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Original Research Article

Antimicrobial activity of *Lactobacillus acidophilus* bacteriocin against clinical isolates

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ABSTRACT

Background: Lactic acid bacteria serve as important human probiotics, as they can produce bacteriocins from different dairy sources, and the antimicrobial activity of their bacteriocins have been documented in the scientific field. This study aimed to assess the antimicrobial activities of *Lactobacillus acidophilus* bacteriocin obtained from different dairy sources.
Materials and Methods: An experimental analytical design was used to isolate *Lactobacillus* species on de Man, Rogosa and Sharpe Media, and identify them phenotypically and evaluated their bacteriocin's antimicrobial activity against four clinical isolates. SPSS Version 22 was used for Statistical purposes.
Results: A total of 14 *Lactobacillus* isolates were verified from various sample sources. These included 6 isolates from camel's milk, 2 from goat's milk, 3 from local yogurt, and 3 from Omani yogurt. The supernatants of most *Lactobacillus* isolates exhibited varying levels of antimicrobial activity during the investigation. Furthermore, the action of the bacteriocin was concentration dependent. The highest level of inhibition was 14 mm for Staphylococcus aureus, 12 mm for *Bacillus subtilis*, 12 mm for *Escherichia coli,* and 10 mm for *Salmonella paratyphi* when using 100 μl of raw bacteriocin. Moreover, the correlation between bacteriocin activity, temperatures, and incubation times was found to be irreversible.
Conclusions: The study reveals inherent antibacterial traits in *Lactobacillus* species from local milk and milk products in Al-Shihr Town. Specifically, *L. acidophilus* variant, synthesizing bacteriocin, effectively suppressed the clinical isolates, showcasing their pathogen-fighting ability. These findings offer potential for developing efficient antimicrobial methods, utilizing *L. acidophilus* bacteriocin as an antibiotic in food and medicine.

1. Introduction

The Lactic Acid Bacteria comprises the following genera: *Lactobacillus,Streptococcus*, *Lactococcus*, Pediococcus, Enterococcus, Leuconostoc, and Bifidobacterium.[1] Lactobacilli are gram-positive bacilli, catalase-negative, microaerophilic, or facultatively anaerobic and non-Spore forming bacteria.[2] *Lactobacillus acidophilus* (L. acidophilus) is a member of the *Lactobacillus* genus that produces Class II bacteriocins. These bacteriocins exhibit pH and temperature stability. Incorporating *L. acidophilus* into food fermentation enhances flavor, scent, and consistency, aided by lactic acid and bacteriocin production.[3] The bacteriocins from *L. acidophilus* have potent antimicrobial effects against related and harmful microorganisms. They have applications in food preservation, quality maintenance, health promotion, and the pharmaceutical industry.[4]

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Over the past century, many studies have emphasized the importance of safely and effectively using *L. acidophilus* as adjunctive therapy for conditions such as acute and chronic diarrhea, including antibiotic-associated diarrhea.[5]

Currently, significant attention focuses on utilizing probiotics, especially *Lactobacillus* strains, to prevent and manage vaginal diseases. The bacteriocin of *L. acidophilus* shows potential in suppressing urogenital pathogens like *Gardnerella vaginalis*, *Streptococcus* agalactiae, and *Pseudomonas aeruginosa*, commonly associated with vaginal diseases.[6]

Recently, Dean and colleagues demonstrated that Gram-positive bacteria release tiny membrane vesicles (MVs) that can effectively deliver antimicrobial agents. They used *L. acidophilus* MVs to transfer bacteriocin peptides to the opportunistic pathogen *L. delbrueckii*, inhibiting its growth and compromising membrane integrity. They suggested that the potential of MVs in complex microbial communities like the gut microbiome, offering opportunities for microbial engineering and innovative biomedical therapies.[7] The bacteriocins can act as antimicrobial agents in powdered food components, purified peptides, or using bacteriocinogenic *lactobacillus* cultures. Combining various *lactobacillus* spp bacteriocins can effectively combat antibiotic-resistant bacteria and improve food product safety, quality, and shelf life.[8]

