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Bioassay activity of crude bacteriocin produced by *Lactobacillus acidophilus* isolate against cold stressed *E.coli O157:H7*

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Summary

The laboratory studies of the cultural isolation revealed that two isolates of Lactobacillus acidophilus were isolated from five soft cheese samples that were manufactured locally according to the farmers production procedure .Colonies of *E.coli* O157:H7 were isolated from raw milk samples by a conventional direct plating on selective enrichment CT-SMAC agar and tested serologically for the presence of O157 and H7 antigens using the commercial available latex agglutination kits .The results revealed that *E.coli* O157:H7 was resist to the crude bacteriocin that was extracted from *L .acidophilus* isolate NO.1 due to the nature of their cell wall, while when the outer membrane of *E.coli* O157:H7 was injured by cooling at 4°C for 6 hours , the permeation of bacteriocin to the cytoplasmic membrane was facilitated and caused inactivation and death of such pathogenic organism .The results revealed that no growth of *E.coli* O157:H7 with no visible turbidity in the nutrient broth with bacteriocin that diluted to 1\2 ,1\4 ,1\8 were observed .Bacteriocin that diluted to 1\8 had been recognized as the minimum inhibitory concentration of the bacteriocin.

Both the dilution factor of bacteriocin and time exposure to bacteriocin at refrigeration storage temperature of milk had a significant ($P<0.05$) influence on its antimicrobial potency against the viability of stressed *E.coli* O157:H7 from minutes 30 to 120 .The viability of *E.coli* O157:H7 subjected to bacteriocin that diluted 10 times(1:10) in both raw and sterile milk was efficiently eliminated after 90 minutes and 60 minutes of exposure at refrigeration temperature respectively.

الاختبار الاحيائي لفعالية البكتروسين المنتج من عزلة *L. acidophilus* ضد بكتريا الايشيريشيا القولونية O157:H7 المجهدة بالتبريد

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الخلاصة

اظهرت الدراســـة المختبرية للعزل الجرثومي الى عزل عزلتين من بكتريـــــــــــا *L .acidophillus* من مجموع (5)عينات من الجبن الطري الذي صنع طبقا لطرائق الانتاج المحلية المتبعة من قبل الفلاحين عزلت مستعمرات الايشيريشيا القولونية O157:H7 من عينات الحليب الخام باستخدام الطرائق التقليدية للزرع المباشر على الوسط الاغنائي الانتقائي السوربيتول ماكونكي(TC-SMAC) واجريت عليها الفحوصات المصلية بحثا عن المستضد الجسمي O157 والسوطي H7 باستعمال العدة التجارية لاختبار تلازن اللاتكس السريع. اظهرت النتائج بان بكتريا الايشيريشيا القولونية النزفية O157:H7 التي عزلت من الحليب الخام ابدت مقاومتها للبكتروسين المنتج من عزلات بكتريا *L acidophilus*.بسبب طبيعة جدار خلاياها بينما عندما تعرض الغشاء الخارجي للبكتريا نفسها الى جهد التبريد عند درجة حرارة (4م°) ولمدة (6) ساعات سمح للبكتروسين من النفاذ الى الغشاء البلازمي وسبب موت هذا المسبب المرضي . اشارت النتائج الى عدم ظهور اي نمو مع عدم وجود اي عكرة مرئية لبكتريا الايشيريشيا القولونية O157:H7 في داخل المرق المغذي المدعم بالبكتروسين المخفف الى 1\2 و 1\4 و1\8 . تم التعرف على اقل تركيز للبكتروسين (MIC)والمثبط لنمو بكتريا الايشيريشيا القولونية O157:H7هو عندما خفف الى 1\8 الذي ادى الى عدم ظهور عكرة مرئية بعد الحضن لمدة18 ساعة عند درجة حرارة (37م°). كان لكل من عامل التخفيف للبكتروسين وعامل المدة الزمنية للتعرض للبكتروسين خلال خزن الحليب بالثلاجة (4م°) تاثيرا معنويا وعلى مستوى ($P<0.05$) على قوته الفعاله لتثبيط بكتريا الايشيريشيا القولونية O157:H7 المجهدة بالتبريد خلال 30 الى 120دقيقة لوحظ ازالة حيويتها ونموها وبشكل كفوءعند تعرضها لفعل البكتروسين المخفف 10 مرات داخل عينات الحليب الخام والمعقم بعد مرور مدة (90) و(60) دقيقة من خزنها بالثلاجة على التوالي.

