

Efficacy of Bacteriocin Extracted from *Lactobacillus acidophilus* (LAK) against *Bacillus cereus* in cow raw milk

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Summary

Fifty seven out of ninety bovine milk samples were collected from different areas inside and nearby Baghdad which were positive for *Bacillus cereus*. The isolates were checked on chromogenic *Bacillus cereus* agar which was specific for detection of *B. cereus* colonies. The colony appearance, gram stain, spore stain and oxidase lipase monitol catalase tests were carried out to identify this bacterium. Bacteriocin extracted from *Lactobacillus acidophilus* was used as a tool to control the growth of undesirable *B. cereus* growth as spoilage and pathogenic bacteria as well as to keep the food more acceptable and safe to consumer. The results of this study showed that bacteriocin (LAK) had 8 a bactericidal effect against *B. cereus* in refrigeration and room temperatures at pH 4, 7 and 9, respectively. High potency of bacteriocin (LAK) was confirmed by minimum inhibitory concentration at 30, 60 and 120 minutes. It was found that the zone of inhibition of *B. cereus* by bacteriocin at 4°C and pH 7 was 26 mm. The present study revealed that bacteriocin was highly effective against locally isolated *B. cereus*.

Keywords: Bacteriocin extract, *Lactobacillus acidophilus*, *Bacillus cereus*, Milk, Cow.

Introduction

Bacillus cereus was first isolated in 1887 from cowshed air by Frankland and Frankland (1). It may be the most common aerobic spore bearer in many types of soil and in sediments, dust, and plants (2). *B. cereus* is also frequently present in food production environments due to the adhesive nature of its endospores (3). This species has been isolated from a variety of foods, such as meat, eggs, spices, dairy, and rice products. The presence of *B. cereus* in pasteurized milk and other dairy products is a major concern of the dairy industry since contamination of milk by this organism can lead to both spoilage and safety problem (4). Its spore-forming and psychro-trophic properties assist *B. cereus* to grow and produce toxins in pasteurized milk at refrigerator temperature. Food-borne outbreaks and cases attributed to *B. cereus* in North America, Europe and Japan were ranged from 1% to 22% for outbreaks covering 0.733% of the cases. The Netherlands and Norway were reported to have the most extensive problem due to *B. cereus*. The threshold number of *B. cereus* is around 10^6 colony forming units (CFU) per gram of food ingested (1 and 5). *B. cereus* has been known

as a potential pathogen responsible for most of the food poisoning outbreaks (6).

Lactobacillus acidophilus strain and a member of the normal intestinal microflora is widely used in fermented dairy products and is of considerable industrial and medical interest because it has been reported to aid in the reduction of the levels of harmful bacteria and yeasts in the small intestine and to produce lactase, an enzyme which is important for the digestion of milk (7). Therefore *L. acidophilus* group of lactic acid bacteria (LAB) is added as dietary adjuncts to commercial fermented milk products and the intake of these bacteria may have beneficial effects on human health (8). The production of bacteriocin occurred in log phase and was highest in the pH range of 5.5-6. Activity declined considerably when cell entered into death phase (9). The first bacteriocin to appear on both the European food additive list and the United States FDA list was intended for the use in the production of pasteurized processed cheese (10). The aims of the present study was to investigate the availability of *B. cereus* in raw milk samples collected randomly from different areas within and outside Baghdad locations and to evaluate the bactericidal potency of crude

bacteriocin produced by *L. acidophilus* (LAK) against the survival of *B. cereus*.

