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The antibacterial activity of bacteriocin produced by *Lactobacillus acidophilus* isolates against sensitive reference strain *Lactobacillus acidophilus* R0052 and its stability to different pH, heating and storage temperatures

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Summary

Two isolates of *Lactobacillus acidophilus* were isolated from locally homemade soft cheese and identified in accordance with their main features as described by bergeys manual of determinative bacteriology .Agar well diffusion bioassay was used for the evaluation of antimicrobial activity of cell free supernatant (culture filtrates) of *Lactobacillus acidophilus* isolates against the sensitive strain *Lactobacillus acidophilus* R0052 and the diameter of the inhibition zone was proportional to the bacteriocin concentration. The antimicrobial activity of the bacteriocin was proportional to the reciprocal of the highest dilution factor producing a detectable zone of inhibition . The storage temperature of the crude bacteriocin had a significant($P< 0.01$) effect on its potency against the sensitive strain .The potential of the antimicrobial activities of the crude bacteriocin that was stored for 10 days at refrigeration temperature (4°C) was significantly ($P< 0.01$) higher than that stored at room temperature. The antimicrobial effectiveness of the crude bacteriocin was heat stable and retaining 100% of its activity after its exposure to 60 °C & 80°C for 10 minutes .Boiling of the crude bacteriocin for 30 minutes had significantly ($P< 0.01$) decreased its potency to 0% .The potency of the crude bacteriocin was stable after four hours of exposure to both neutral (pH7) and acidic (pH4) conditions but was inactivated at alkaline (pH9) condition.

الفعالية التثبيطية للبكترويسن المنتج من عزلات بكتريا*Lactobacillus acidophilus* ضدالبكتريا القياسية الحساسة *Lactobacillus acidophilus R0052* وثبوتيته خلال مختلف قيم الاس الهدروجيني ودرجات حرارة التسخين والخزن

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الخلاصة

عزلت عزلتان من بكتريا *Lactobacillus acidophilus* من الجبن الطري المحلي وشخصت هذه البكتريا وذلك بمطابقة مميزاتها الرئيسة مع التي ذكرت في دليل Bergy′s لعلم البكتريا . واستعمل الاختبار الاحيائي للانتشار في الحفر عبر الاكار في تقييم الفعالية التثبيطية لراشح عزلات بكتريا *Lactobacillus acidophilus* ضد سلالة *Lactobacillus acidophilus* R0052 القياسية الحساسة للبكتروسين وكان قطر منطقة التثبيط متناسبا طرديا مع الفعالية التثبيطية للبكتروسين حيث ان الفعالية التثبيطية كانت متناسبة مع مقلوب اعلى عامل تخفيف تعطي منطقة تثبيط واضحة . كانت درجة حرارة خزن البكتروسين الخام مؤثرة وبصورة معنوية وعلى مستوى ($P< 0.01$) في قوته الفعالة ضد السلالة الحساسة حيث ان شدة الفعالية التثبيطية للبكتروسين الخام الذي خزن لمدة (10) ايام عند درجة حرارة الثلاجة اعلى وبمستوى معنوية ($P< 0.01$) من البكتروسين الذي خزن على درجة حرارة الغرفة ولمدة نفسها. كانت الفعالية التثبيطية للبكتروسين الخام مستقرة حراريا واحتفظ على (100%) من قوته الفعالة بعد تعريضة للتسخين عند درجة حرارة (60 م°) و (80 م°) لمدة 10 دقائق . كان غليان البكتروسين الخام لمدة 30 دقيقة له تاثير كبير من خلال خفض قوته الفعالة الى (0%) وبصورة معنوية بمستوى ($P< 0.01$) . كانت شدة البكتروسين الخام الفعالة مستقرة بعد (4) ساعات من التعرض للظروف المتعادلة (pH7) والحامضية (pH4) وكانت غير فعالة عند التعرض للظروف القاعدية (pH9) .