Unfortunately, Yemen faces significant challenges related to antibiotic misuse and resistance. It was demonstrated that 60% of Yemeni outpatients aged 0-15 years took antibiotics without a prescription.[9] Furthermore, another study revealed that 73.3% of local pharmacies in Yemen provided antibiotics to customers without requiring a medical prescription.[10] Likewise, A recent survey in Yemen revealed high antimicrobial resistance rates, with 74% of prescribers resorting to broad-spectrum antibiotics, and 81% of cases lacking antimicrobial sensitivity testing for antibiotic selection.[11]

Fortunately, milk and milk products are essential components in many Yemeni dishes, and they are widespread in this society as dietary staples. Hence, this study aims to investigate the in vitro antimicrobial potential of bacteriocins elaborated by *Lactobacillus* species isolated from local milk and yogurt samples against four pathogenic bacteria.

2. Materials and Methods

2.1. Research design

An experimental analytical study was conducted at the laboratory of Burum Seafood Company Limited, in Al-Shihr Town, Hadhramaut Governorate, Yemen, during the month of January in the year 2020.

2.2. Samples collection

Six lactating camels were selected from various locations within the study area. After thorough hand washing and cleaning of the udders with water, the camels were milked, and individual milk samples were obtained. These samples were stored in sealed plastic tubes, cooled, and 10 ml of milk was extracted from each tube to create the overall sample. Subsequently, an additional 10 ml was drawn from the total sample, resulting in the representative sample, which was then stored for isolation. An identical approach was applied for collecting milk samples from six lactating goats. Furthermore, 25 containers of Hana yogurt (Yemeni brand) were procured from major stores in Al-Shihr Town. Following refrigeration, 10 grams of yogurt were taken from each container, and an additional 10 grams were sampled from the total to constitute the representative sample for isolation. The same process was repeated to gather samples from A·Safwah yogurt (Omani brand).

2.3. Isolation and identification of lactobacillus species

The isolation process for *lactobacillus* species from milk and yogurt samples involved the preparation of dilutions comprising 10 ml/g of sample and 50 ml of 0.9% sterile saline solution and with a dilution of 1×10^{-5}. Eventually, 0.1 ml from each dilution was plated onto de Man, Rogosa and Sharpe Agar (MRS), followed by an incubation period of 24 hours at 37°C under anaerobic conditions. The subsequent steps involved the selection and purification of colonies, as well as the identification of the isolates based on their morphological and biochemical characteristics that include gram stain, motility test, Catalase test, Sugar fermentation test, following the method outlined by Krieg and Holt.[12] The confirmed bacteria were then subcultured in MRS broth and incubated under suitable anaerobic conditions to promote growth and facilitate the release of the bacteriocin. Following incubation, the inhibitory activity of the bacteriocin was assessed against Staphylococcus aureus, using the method described by Paluszak and colleagues to select the proper isolate with highest antimicrobial activities.[13]

2.4. Preservation of lactobacillus isolates

The *lactobacillus* isolates were introduced to MRS broth with 1% calcium carbonate and incubated for 24-hours at 37°C, cultures then refrigerated to be used for next steps.[14] Bacterial strains were activated and preserved in either broth or slant forms on MRS agar after 24-hours of incubation at 37°C. MRS Broth cultures were refreshed weekly, while slants were renewed monthly.