Materials and Methods

Ninety bovine raw milk samples were collected randomly at weekly intervals (10 samples a week) in sterile 100 ml plastic bags from different regions of Baghdad city and its surrounding districts then transported to the laboratory in ice-cooled box. Brilliance *B. cereus* agar (Chromogenic agar) was one of the solid culture mediums that used for the isolation, identification and enumeration of *B. cereus* and the authors addressed that the new chromogenic media represent a good alternative to the conventional standard media (11). It was prepared according to the manufacturer instructions (Oxoid / UK). After exposure each milk sample to heat treatment at 80°C for 12 minutes plating was done within 3-6 hrs of arrival of the samples to the laboratory. Isolation of *B. cereus* was carried out by spreading 0.1 ml of 10-1 and 10-2 of milk samples on the Brilliance *B. cereus* agar (Chromogenic agar), and then incubated at 37 °C for 24 hours after the incubation period the colonies were counted in a Colony Counter (UK). The crude bacteriocin was obtained from the bacteriocin producing strains *Lactobacillus acidophilus* LAK which was grown in MRS broth under anaerobic condition at 37°C for 24 hours the supernatant fluid was separated from cells by centrifugation at 5000 rpm for 30 min. Late the supernatant was collected and pH was adjusted to 6.8- 7 with sterile in NaOH to exclude the effect of organic acids was filtered through a syringe filter with pore size of 0.45 µm , then heating for 10 min at 70°C to inactivate antibacterial peptides (protease) and to destroy cells and were stored at 4°C in a refrigerator (12).

The antimicrobial activity of bacteriocin against *B. cereus* was determined using agar well diffusion assay. *B. cereus* was isolated and identified from raw milk samples after 24 hours of aerobic incubation at 37°C on chromogenic agar. Five colonies of *B. cereus* were transferred to tube containing 5 ml of sterile nutrient broth and was incubated at 37°C for 24 hr. one ml of cultured nutrient broth containing *B. cereus* counts of

approximately 1×10^6 cfu/ml was transferred into a sterile Petri dish and then pour plated with Muller-Hinton agar and kept in the refrigerator for 2-3 hr. Five wells (6 in diameter) were made inside the soft agar seeded with *B. cereus* 50 µl of crude bacteriocin were added into each well. The plate then was incubated overnight at 37°C. After the incubation, the diameter of the clear inhibition zone was measured using the standard ruler and the presence of 2 mm or more clear zone of inhibition around the wells was regarded as a positive results.

The levels of resistance to natural antimicrobial agent such as bacteriocin was determined by the dilution method proposed by (13) with slight modifications. Serial dilutions of bacteriocin were made in a liquid media which was inoculated with a standardized number of *B. cereus* 10^6 and incubated aerobically at 37°C for overnight. The minimal inhibitory concentration (MIC) was considered as the MIC at which the turbidity due to the bacterial growth that measured by spectrophotometer was not observed and this value was recorded by (13). Growth (biomass) was measured by determining its turbidity in terms of optical density (OD) at 540 nm by spectrophotometer according to the manufacturer instructions (Optima sp 300, Japan). Effect of bacteriocin on *B. cereus* in a liquid medium was determined by deducing the dead or growth inhibition of indicator organism at various dilutions of crude bacteriocin which were added to indicator organism 6.3 cfu/ml which inoculated in sterile nutrient broth and incubated overnight at 37°C. Indicator cells without bacteriocin were used as (14). Statistical analysis was done according to simple student t-test and Two-way ANOVAs (15).

Results and Discussion

Isolation of *Bacillus Cereus* from bovine raw milk samples were collected from different districts inside and surround Baghdad. In the current study, ninety raw milk samples were collected, a total of 10 samples from each district. The prevalence of *B. cereus* in raw milk samples from the above mentioned different locations inside and surround Baghdad are shown in (Table, 1).