Introduction

Some strains of *L. acidophilus* are commercially used in many dairy products and considered to have probiotic characteristics, besides that occur naturally in the human and animal gastrointestinal tract, mouth, and vagina (1). Bacteriocins are bacterial peptides that inhibit or kill microorganisms that are usually, closely related to the producer strain (2).

Bacteriocins consist of a biologically active protein moiety, have a bactericidal mode of action and attach to specific cell receptor. Both Gram negative and Gram positive bacteria produce bacteriocins, the Gram –negative bacteriocins are Colicins, which produced by strain of *E.coli* (3). Most of the Gram - positive bacteriocins are membrane active compounds that increase the permeability of cytoplasmic membrane (4). Bacteriocins are of great importance to human as they can play a considerable role in food preservation and human therapy and they can be used as an alternative or replacement to various antibiotics (5) , this can limit the use of antibiotics and thus reduce the development of antibiotic resistance (6).The main objective of this study was to isolate *L .acidophilus* from raw dairy products and studying the antibacterial spectrum of the bacteriocin and its stability to different pH, heating and storage temperatures .

Materials and Methods

ten grams portion from the surface and the core of each soft cheese sample were extracted aseptically and added to 90 ml of sterile MRS broth versus aqueous 2% Sodium citrate (Dual - purpose medium) enrichment medium which pre warmed to 40°C and homogenized for 5 min in a stomacher , and then the homogenates were incubated at 37 °C for 24- 48 hrs, then the enriched broth was serially diluted prior to being pour plated on MRS agar. Isolation of *lactobacillus acidophilus* by plating serial decimal dilutions on the MRS agar at pH 6.8 using peptone water 0.1 % (vol /vol) as a diluents was performed . The identification of the *lactobacillus acidophilus* was performed according to their morphological cultural and biochemical characteristics using standard biochemical methods (7) and Bergeys manual of determinative bacteriology (8) . All the isolates of *lactobacillus acidophilus* , confirmed through the biochemical tests and compared with the results of standard strain *lactobacillus acidophilus* R0052 that procured from the School of Animal Sciences ,Louisiana state university (USA) (a freeze dried powder imported from Rosell institute Montreal, Canada). The crude bacteriocins were obtained from the bacteriocin producing strain Lactobacillus *acidophilus* which was grown in MRS broth under anaerobic condition at 37 °C for 24 hrs and the supernatant fluid was separated from cells by centrifugation at 10,000 rpm for 20 minutes. The supernatant was collected and pH adjusted to 7 with sterile 1N NaOH so as to rule out inhibition through production of organic acids and then filtered through a syringe filter with pore size of 0.45 µm , then heating for 5 minutes at 70 °C to kill cells and prevent inactivation of antibacterial peptides by protease and then stored at 4 °C in a refrigerator . Inhibitory activity of crude bacteriocin against sensitive strain was assayed according to (9). The cell free supernatant (CFS) of isolates grown in MRS broth for 24 hrs was exposed to various heat treatments (5ml of culture supernatant was heated for 10 min at 60 °C , 80 °C and 100 °C and for 30 & 60 min at 100 °C in water bath and cooled rapidly) . The agar well diffusion method was performed to detect residual activity. The sensitivity of the crude bacteriocin to different pH values was estimated by adjusted the pH of the culture filtrate to 4 , 7 , and 9 with sterile 1N NaOH then kept for 4 hours at room temperature and the antimicrobial activity was then determined by the agar well diffusion method and the original culture supernatant was used as a control sample. The culture supernatants were stored at 4 °C for 21 days and the stability of the antimicrobial activity under shelf life condition was assessed at different refrigeration storage intervals of 1, 2, 7, 10, 14 and 21 days .Three replications were conducted for each of the above experimental condition.