2.5. Preparation of bacteriocin extracts

The extraction of bacteriocin from the chosen *lactobacillus* isolates was carried out according to the procedure outlined by Abo–Amer.[15] This involved cultivating *lactobacillus* isolates in 100 ml of MRS broth under anaerobic conditions at 37°C. The broth was then centrifuged at 5000 RPM for 10 minutes to collect the supernatant. Subsequently, 50 ml of the supernatant containing bacteriocin underwent treatment with ethyl acetate and was vigorously shaken for 10 minutes before being allowed to settle. The upper layer, which represents the supernatant mixture, was separated into a new container. To exclude the influence of organic acids and hydrogen peroxide and to confirm the proteinaceous nature of the bacteriocin, the supernatant was treated with 1 ml of methanol to adjust the pH to 6. Finally, to neutralize the acidic activity, 1N sodium hydroxide was used, followed by treatment with 5 g/ml of dipotassium phosphate. The mixture was then filtered through a 0.22 mm diameter filter, and the resulting filtrate was stored in a sterile container in the refrigerator for testing purposes.

2.6. Pathogenic Strains

The current study utilized four strains of clinically identified pathogenic organisms. These strains had been previously isolated from clinical specimens and identified by the Central Public Health Laboratory in Mukalla City, Hadhramaut Governorate, Yemen. The pathogenic strains comprised Staphylococcus aureus (*S. aureus*), *Bacillus subtilis* (*B. subtilis*), *Escherichia coli* (*E. coli*), and *Salmonella paratyphi* (*S. paratyphi*).

2.7. Inhibitory efficacy of lactobacillus bBacteriocin against pathogenic strains

The inhibitory efficacy of the bacteriocin produced by isolated *Lactobacillus* species were assessed using the Disc Assay Method.[16] A volume of 50 microliters of bacteriocin extract was applied to sterilized Whatman No. 1 paper discs, each measuring 5 mm in diameter. Similarly, a disc was immersed in sterile MRS broth, serving as a negative control. The agar plates were pre-inoculated with Pathogenic Strains prior to placing these discs as indicated by Syukur and colleagues.[17] Subsequently, the bacteriocin from confirmed *L. acidophilus* was tested three times, to assess its activity at various concentrations, temperatures, and incubation periods. Following these tests, the extent of growth inhibition around the discs was evaluated by measuring the transparency levels using a ruler, and the arithmetic means of the results was calculated to be used as representative results.

2.8. Data analysis

The data was input and processed using SPSS version 22 software. The correlation between bacteriocin and other factors was determined using the Pearson correlation coefficient, with a 95% confidence interval, and statistical significance was defined as a P-value ≤ 0.05.

3. Results

The study was successful in identifying 14 isolates within the *Lactobacillus* genus. Variations in the number of isolates were observed across different sources, including milk (from camels and goats) and yogurt (both Yemeni and Omani brands). Specifically, the total isolates included 6 from camel's milk, 2 isolates from goat's milk, 3 from Yemeni yogurt, and 3 from Omani yogurt. These proportions corresponded to percentages of 42.9%, 14.3%, 21.4%, and 21.4% of the total isolates obtained, respectively (Figure 1).

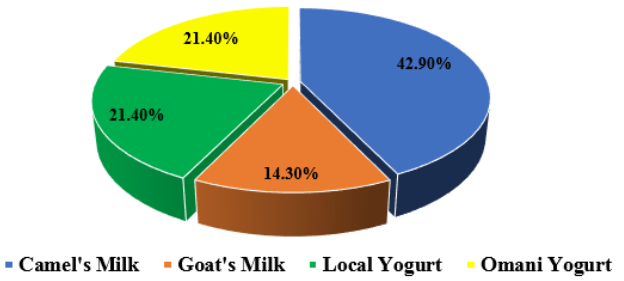

Fig. 1: Distribution of *Lactobacillus* isolates from different Sources.

Throughout the course of the study, it was observed that the supernatants of all *lactobacillus* isolates, except for isolates Lac 8 and Lac 11, displayed varying degrees of inhibitory activity against the growth of *S. aureus*. The comparative analysis of the values within our identical dataset revealed that the supernatant from isolate labeled as Lac 3, obtained from camel's milk, exhibited the largest inhibitory zone. This isolate showed 14 mm inhibition zone against *S. aureus*, indicating the most potent inhibitory effect among the *Lactobacillus* isolates. Consequently, we selected this isolate for further studies and extracted bacteriocin from it throughout the entire study (Table 1).