Fifty seven out of ninety (63.33%) raw milk samples had positive results for presence of *B. cereus* while the maximum count of *B. cereus* was 760.44 cfu/ml and the mean value was 1.768 ± 0.430 cfu/ml. Four out of ten (40%) milk samples were positive which were collected from the College of Agriculture Farm and Al-Rashdia were positive with the maximum counts of 300, 700 cfu/ml and mean values of 0.980 ± 0.390 and 1.069 ± 0.429 cfu/ml, respectively. However six of milk samples were positive (60%) in the College of Veterinary Medicine Farm and Al-Tarmia with the maximum counts 900, 800 cfu/ml and mean values of 1.713 ± 0.437 cfu/ml and 1.587 ± 0.405 cfu/ml respectively. The outcomes of Al-Radhwania east and Al-Bakria farms, eight out of ten (80%) milk samples were positive with the maximum ranges of 1530 cfu/ml and 730 cfu/ml and means values of 2.344 ± 0.070 cfu/ml and 2.285 ± 0.292 cfu/ml respectively. While in Al-Radhwania west farm five milk samples were positive (50%) with Maximum count of 900 cfu/ml and mean values 1.482 ± 0.472 cfu/ml. Nine of milk samples were positive (90%) in Al-Sowera with a maximum counts 544 cfu/ml and a mean value 2.374 ± 0.316 cfu/ml. These results revealed different variations in the counts of *B. cereus* isolates. These differences in the numbers were either due to impact of the sanitation system applied in these locations or belonging to the animal management regime which may include grooming and the type of food and water offered to these animals as well as the environment surrounding these locations (15). All notes of milk samples obtained from different location inside and surround Baghdad were similar to the findings (Table, 1). The results obtained from the Farm of Agriculture College, Al- Radhwania west and Al-Rashdia had revealed significant ($P < 0.05$) versus those of the Farm of Veterinary College, Al-Radhwania east, Al-Bakria and Al-Sowera. While the observations of Abu-Graib, and Al-Tarmia farms were not significant with those of all farms mentioned earlier. These findings of the present study were in agreement with those of other

scientific literature (16 and 17) who isolated *B. cereus* from raw, pasteurized milk and other dairy products. The highest mean values of *B. cereus* isolates were obtained from the AlSowera, Al-Radhwania east and Al-Bakria which were 2.374 ± 0.316 , 2.344 ± 0.070 and 2.285 ± 0.292 cfu/ml, respectively.

As compared with the other locations, the investigation of *B. cereus* isolates indicated that such cows produce low milk quality in the areas which need farther attention for better cleaning and management however, the udder hygiene represented by milk characters should be routinely checked for the presence of somatic cell counts preferably every 6 month to identify the infecting animals with subclinical mastitis spicily those with *B. cereus* (18).

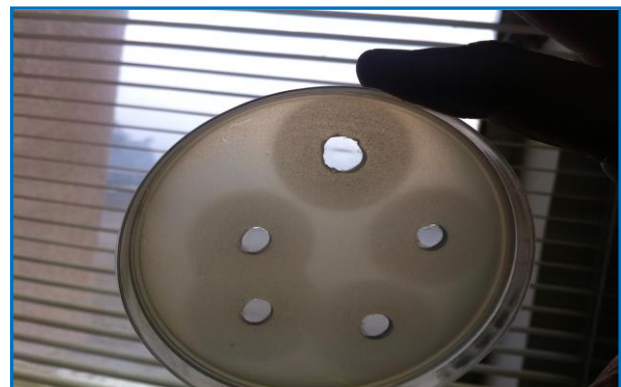
Furthermore, the highest incidence of *B. cereus* was observed in the location of Al-Sowera having 9 (90%) which were infected with *B. cereus*. Also, Al-Bakria and Al-Radhwania east locations had high infection with *B. cereus* with eight (80%) while moderate infection was noted in Abu-Graib area which was seven (70%). The other locations had low incidence with *B. cereus* such as Veterinary College Farm and Al-Tarmia location having six infected (60%) samples. While Al-Radhwania west location had lower count as compared with other locations reflecting five (50%) samples positive out of ten, with exception of the College of Agriculture Farm and Al-Rashdia which had the lowest incidence with *B. cereus* which were four (40%) out of ten positive samples. The results of the present study were in discrepancy with the findings recorded by others (19 and 20) who attributed the discrepancy to the difference in the breed of cows, of the environment and to the technique of isolation of *B. cereus*. The same outcomes to the present results were found in the research performed by (21). It is worthy to demonstrate that *B. cereus* spores in raw milk represents the major source of *B. cereus*. *Bacillus* had been isolated from 58% of raw milk samples tested and 94% of the positive milk samples was stored at 6°C for 14 days.

