad antimicrobial spectrum against the food-borne pathogen *S. aureus*. The antimicrobial compound was inactivated by proteolytic enzymes, indicating then to be bacteriocin-like substance according to (25, 26). Moreover, the slight bacteriocin inactivated by lipase and α- amylase might be an indication that a lipids or carbohydrate components are involved in the antimicrobial activity. Similar results were obtained by (27, 28, 2, 29).

Maximum bacteriocin activity was marked at the stationary growth phase, which suggests that the antimicrobial peptide is a secondary metabolite (30, 31, 28, 32).

The heat stability of bacteriocin discaused here indicates that it could be used as biopreservative in combination with thermal processing to preserve the food products (pasteurization, drying, and freezing).

Our pH and temperature results were consistent with those reported by (27, 5, 32, 33). *Pc. pentosaceus* sp. Bacteriocin was destroyed after exposure to UV light; these resultsconfirmed the proteins status of the bacteriocin (21). The interaction between some food ingredients such as inorganic salts, spices, and our bacteriocin can suggest their synergistic effect when added with specific concentrations in foods (22, 34, 2).

The preservation capacity of the bacteriocin in terms of the periods and temperature of storage was quite interesting, as it maintaining full stability for tree month at 4°C and -20°C, indicating that the stability of bacteriocin to different conditions reflects that such compounds can withstand the conditions normally encountered in food processing so would remain effective during processing (27, 8).

Contradictory results were mentioned by (35, 36, 23). The bacteriocin from isolate *Pc. pentosaceus* sp. was also tested for preservative effect against *S. aureus* in the goat milk. Maximum reduction of target microorganism population of 90% was observed; the results indicate that bacteriocin possessed several desirable characteristics of a biopreservative. Similar results were obtained by *Lactobacillus* sp. and *Bacillus cereus* in juice (27), *Lactococcus lactis* LBII and *L. monocytogenes* in the pastaurized milk (37), *Lactococcus lactis*W8 and *L. monocytogenes* in fermented milk (Dahi) (23). The bactericidal mode of action of our bacteriocin determined by the TEM may probably be due to pore formation (38) as typically reported for LAB bacteriocins (39, 40).

5. Conclusion

The study revealed that bacteriocin from *Pc. pentosaceus* sp. isolated from algerian goat milk possesses a spectrum of inhibitory activity against the food–borne pathogen *S. aureus.* Therefore, it has a potential for application as a biopreservative in the dairy milk products. Since lactic acid fermentation is employed mostly for development of products, especially for flavor and taste of the fermented products, for enhancing the microbial quality and safety of processed foods.

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