Introduction

E.coli O157:H7 was first recognized as a human pathogen in 1982 when two outbreaks of haemorrhagic colitis (HC) in Michigan and Oregon (USA) were associated with the

consumption of under cooked ground beef that had been contaminated with such organism (1).Since then ,this pathogen has caused many food borne outbreaks of Haemorragiccolitis (HC) and Haemolytic Ureamic Syndrome (HUS) worldwide (2) .Human beings and cattle carry the pathogen in their intestines and their faeces are therefore considered as a source of contamination of foods ,water and environment .The faeces may contaminate udders and milking equipment's and get into milk during milking and handling under inadequate hygiene practices(2) .Most of food borne infections associated with this pathogen included consumption of raw milk (3).Various modifications of agar diffusion assays as well as test procedures in liquid media were widely used and partly standardized for testing the activity of antimicrobial agents (4).The use of lactic acid bacteria (LAB) especially the isolates from dairy products such as *L . acidophilus* and \or their antagonistic metabolism such as bacteriocin is an example of biopreservation (5). The use of friend microorganisms or their natural products is preferred to chemical in the field of food preservation due to their antagonistic activity against many food borne pathogens and spoilage bacteria (6). Bacteriocins from *Lactobacillus acidophilus* are known to be bactericidal to closely related gram positive bacteria and few reports are available on their activity against gram negative microorganisms (7) Bacteriocins bactericidal mode of action usually acting on the bacterial cytoplasmic membrane .(8) A large numbers of studies were published in recent decades on bacteriocins against gram positive bacteria but few researches concerning LAB bacteriocins against gram negative bacteria and this is the main reason for this study to highlight its effect against *E.coli* O157:H7. The main objectives of this study were to isolate *L .acidophillus* from locally homemade soft cheese and to evaluate the antimicrobial potency of crude bacteriocin and its minimum inhibitory concentration against the survival of both unstressed and cold stressed *E.coli* O157:H7, beside that studying its effectiveness in both raw and sterile milk on the survival of *E.coli* O157:H7 at refrigeration storage.

Materials and Methods

The antimicrobial activity of bacteriocin against indicator organism was determined using a well diffusion assay after subjecting *E.coli* O *157: H7* to a stress condition at low refrigeration temperature (4°C). Enterohaemorrhagic *E.coli* O *157: H7* was isolated and identified from raw milk samples after 24 hours of aerobic incubation at 37°C on Cefixine – Tellurite Sorbitol MacConky agar (CT-SMCA) .Five identified colonies of *E.coli* O *157: H7* were selected and subcultured onto nutrient agar streak to obtain pure colonies by incubating at 37 °C for overnight then five colonies inoculated directly in 10 ml of sterile nutrient broth . The inoculated nutrient broth was incubated aerobically at 37 °C for 24 hours. *E.coli* O *157: H7* in the cultured nutrient broth was subjected to a cold stress by cooling it to 4°C at refrigeration storage over the five time points of 1,2,4,6, and 24 hours. Injured cells of *E.coli* O *157 H7* due to cold stress were determined by inoculating a loopful of cultured nutrient broth in the non-selective nutrient agar at 1,2,4,6 and 24 hours of refrigeration storage of cultured nutrient broth. Bacteriocin activity bioassay by spectrophotometer Growth (biomass) was measured by determining its turbidity in terms of OD (optical density) at 600 nm by spectrophotometer according to the manufacturer instructions (Optima sp 300 , Japan). Effect of bacteriocin on stressed *E.coli O157:H7* in a liquid medium was determined by deducing the killing or growth inhibition of indicator organism at various dilutions of crude bacteriocin which were added to indicator organism (10^6 cfu/ml) which inoculated in sterile nutrient broth and incubated overnight at 37 °C. Indicator cells without bacteriocin were used as an experimental control. Raw and sterile milk samples that were screened to be free from *E coli O157:H7* contamination were experimentally inoculated with a fixed number of stressed *E coli O157:H7* (10^6 CFU) and then the efficacy of different dilutions of crude bacteriocin against the same microorganism in raw milk sample were. The experimental design was a repeated measure design. Three replications were conducted for each experimental condition.

Results

The cultural characteristics of *E.coli* O *157 H:7* in the nutrient broth that was subjected to a rapid cold stress by cooling it to refrigeration temperature (4°C) over the five time points of 1,2,4,6, and 24 hours of refrigeration storage are shown in Table 1. In accordance with the present results, growth of bacteria on non selective media was observed after 1, 2 and 4 hours of refrigeration storage of nutrient broth while in contrast no growth was observed after 6 hours of exposure to refrigeration temperature. Also our result indicated the observation of growth when the nutrient broth was subjected to a refrigeration temperature over 24 hours of incubation at 4°C (Table 1).