Table, 1: The prevalence of *B. cereus* in cow raw milk samples from different locations inside and surround Baghdad.

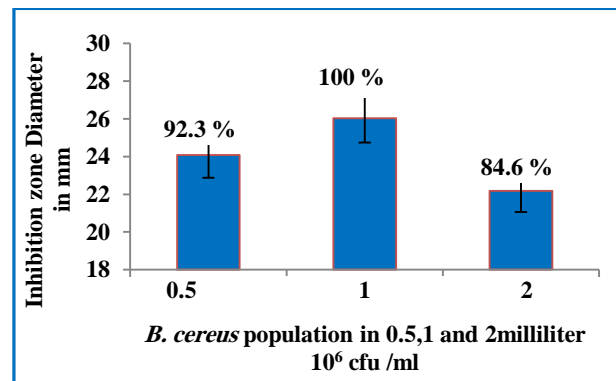
Location	No. of samples	No. of (+)Ve samples	Isolation %	Counting of <i>B. cereus</i>	
				Maximum cfu/ml	Mean cfu/ml
Agriculture) College Farm	10	4	40% H	300	0.980± 0.390B
	10	6	60% D	900	1.713± 0.437 A
Veterinary College Farm	10	7	70% C	440	2.081± 0.268 AB
	10	8	80%B	730	2.344± 0.070 A
Abu-Graib	10	8	80%B	730	2.344± 0.070 A
Al-Radh east	10	5	50%E	900	1.482± 0.472 B
Al-Radh west	10	8	80%B	1530	2.285± 0.292 A
Al-Bakria	10	9	90% A	544	2.3 74± 0.316 A
Al-Sowera	10	4	40% H	700	1.069± 0.429 B
	10	6	60% D	800	1.587± 0.405 AB
	90	57	63.33%	760.44	1.768± 0.430

There were capital letters in the (Table, 1) indicated that the similar letter were not significant while the different were significant (P<0.05).

Bacteriocin activity bioassay against *B. cereus* by well diffusion method: Inhibitory zone measurement was done on solid media by pouring method including 1ml of 10⁶ cfu/ml, with mean inhibition zone of 26 mm. Inhibitory zone was 24mm when it was carried out in 0.5 ml of 10⁶ cfu/ml, in case of 2 ml of 6 cfu/ml, the mean of inhibitory zone were 22 mm. These results indicated that the potency of bacteriocin against *B. cereus* was effective to kill *B. cereus* (Fig. 1 and 2). The potency of bacteriocin was measured in *E. coli* as a Gram negative bacteria. The result of 12 mm and 6mm was recorded as a maximum inhibitory zones, however, the readings of inhibitory zone of bacteriocin against *B. cereus* had effective potency comparing those of *E. coli*. The results in this study were similar to those reported by (22). The potency of bacteriocin against numbers of *B. cereus* was measured after incubation at 37°C for 0, 30, 60 and 120 min. The number of *B. cereus* counts began at more than 6.3 cfu/ml. Their number after 30 min. incubation at 37°C with bacteriocin was low to 5.9 cfu/ml, and after an hour of incubation. Consequently, the reduction was down to 5.6 cfu/ml. The count numbers after two hours of incubation reached less than 5.3 cfu/ml. These outcomes showed that the bacteriocin were highly effective against *B. cereus* counts. The bacteriocin in this study was observed to have broad spectrum inhibitory activity against *B. cereus* (Fig. 3).

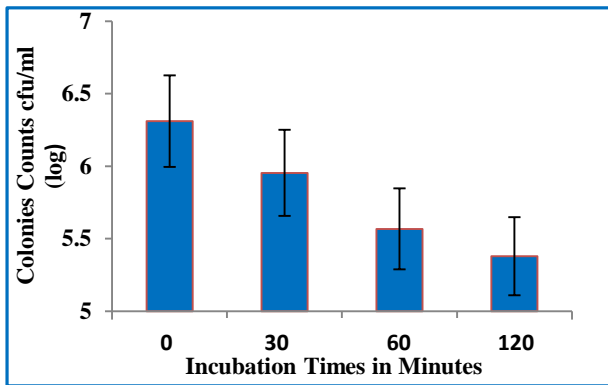


Figure, 1: Inhibition Zone of Bacteiocin in *B. cereus* on chromogenic agar.