Results

Two isolates of *lactobacillus acidophilus* were isolated from five samples of locally produced homemade soft cheese that were manufactured from raw milk. The two isolates were identified as Gram positive long rods and arranged in pairs or chains .The macroscopic appearance of the colonies was with creamy or beige pigments and little sticky in consistency with smooth surface large size (1-2 mm). The bacterial isolates tested were unable to produce

Catalase ,Oxidase and Gelatinase enzyemes. *L. acidophilus* isolates were unable to grow at the incubation temperature of 15°C but were capable of growing well at 45°C. Results of salt tolerance of *L .acidophilus* isolates revealed that no growth was observed after incubation period in either 4% or 6.5% of Sodium chloride. Carbohydrates such as lactose , Glucose , Xylose , Sucrose , Maltose , Galactose and Fructose were fermented with acid production while Mannitol , Ribose and Raffinose were not fermented by *Lactobacillus acidophilus* isolates . The antimicrobial spectrum exhibited by the crude bacteriocin of *L .acidophilus* isolates against the sensitive strain *L .acidophilus* R0052 is shown in Table 1. The average diameter of the inhibition zone of crude bacteriocin that was produced by *L .acidophilus* isolates NO. 1 against the sensitive strain *L .acidophilus* R0052 was 12 mm while that produced by *L .acidophilus* isolate NO.2 against the same sensitive strain was 10mm .

Table 1- The antimicrobial spectrum by the undiluted crude bacteriocin *of Lactobacillus acidophilus* isolates against the sensitive strain L. *acidophilus* R0052:

Samples	Isolates	Sensitive strains	Bacteriocin activity	Inhibition Zone diameter(mm) Mean± S.E
Soft cheese	*lactobacillus acidophilus* NO 1	*Lactobacillus acidophilus* R0052	(+Ve)	12 a ±0.04
	lactobacillus acidophilus NO 2	*Lactobacillus acidophilus* R0052	(+Ve)	10 b ±0.02

Different letters in a column revealed significant differences (P <0.05) between the diameters of the inhibition zone. SE = Standard error. (+Ve) = Positive result (with inhibition Zone)

The antimicrobial spectrum exhibited by both the crude and the diluted bacteriocins of *L .acidophilus* isolate NO.1 against the sensitive strain *L .acidophilus* R0052 is shown in table 2 . The average diameter of the inhibition zone of the crude bacteriocin that was produced by *L .acidophilus* isolate NO.1 against sensitive strain *Lactobacillus acidophilus* R0052 was 12mm .

Table 2- The antimicrobial spectrum by the crude and diluted bacteriocin of *L .acidophilus* isolate NO.1 against the sensitive strain *L. acidophilus* R0052.

lactobacillus acidophilus Isolate	Dilution	sensitive strains	Bacteriocin activity	Inhibition Zone diameter (mm)	AU/ ml ±S.E
Lactobacillus acidophilus NO 1	Crude bacteriocin	*Lactobacillus acidophilus* R0052	(+Ve)	12	-
	Serial two fold				
	1/2	*Lactobacillus acidophilus* R0052	(+Ve)	11	40 a ± 1.4
	1/4	*Lactobacillus acidophilus* R0052	(+Ve)	10	80 b ± 1.2
	1/8	*Lactobacillus acidophilus* R0052	(+Ve)	8	160 c ± 1.12
	1/16	*Lactobacillus acidophilus* R0052	(-Ve)	0	0 d ± 0.0

by the same letter Values in a column not followed differ significantly (P< 0.05)
AU= Arbitrary Unit; 8 = Reciprocal of the highest dilution (1/8); 20 = 1000 µl /50 µl (conversion factor).
(-Ve) = Negative result (no Inhibition Zone). (+Ve) = Positive result (with inhibition Zone).