The Lac3 bacterial isolate underwent morphological and biochemical investigations. Morphology served as a preliminary step for characterization, and verification was carried out using biochemical tests to confirm the classification of the isolated bacteria within the *Lactobacillus* genus. The combined morphological and biochemical analyses indicated that the Lac 3 isolate was of *L. acidophilus* origin (Table 2).

During the journey of investigation, the pathogenic indicator exhibited variability in their response to

Table 1: Antibacterial activity of Bacteriocin from Various Sources against *S. aureus*

Source	Number	Inhibition Zone (mm)
Goat's Milk	Lac1	10
	Lac2	8
	Lac3	14
Camel's Milk	Lac4	10
	Lac5	11
	Lac6	7
	Lac7	13
	Lac8	0
Hana Yoghurt (Yemeni Brand)	Lac9	10
	Lac10	7
	Lac11	0
A' Safwah Yoghurt (Omani Brand)	Lac12	13
	Lac13	9
	Lac14	11

bacteriocin extracted from *L. acidophilus.* A quantity of 25 μl of bacteriocin caused inhibition of *S. aureus* with a diameter of 10 mm, while the concentrations 50 μl and 70 μl gave 11 mm and 13 mm respectively. On other hand, the bacteriocin had a lesser effect on *B. subtilis,* where the addition of 25 μl, 50 μl and 70 μl resulted in a growth inhibition diameter of 7 mm, 8 mm, and 10 mm respectively.

Table 2: Morphological and biochemical investigations of lac 3 isolate

Characteristics	Result
Shape and Color of colonies	Convex and creamy-colored.
Gram Saining and Morphology	Gram Positive Bacilli, Straight spindle-shaped unbranched and some are single or paired.
Motility	Non motile
Catalase Test	Negative
Glucose Fermentation	Positive
Sucrose Fermentation	Positive
Lactose Fermentation	Positive
Galactose Fermentation	Negative
Fructose Fermentation	Negative
Maltose Fermentation	Positive

The largest inhibition zones, 14 mm for *S. aureus* and 12 mm for *B. subtilis*, were observed when adding 100 μl of raw bacteriocin. Furthermore, the data indicated that the bacteriocin led to the inhibition of gram-negative pathogens. The addition of 25 μl, 50 μl and 70 μl resulted in growth inhibition zones of 6 mm, 7 mm, and 9 mm for *S. paratyphi* and 7 mm, 8 mm, and 11 mm for *E. coli*. However, the highest inhibition zone was observed after adding 100 μl of bacteriocin, with a result of 10 mm for *S. paratyphi* and 12 mm for *E. coli*. The inhibitory effect of bacteriocin is concentration-dependent, where the inhibitory impact on Gram-negative bacteria was slightly limited compared to its effect on Gram-positive bacteria, using an equivalent amount of bacteriocin.

Statistical analysis revealed a significant direct relationship between different concentrations of bacteriocin extracts against tested pathogenic bacteria (Table 3).

The impact of the incubation periods revealed that the highest inhibitory zone of the bacteriocin occurred within a 24-hour incubation period, corresponding to the middle to late logarithmic growth phase. Subsequently, the inhibitory activity slightly diminished with longer incubation periods. The data exhibited that the outcomes obtained from different time periods for the tested bacteria were not statistically significant at 0.05%.

In terms of the Pearson correlation coefficient, there was a weak negative relationship between the incubation time and the bacteriocin action for *S. aureus*, *E. coli*, and *S. paratyphi.* In this relationship, a slight increase in the incubation time, particularly longer than 24 hours, was associated with decrease in the effectiveness of bacteriocin activity; however, this relationship lacks significance. Furthermore, for *B. subtilis*, a moderately positive relationship was observed (0.4) between the incubation period and the action of bacteriocin (Table 4).