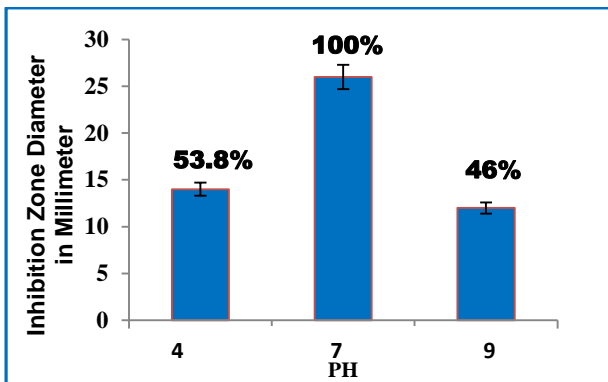


Figure, 2: Effect of Bacteriocin activities *B. cereus* count in Bacteriocin activities.

These findings were in agreement with (23 and 24) who reported that production of bacteriocin is highly affected by the many factors such as the species of microorganisms, ingredients and pH of medium, incubation temperature and time. The zone of inhibition by bacteriocin for *B. cereus* count at pH 4 was 14mm (53%) as a mean reading while in pH 9 the mean reading was 13mm (50%). In pH 7 the width of the zone of the inhibition ranged 30 mm and 26 mm as a mean (100%) (Fig. 4).



Figure, 3: Effectiveness of crude bacteriocin on bioavailability of *B. cereus* in nutrient broth kept at refrigeration temperature for 120 minutes.



Figure, 4: Potency of crude bacteriocin of *L. acidophilus* LAK at different pH values against *B. cereus*.

These results relating to inhibition zone indicated that the pH 7 was the optimum pH for perfect bactericidal activity of bacteriocin. It was found that both alkaline pH 9 and acidic pH 4 possessed decreased effect of the bactericidal activity of bacteriocin (Fig. 4). Similar findings were

reported by (25) who noticed that the bactericidal activity at the pH 9 was high against *Bacillus subtilis* (12mm) and the concentration employed was 100111. It was evident that the bactericidal effect of bacteriocin was highly dependent on the pH. Notably an increase in the level of pH above 9 showed a reduction in the inhibitory activity. The levels of pH were between 4 to 9 which was the optimum pH for favorable inhibitory activity of bacteriocin from *Lactobacillus* spp. against a wide spectrum of various pathogenic organisms.

The minimum inhibitory concentration of bacteriocin results were shown in the readings of optical density at concentration 1/1 were 1.011 ± 0.0011 before incubation and 1.020 ± 0.0015 after incubation at 37°C for 24 hrs. Optical density at 1/2 was 1.076 ± 0.0052 before incubation and after the reading of optical density at 1/4 was 1.118 ± 0.0021 and after incubation was 1.124 ± 0.00348 incubation the optical density reading was 1.092 ± 0.003 . In 1/8 bacteriocin dilution the optical density (OD) of *B. cereus* growth was 1.130 ± 0.0038 before incubation and 1.157 ± 0.00176 after incubation. The reading of OD before incubation at dilution 1/16 was 1.144 ± 0.0017 and after incubation was 1.647 ± 0.038 . The control reading of OD before and incubation was 1.661 ± 0.038 and 1.991 ± 0.0095 , respectively (Table, 2).

Table, 2: Turbid metric Assay for the Minimum Inhibitory Concentration test in (log) by using Spectrophotometer.