Table 1- Influence of cold stress on the viability of *E.coli O157:H7* stored at refrigeration temperature (4°C) over the five time points of 1 ,2 ,4 ,6 ,24 , hours :

Refrigeration storage at 4°C	Refrigeration (hrs)	Results
	1	(+)Ve
	2	(+) Ve
	4	(+) Ve
	6	(-) Ve
	24	(+) Ve

The antimicrobial spectrum exhibited by the crude bacteriocin that was obtained from *Lactobacillus acidophilu*s isolate NO.1 against sensitive strain *Lactobacillus acidophilu*s R0052 and test organism *E.coli O157:H7* is shown in Table 2. The average diameter of the inhibition zone of crude bacteriocin that was produced by the *Lactobacillus acidophilu*s isolate NO.1 against the sensitive strain *Lactobacillus acidophilu*s R0052 was 12 mm and showed a potency of 100% while the inhibition zone against the test organism *E.coli O157:H7* was 0.0 mm which means was resistant to the crude bacteriocin (Table 2) 0% potency. While the inhibition zone against stressed *E.coli O157:H7* for 6 hr at 4°C was 10 mm and showed a potency 83.3% .

Table-2- The antimicrobial spectrum of crude bacteriocin produced by *L. acidophilus* isolate NO. 1 against sensitive strain *Lactobacillus acidophilus* R0052 and *E.coli O157:H7*:

Bacteriocin of *Lactobacillus acidophilus* isolate NO. 1	Indicator / sensitive strain	bacteriocin activity	Inhibition Zone diameter (mm) Mean ±S.E	% of potency
	Sensitive reference strain R0052	(+Ve)	12 ±0.04	100 a
	E.coli O157:H7	(-Ve)	0 ± 0.0	0 c
	Stressed *E.coli O157:H7* (6 Hours at4 °C)	(+Ve)	10 ±0.02	83.3 b

Percentages of potency in a column not followed by the same letter differ significantly (P <0.01) .
(-Ve) = Negative result (no Inhibition Zone).
(+Ve) = Positive result (with inhibition Zone).

Various dilutions of *Lactobacillus acidophilus* crude bacteriocin against the indicator strain *E.coli O157:H7* (10^6 CFU/ml) for the quantitative determination of Minimal Inhibitory Concentration (MIC) by measuring the Optical Density(OD) by the spectrophotometer are shown in Table 3 . The results revealed that no growth of *E.coli O157:H7* with no visible

turbidity in the broth was observed with bacteriocin that diluted to 1/2 , 1/4 and 1/8. In addition to that no growth was observed by streaking a loop from each of the above mentioned dilutions of bacteriocin on the nutrient agar.

The Minimum inhibitory concentration (MIC) of the bacteriocin that diluted to 1/8 with optical density reading 1.374 was recognized .

The growth of *E.coli O157:H7* with bacteriocin that diluted to 1/16 showed a changes in turbidity with optical density reading 1.613 was observed while the growth of the same bacteria in nutrient broth without
bacteriocin (as a control) was also observed and gave the optical density reading of 1.999 (Table 3).

Table- 3- Turbidmetric assay for Minimum Inhibition Concentration test by using spectrophotometer:

Tube number	Dilutions Of bacteriocin	O .D. $_{600\ nm}$ Before incubation (mean of replications)	O .D. $_{600\ nm}$ After incubation overnight (mean of replications)	Growth detection (By streaking)
1-	1/2	1.659 A	1.648 a A	(-ve) growth
2-	1/4	1.595 B	1.592 b B	(-ve) growth
3-	1/8	1.378 C	1.374 c C	(-ve) growth
4-	1/16	1.136 D	1.613 a E	(+ve) growth
5-	Nutrient broth & *Ecoli* O157: H7 suspension (as control)	1.722 F	1.999 d G	(+ve) growth

Different small letters in a column revealed significant differences (P<0.05) between dilutions factor of bacteriocin. Horizontal different capital letters revealed significant differences (p<0.05). O .D : Optical density

The viability of *E.coli O157 :H7* subjected to diluted bacteriocin in raw and sterile milk when incubated at refrigeration temperature (4°C) over the four time points of 30, 60 , 90 and 120 minutes are shown in table 4. The viability of *E.coli O157 :H7* subjected to bacteriocin that diluted 10 times (10^{-1}) in raw milk was lost after 90 minutes of incubation at refrigeration temperature (4°C) where no growth was observed after both 90 minutes of refrigeration incubation (Table 4) .