The influence of temperature on the activity of *L. acidophilus* bacteriocin was examined through inhibitory efficacy testing against indicator bacteria after exposure to different temperature conditions for 24 hours. The results indicate that the bacteriocin maintained its efficacy when subjected to the temperature ranges used in this study. The highest inhibitory efficacy was observed at 30°C against all test bacteria, while efficacy decreased with higher temperatures.

The statistical analysis of our investigation indicated that the results from various temperature levels used in this study for the indicator bacteria were not statistically significant at a significance level of 0.05%. The correlation between the temperatures used and the average inhibition zone for the four bacteria according to the Pearson correlation coefficient, showed a strong inverse relationship between the two variables. This means that as temperatures increase, the action of bacteriocin decreases (Table 5).

4. Discussion

In our study, we isolated 14 *Lactobacillus* strains from various dairy products, including camel's milk, goat's milk, Yemeni yogurt, and Omani brand yogurt, These findings were aligning with Erbilir and Erdogrul's (2006) findings, where they isolated 21 strains from different dairy products.[18] Additionally, Hassan et al. (2020) successfully isolated *Lactobacillus* species, including L. plantarum and *L. helveticus*, from yogurt.[19] As well as, Khan and Chaturvedi (2022) reported a successful isolation of *L. acidophilus* from Indian curd and milk.[20]

Table 3: Antibacterial effect of bacteriocin against pathogenic bacteria

Pathogenic Strains	Amount of Bacteriocin (μl) at 37°C				Pearson correlation coefficient	P-value
	25	50	70	100		
	The mean diameter of inhibition zones (mm) ± standard deviation					
S. aureus	10 ± 0.0	11 ± 1.0	13 ± 1.0	14 ± 1.0		
B. subtilis	7 ± 1.0	8 ± 1.0	10 ± 0.0	12 ± 1.0	0.9	0.01
S. paratyphi	6 ± 1.0	7 ± 0.0	9 ± 1.0	10 ± 1.0		

P Value: ≤ 0 05(significance)
Pearson Coefficient: (-): Inverse Relationship, (+): Direct Relationship [0.80-1.000 Very strong, 0.60 – 0.799 Strong, 0.40 – 0.599 Moderate, 0.20–0.399 Weak, and 0.00–0.199 Very weak].

Table 4: Effect of incubation period on antibacterial efficacy of bacteriocin against pathogenic bacteria

Incubation period (Hours)	**12**	**24**	**36**	**48**	**60**	**Pearson correlation coefficient**
Pathogenic Bacterial indicator s		**The mean of inhibition zones (mm)**				
S. aureus	8	17	15	14	8	-0.1
B. subtilis	9	15	13	10	6	0.4
S. paratyphi	8	16	14	11	9	-0.1
E. coli	7	18	16	12	8	-0.1
P value	0.2	1.0	1.0	0.6	0.8	-

Value: ≤ 0 05(significance)
Pearson Coefficient: (-): Inverse Relationship, (+): Direct Relationship [0.80-1.000 Very strong, 0.60 – 0.799 Strong, 0.40 – 0.599 Moderate, 0.20–0.399 Weak, and 0.00–0.199 Very weak].

Table 5: Impact of temperature on antibacterial efficacy of bacteriocin against pathogenic bacteria

Temperatures (°C)	**30**	**35**	**40**	**45**	**50**	**Pearson correlation coefficient**
Pathogenic Bacterial indicators		The mean of inhibition zones (mm)				
S. aureus	16	15	14	13	12	-1.0
B. subtilis	14	13	13	12	10	- 0.9
S. paratyphi	15	14	14	14	12	-0.8
E. coli	15	14	13	12	12	-0.9
P value	0.26	0.2	0.13	0.5	0.5	-

Value: ≤ 0 05(significance)
Pearson Coefficient: (-): Inverse Relationship, (+): Direct Relationship [0.80-1.000 Very strong, 0.60 – 0.799 Strong, 0.40 – 0.599 Moderate, 0.20–0.399 Weak, and 0.00–0.199 Very weak]