The average diameter values of the inhibition zone (mm) of supernatant fluids that were stored at either refrigeration temperature (4°C) or room temperature (22-25°C) against

sensitive strain *Lactobacillus acidophilus* R0052 over the six time points of 1,2,7,10,14 and 21 days are shown in Table 3. There was a significant (P<0.01) decrease in the potency (66.6%) of the crude bacteriocin after 10 days of storage at room temperature. The highest potency (100%) at which the diameter value of inhibition zone was 12 mm was observed after 2 days of storage at both the refrigeration and room temperatures while the lowest potency (91.6% and 66.6%) at which diameter values of the inhibition zone were 11mm and 8mm was observed after 10 days of storage respectively. The antimicrobial potency of the crude bacteriocin was lost (0%) after14 days of storage at both the refrigeration and room temperatures. The average diameter values of the inhibition zone (mm) of supernatant fluids that were exposed to different heating temperatures for different periods of time against the sensitive strain *L .acidophilus* R0052. The antimicrobial activities of the crude bacteriocin that was obtained from isolate NO.1 against sensitive strain *L .acidophilus* R0052 were not affected (Heat stable) and 100% of its potency was retained by exposing to heating temperatures of 60°C & 80°C for 10 minutes while 91.6% of its potency was retained by boiling (100°C) for 10 minutes .The potency of the same crude bacteriocin was lost (0%) after exposing to heating temperature of 100 °C for 30 and 60 minutes .Boiling of the crude bacteriocin for 30 minutes or more had a significant (P < 0.01) effect by decreasing its potency to 0% Table 4.

Table 3 - Effect of both refrigeration & room storage temperatures on the activity of crude bacteriocin of *L. acidophilus* isolate NO.1 against sensitive strain *L .acidophilus* R0002

Storage intervals (days)	Refrigeration storage 4°C		Room storage temperature 25°C	
	Inhibition zone diameter(mm) mean ±SE	% of potency	Inhibition zone diameter(mm) mean ±SE	% of potency
1	12±0.04	100 A	12±0.04	100 A
2	12±0.04	100 A	12±0.04	100 A
7	11±0.03	91.6 A	10±0.02	83.3 B
10	11±0.03	91.6 A	8±0.01	66.6 C
14	0±0.0	0 b	0±0.0	0 D
21	0±0.0	0 b	0±0.0	0 D

Table 4- Effect of different heating temperatures for different time of exposure on the antimicrobial activity of crude bacteriocin of *L.acidophilus* isolate NO.1 against sensitive strain *Lactobacillus acidophilus* R0052:

Heating temp (°C)	Period of Heating (min)	Sensitivity	Inhibition Zone diameter(mm) Mean± S.E	% of potency
60	10	Resistance	12±0.04	100 a
80	10	Resistance	12±0.04	100 a
100	10	Resistance	11±0.03	91.6 a
100	30	Sensitive	0± 0.0	0 b
100	60	Sensitive	0± 0.0	0 b

Percentages of potency in a column not followed by the same letter differ significantly (P <0.01) . 0mm= Negative result (no inhibition zone).
Sensitive = Loss of bacteriocin activity
Resistance = No change in bacteriocin activity.

The average diameter values of inhibition zone (mm) of crude bacteriocin that were exposed to different pH values for four hours at room temperature against strain *L. acidophilus* R0052 are shown in Table 5 . In accordance with present data the potency of the crude bacteriocin was highly affected by the exposure to an alkalin condition (pH9) and

slightly affected by the exposure to an acidic condition (pH4) with a significant (P< 0.01) decrease in its potency to 0% (inactive) and 83.33% respectively .The potency of crude bacteriocin was stable (100%)at neutral (pH7) after four hours of exposure Table 5 .

Table 5- Potency of crude bacteriocin of *L .acidophilus* isolate NO.1 at different pH values against the sensitive strain *Lactobacillus acidophilus* R0052 :

pH values at 25°C ±1	Sensitivity	Inhibition Zone diameter(mm) Mean± S.E	% of potency
4	Resistance	10±0.02	83.33 b
7	Resistance	12±0.04	100 a
9	Sensitive	0± 0.0	0 c

Percentages of potency in a column not followed by the same letter differ significantly (P <0.01) .
Resistance = No change in bacteriocin activity
Sensitive = Loss of bacteriocin activity.
0mm= Negative result (no inhibition zone).