Table - 4- Effectiveness of diluted crude bacteriocins in raw and sterile milk samples stored at refrigeration temperature against stressed *E.coli* O157:H7

bacteriocin dilution	Contact time (at refrigeration temperature 4 C°)				
	Source of milk	30 minutes	60 minutes	90 minutes	120 minutes
10^{-1}	Raw	(+Ve)	(+Ve)	(- Ve)	(- Ve)
	Sterile	(+Ve)	(- Ve)	(- Ve)	(- Ve)
10^{-2}	Raw	(+Ve)	(+Ve)	(+Ve)	(+Ve)
	Sterile	(+Ve)	(+Ve)	(+Ve)	(+Ve)
Control	Raw	(+Ve)	(+Ve)	(+Ve)	(+Ve)
	Sterile	(+Ve)	(+Ve)	(+Ve)	(+Ve)

(+Ve) = growth (-Ve) = no growth

Discussion

For discussing the growth obtained after 24 hours of refrigeration storage,(Table 1) one can say that could be due to the capacity of the bacteria to recover or repair subsequently after subjecting to cold injuries and ability of the bacteria to develop mechanisms that permitted low temperature growth in order to cope with low temperature stress and this involved membrane modification and maintenance of the structural integrity of macromolecular such as protein. Gram –negative bacteria are resistant to several antimicrobial agents due to the presence of outer membrane (9). Two isolates of *L.acidophillus* were isolated from five samples of locally home made soft cheese and identified in accordance with the main features described in bergeys manual of determinative bacteriology. The average diameter of the inhibition zone of crude bacteriocin that was produced by the *Lactobacillus acidophilu*s isolate NO.1 against the sensitive strain *Lactobacillus acidophilu*s R0052 was 12 mm and showed a potency of 100% while the inhibition zone against the test organism *E.coli O157:H7* was 0.0 mm which means was resistant to the crude bacteriocin (Table 2) (0% potency). Generally many bacteriocins of LAB were active against Gram positive bacteria but Gram- negative bacteria were a little insensitive to bacteriocins . The effect of bacteriocin on target cell in liquid medium was determined by deducing the killing or growth inhibition of the indicator organism . The indicator strain *E.coli O157:H7* inoculated in nutrient broth with various dilutions of crude bacteriocin were incubated aerobically for overnight at 37 C°. The growth – inhibitory effect of the diluted crude bacteriocin was determined after the incubation period by noticing any changes in visible turbidity through measuring the absorbance or optical density at 600nm.

The inhibitory effect of the diluted crude bacteriocin against the growth of the indicator bacteria was studied and compared with the growth of the same bacteria as control (without the addition of bacteriocin to it) (Table 3). Quantitative measurements of bacteriocin activity are essential for most of the activities with respect to their characterization. Several methods have been used for the quantitation of the lantibiotic Nisin due to its importance as food preservative, standard units and methods have been defined for this substance and a commercial standard preparation is available. Methods for the measurement of bacteriocin activity are usually derived from those of antibiotics modifications (spot, well, disc diffusion) of agar diffusion assay (11) and photometric methods have been widely used.

The criteria used for the determination of inhibitory effects by turbidometry are based on the changes of visible turbidity and is carried out by the measurement of absorbance, i.e., optical density of the indicator culture in liquid medium. One of the most basic turbidometric applications is to measure the end absorbance value after a specified incubation period in parallel in the cultures with and without the bacteriocin (12). The dilution factor of bacteriocin had a significant ($P<0.05$) effect on the antimicrobial potency of the bacteriocin against stressed *E.coli O157 :H7* Table 4 .The time of exposure to bacteriocin at refrigeration storage temperature had a significant ($P<0.05$) influence on the viability of *E.coli O157 :H7.* From minutes 30 to 120. A significant ($p<0.05$) increase in the antibacterial potency of bacteriocin that diluted 10 minutes in raw milk against *E.coli O157 :H7* was found after 90 minutes of exposure at refrigeration temperature , in addition to that, our results suggest that bacteriocin produced by *L. acidophilus* was not affected by carbohydrates , fat and proteins of raw milk and had a potential effect to be used in raw milk. The antibacterial properties of the bacteriocin can reduce the cell survival of other undesired microorganisms in raw milk as well as perform essential roles in milk preservation for human consumption. A significant ($P<0.05$) increase in the antibacterial potency of the bacteriocin that diluted 10 times in sterile milk against stressed *E.coli O157 :H7* was found after 60 minutes of exposure at refrigeration temperature (Table 4) . In addition to that the results obtained by the present study suggest that the bacteriocin in raw milk samples could be influenced by microbial popularity and this can reduce the antibacterial properties of the crude bacteriocin.

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