Our investigation revealed variations in the inhibitory activity of Bacteriocin derived from *Lactobacillus* isolates during preliminary screening procedure. These variations can be attributed to differences in their physiological effectiveness and disparities in gene composition, affecting enzyme activity and metabolic efficiency.[8,21] Moreover, we identified the most potent bacteriocin from a *Lactobacillus* strain isolated from camel's milk as *L. acidophilus*, this result was in line with Ahmed and Kanwal (2004) study, which demonstrated its effectiveness in converting lactose sugar into lactic acid.[22] Similar results were reported by Pyar and Peh in 2014[23] and Mithun, Dipak, and Sheela in 2015.[24]

Furthermore, our study highlighted the concentration-dependent activity of bacteriocin. This result is consistent with the findings of Piazentin et al. (2022) on bacteriocin derived from Lactococcus lactis and Enterococcus faecium 135.[25] Moreover, we observed that gram-positive bacteria were more susceptible to *L. acidophilus* bacteriocin than gram-negative bacteria. This finding is consistent with the results reported by Chitra and Kumar (2018), who demonstrated that gram-positive foodborne pathogens were more susceptible to crude bacteriocin derived from *L. brevis* compared to gram-negative pathogens.[26] Additionally, previous studies on nisin, produced by L. lactis, have shown that it inhibits the biosynthesis of peptidoglycan and can inhibit cell wall biosynthesis either independently or in conjunction with pore formation.[27,28]

Regarding incubation periods, our findings confirm that the maximal efficacy of bacteriocins is typically observed within an incubation period of 18 to 24 hours, which corresponds to the logarithmic growth phase, similar to many anti-cell wall antibiotics.[29] As well as we found a moderately positive relationship between the incubation period and bacteriocin activity on *B. subtilis*, likely due to its longer generation time (120 minutes) allowing for an extended window of bacteriocin effects.[30] Conversely, shorter generation times in *S. aureus*, *E. coli*, and *S.*

paratyphi may contribute to a weak negative relationship, where faster growth leads to decreased bacteriocin effectiveness during longer incubation.

Our research also revealed that the highest inhibitory efficacy was observed at 30°C against all tested bacteria, while efficacy decreased with higher temperatures. These findings were aligning with prior research by Hassan et al. (2020) on bacteriocins extracted from L. helveticus and L. plantarum isolated from traditional Pakistani yogurt.[19] Moreover, Bromberg et al. (2005) noted the thermal stability of lactic acid bacterial filtrates due to hydrophobic regions, high glycine content, and stable protein structures in bacteriocins.[31]

5. Conclusion

The findings of our research shed light on the natural antimicrobial properties produced by the *Lactobacillus* genus found in locally sourced milk and milk products from Al-Shihr town in the Hadhramaut Governorate, Yemen. Specifically, a unique strain of *L. acidophilus* that produces bacteriocin inhibited the growth of a range of bacterial species. Our study demonstrated that the bacteriocin was concentration-dependent, its stability was slightly affected by high temperatures, and its action was more potent in the late stage of the middle to late logarithmic phase. These findings hold promise for the development of effective antimicrobial strategies, including the use of *L. acidophilus* bacteriocin as an antibiotic in both food applications and the pharmaceutical industry.

Our investigation is limited by the absence of bacteriocin purification technology. This research emphasizes the imperative need for further exploration in this area, especially in places like Yemen, where there is a high rate of antimicrobial resistance.

6. Authors' Contributions

Wedad M. Alhaik, Yasser M. Matran, and Ahmed M. Al-Haddad aided in selecting the title and designing the study frame. Wedad M. Alhaik and Ibrahim M. Bawazir conducted the entire laboratory experiment, analyzed the data, and interpreted the results. Yasser M. Matran and Wedad M. Alhaik drafted the initial manuscript, while Ahmed M. Al-Haddad finalized and validated the manuscript. Finally, the authors reached an agreement and confirmed the submission of the paper.

7. Conflicts of Interest

The authors disclose that they have no competing interests.

8. Source of Funding

None.

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