Discussion

Two isolates of *lactobacillus acidophilus* were isolated from five samples of locally produced homemade soft cheese that were manufactured from raw milk. This is in agreement with other scientific study by Mathara *et al* (10) who isolated *L. acidophilus* from fermented dairy products . All cultural characteristics and biochemical reactions of *L. acidophilus* isolates were in accordance with the main features described in Bergeys manual of determinative Bacteriology (7). All the results of the bacterial isolates were confirmed as *L . acidophilus* by comparing them with the results of *lactobacillus acidophilus* R0052 strain. Most methods of measuring bacteriocin activity rely on using a sensitive bacterium (indicator) and determining the degree of inhibition of this indicator by the medium containing the bacteriocin . The determined action of bacteriocin activity by agar well diffusion bioassay is similar to the technique for measuring the antibiotic sensitivity or potency.

The crude bacteriocin that was produced by *L .acidophilus* isolate NO.1 exhibited significantly (P < 0.05) the highest antimicrobial activity and effectiveness against the sensitive strain compared to that produced by *L .acidophilus* isolate NO. 2 (Table 1). Bacteriocins from LAB are proteinaceous compounds which have inhibitory effect against closely related species and other Gram- positive bacteria (11).

Bacteriocin forms the pores in the membrane of sensitive cells and deplete the trans-membrane potential and /or the pH gradient resulting in the leakage of cellular materials (12) .Vinderola *et al* (13) reported an important inhibitory activity of culture filtrates of *Lactobacillus acidophilus* obtained from milk on the growth of lactic acid starter. The crude bacteriocin to be tested was diluted a twofold dilution series. There was a significant (P< 0.05) decrease in the antimicrobial effectiveness of the bacteriocin as the dilution factor increased (Table 2) , this means that the diameter of the inhibition zone was proportional to the bacteriocin concentration. The highest dilution factor generated an inhibition zone of 8mm which indicated the strength of the antimicrobial action against the sensitive strain was 1/8 while the dilution factor that did not bring about a clear inhibition against the same sensitive strain was 1/16 .

The bacteriocin activity was therefore proportional to the reciprocal of the highest dilution factor producing a detectable zone of inhibition. Bcateriocin activity was calculated by (Arbitrary units) AU /ml . Highest dilution(last serial) that showed a distinct zone of inhibition x 20 (1000µl \ 50 ml). The potential of the antimicrobial activities of the crude bacteriocin that was stored at refrigeration temperature were significantly (P<0.01) higher than that stored at room temperature (Table 3). There was a significant (P<0.01) decrease in the potency (66.6%) of the crude bacteriocin after 10 days of storage at room temperature which may be due to the probable action of proteolytic enzymes which were present in the supernatant fluid in accordance with earlier mentioned reports (14) . The storage temperature of the crude bacteriocin had a significant (P<0.01) effect on its potency against the sensitive

strain *Lactobacillus acidophilus* R0052. Roberfroid ,(15)reported that *Lactobacillus plantarum* bacteriocin antimicrobial activity in supernatant was stable and no decrease in its activity was detected after 5 days of storage at 25 °C and was not lost by long term storage at 4 °C.

Supernatant fluids that were exposed to different heating temperatures for different periods of time against the sensitive strain *L .acidophilus* R0052 (Table 4) . were chosen based on their usual processing operation of food. Heat sensitivity of this bacteriocin could be attributed to their non- complex and linear structures .The heat stability could be attributed to the formation of small globular structures and occurrence of strongly hydrophobic regions , stable cross- linkages and high glycine content. In general heat stability is an advantage temperature stability being a very important parameter if a bacteriocin is to be used as a food preservative because many processing procedures involve a heating step. (16). The potency of the crude bacteriocin was highly affected by the exposure to an alkalin condition . The potency of crude bacteriocin was stable (100%) at neutral (pH7) after four hours of exposure (Table 5).Most of the bacteriocin produced by lactic acid bacteria were stable at acidic and neutral pH and were inactivated at a pH above 8 (16).

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