Tube Number	Dilutions of Bacteriocin	O.D. 540 nm Before incubation (mean of replication)	O.D. 540 nm After incubation overnight (mean of replications)	Growth detection By streaking
1	1/1	1.011 ± 0.0011	1.020 ± 0.0015	(-ve) growth
2	1/2	1.076 ± 0.0052	1.092 ± 0.003	(-ve) growth
3	1/4	1.118 ± 0.0021	1.124 ± 0.00348	(-ve) growth
4	1/8	1.130 ± 0.0038	1.157 ± 0.00176	(-ve) growth
5	1/16	1.144 ± 0.0017	1.647 ± 0.038	(+ve) growth
6	Nutrient broth and <i>B. cereus</i> suspension (as control)	1.661 ± 0.038	1.991 ± 0.0095	(+ve) growth

Different capital 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, -Ve= Negative growth, +Ve= Positive growth

The OD readings before and after incubation were significantly different at ($P < 0.05$) in bacteriocin dilution within 1/1, 1/2 and 1/4, 1/8. The readings of OD in bacteriocin dilution 1/16 before and after

incubation were significantly different at ($P < 0.05$) with all the fore-mentioned dilutions. The bacteriocin extracted from *L. acidophilus* plays an important role in controlling the total bacterial count, aerobic-

spore formers and psychrotrophic bacterial counts in pasteurized milk. Moreover, it can extend shelf-life of pastured milk up to 12 days especially at concentration 320 and 160 IU/ml (26). On the other hand, some contaminants like aerobic spore formers and psychrotrophic microorganisms may multiply in food during its storage, affecting its safety and quality. Such damage may involve pasteurized milk leading to spoilage problems as bitter cream, off-flavors or sweet curdling. Other harm reflecting the decrease shelf-life and substantial economic losses of milk products (27). Bacteriocins are used as tool to control the growth of undesirable microbial growth including spoilage and pathogenic bacteria and to keep the food quality more acceptable and palatable to the consumer (28).

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فعالية الباكتريريوسين المستخرج من ملبنة اسيدوفيلوس (لاك) ضد العصيات الشمعية المكونة للأبواغ في حليب الأبقار الخام

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الخلاصة

جمعت عينات حليب البقر السبعة والخمسين الإيجابية للعصيات الشمعية المكونة للأبواغ من أصل تسعين عينة من مناطق مختلفة من داخل ومحيط بغداد. كُشف عن العزلات في أجار اللونية الخاصة للكشف عن مستعمرات العصيات الشمعية المكونة للأبواغ اعتماداً على مظهر المستعمرة. استعملت الإختبارات صبغة غرام وصبغة البوغ وإختبارات الكاتاليز والاكسيديز فضلاً عن إختبارات انزيم اللايباز وسكر المانيتول لتحديد نوع هذه البكتريا. الباكتريريوسين المستخرج من ملبنة اسيدوفيلوس استعمل للسيطرة على نمو هذه العصيات غير المرغوب بها والتي سببت تلف الأغذية ولتثبيط نمو البكتريا المسببة للأمراض وللحفاظ على الغذاء ليكون صحياً للمستهلك. بينت النتائج أن الباكتريريوسين (لاك) له تأثير ضد جراثيم العصيات الشمعية المكونة للأبواغ في التبريد ودرجة حرارة الغرفة وكذلك درجة الأس الهيدروجين 4 و7 و9. تأكدت الفعالية العالية للباكتريريوسين بوساطة التركيز المثبط الأدنى للعصيات الشمعية المكونة للأبواغ في الدقائق 30 و60 و120. إن قياس أعلى تثبيط بالباكتريريوسين هو 26 ملم للعصيات الشمعية المكونة للأبواغ في 4 درجة مئوية وعند الأس الهيدروجين 7. نستنتج من هذه الدراسة أن الباكتريريوسين ذو فعالية قوية ضد العصيات الشمعية المكونة للأبواغ المعزولة محلياً.

الكلمات المفتاحية: الباكتريريوسين المستخرج، ملبنة اسيدوفيلوس، العصيات الشمعية، الحليب، الأبقار.