

Antimicrobial activity of bacteriocins against common mastitis agents with widely antibacterial resistance: potential alternatives to antibiotics for prevention and treatment

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Abstract

Introduction: While bovine mastitis remains a leading cause of global economic deficits in the dairy industry, the widespread reliance on antibiotic therapies for its management is increasingly constrained by the global threat of antimicrobial resistance. Therefore, identifying alternative antimicrobial compounds has become increasingly important. This study evaluates the antimicrobial efficacy of bacteriocins synthesized by *Lactobacillus plantarum* and *Lactobacillus acidophilus* as targeted biotherapeutic agents against predominant mastitis-inducing pathogens.

Methods: The *Lactobacillus* were cultivated in de Man Rogosa Sharpe broth, and their bacteriocins were subsequently recovered through centrifugation and filtration processes to eliminate bacterial cells. Following concentration via Ultra-4 centrifugal filtration devices, the recovered filtrates were preserved at 4°C prior to evaluating their antimicrobial profile against key mastitis pathogens, specifically *Corynebacterium ulcerans*, *Streptococcus uberis*, *Streptococcus agalactiae*, *Klebsiella pneumoniae*, and *Escherichia coli*, employing a comprehensive diagnostic battery of agar well and disc diffusion assays alongside minimum inhibitory (MIC) and minimum bactericidal (MBC) concentration determinations.

Results: Bacteriocin derived from *L. plantarum* exhibited stronger inhibitory effects on mastitis-associated pathogens than that produced by *L. acidophilus*. Among the tested microorganisms, *S. agalactiae* and *C. ulcerans* demonstrated the highest susceptibility, whereas *S. aureus* was the least susceptible. MIC and MBC analyses revealed comparable effective concentrations for both bacteriocins.

Conclusion: The findings indicate that bacteriocins from *L. plantarum* and *L. acidophilus* possess promising antimicrobial properties and may serve as valuable alternatives for the prevention and management of bacterial mastitis.

Keywords: Antimicrobial resistance; Bacteriocins; Bovine mastitis; *Lactobacillus plantarum*; *Lactobacillus acidophilus*

1.Introduction

Characterized by the pathogenic invasion of the mammary gland, mastitis induces severe deterioration of milk-secreting tissues and subsequent glandular dysfunction, ultimately driving a substantial decline in dairy cattle and caprine milk yields that culminates in profound economic deficits across the dairy sector [1]. This pathological condition is most frequently precipitated by key bacterial etiologies, predominantly *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Escherichia coli* [2]. Recent evidence has also demonstrated that *Escherichia coli* isolates recovered from bovine mastitis frequently harbor important virulence genes such as increased serum survival (ISS), highlighting their pathogenic potential and the need for effective alternative antimicrobial strategies [3].

These bacterial culprits could strongly influence the severity and management of the disease [4]. Mastitis manifests in clinical and subclinical, with the latter often going undetected due to the absence of overt clinical symptoms. The intensity and manifestation of the disease can depend on various factors, including the age and health of the animal, milking practices, and the specific bacteria involved [2]. Traditionally, in veterinary medicine, antibiotics such as penicillin, ampicillin, tetracycline, gentamycin, and many others are used to fight clinical and subclinical infectious diseases and prevent Mastitis [5]. In spite of that, the emergence of antibiotic-resistant strains has sparked significant concerns [6, 7]. Transcending the boundaries of veterinary medicine, this resistance phenomenon constitutes an equally critical global threat

to human public health sectors [5]. As a result, there is a compelling need to explore alternative strategies for managing mastitis [4, 6]. Several natural antimicrobial compounds, including plant essential oils, have also demonstrated inhibitory effects against mastitis-associated bacteria, further supporting the development of non-antibiotic therapeutic approaches [8]. One of these alternatives includes the use of novel antimicrobial agents such as bacteriocins, which have shown great promise in their precision targeting of mastitis-causing bacteria [9].

Synthesized by specific bacterial lineages, including *Lactobacillus* species, bacteriocins represent a distinct class of ribosome generated antimicrobial peptides and proteins endowed with potent antagonistic activities and compounds serve as a remarkable defense mechanism within microbial communities, inhibiting the growth or even causing the death of closely related or competing bacterial strains so they are gaining attention not only for their antimicrobial properties but also for their precision in targeting specific bacterial species, thus offering an alternative to traditional antibiotics [10].

The antimicrobial activity of bacteriocins is mainly attributed to disruption of bacterial cytoplasmic membrane integrity, leading to cell lysis and death. In addition to pore formation and inhibition of peptidoglycan synthesis, bacteriocins may also degrade DNA or RNA, inhibit protein synthesis, disrupt essential metabolic processes, induce autolysis, and interfere with bacterial communication systems. Their specific mode of action makes bacteriocins promising antimicrobial agents that effectively control pathogenic bacteria while minimizing adverse effects on beneficial microbiota [10].

A number of lactic acid bacteria naturally produce antibacterial peptides known as bacteriocins, which are synthesized through ribosomal pathways. These compounds exert their antimicrobial effect mainly by permeabilizing the membranes of target bacterial cells, ultimately leading to their elimination [10]. Often, bacteriocins do not disrupt membrane lipids directly; instead, they

bind to a particular membrane protein known as the "bacteriocin receptor and the interaction between the peptide and receptor protein results in membrane leakage and cell death [11]. Although recently championed as viable alternatives to conventional antibiotics, the therapeutic deployment of bacteriocins extends far beyond intramammary infections; indeed, contemporary research underscores their efficacy in mitigating dermatological pathologies induced by methicillin-resistant *Staphylococcus aureus* (MRSA), highlighted by evidence that the individual or joint administration of the broad-spectrum bacteriocins Garvicin KS and micrococcin P1, both autonomously and in synergy with penicillin G, elicits potent in vitro antagonistic outcomes and their therapeutic efficacy was further confirmed using a murine skin infection model, in which they demonstrated efficient eradication of MRSA, including strains resistant to multiple drugs and also these bacteriocins also showed promise in limiting the development of resistance, offering a compelling alternative to traditional antibiotic treatments [12].

Concurrently, extensive literature has investigated the multifaceted utility of bacteriocins as robust therapeutic agents; notably, empirical evidence demonstrates that bacteriocin-producing *Lactobacillus* significantly impair the viability and persistence of *Escherichia coli* and *Listeriae* within simulated gastrointestinal environments. Furthermore, a multitude of in vitro profiles substantiate the antagonistic performance of these peptides against a diverse spectrum of clinical pathogens, including those implicated in cutaneous inflammation, thereby underscoring the versatile applicability of bacteriocins in mitigating microbial infections and justifying the continued development of this promising paradigm for disease management [13].

In contrast, recent studies have expanded the understanding of the potential applications of *Lactobacillus brevis* DF01-derived bacteriocin by demonstrating its inhibitory effects on biofilm formation by major foodborne pathogens, including *Escherichia coli* and *Salmonella typhimurium*. In a separate investigation, nisin which is an antimicrobial bacteriocin produced

by *Lactococcus lactis* was assessed for its potential to trigger apoptosis in colon cancer cell lines [14]. Against this backdrop, the current study evaluates the antagonistic efficacy of bacteriocins synthesized by *Lactobacillus plantarum* and *Lactobacillus acidophilus* against a comprehensive panel of prominent mastitis-associated etiologies, including *Corynebacterium ulcerans*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Streptococcus agalactiae*, and *Streptococcus uberis*.

2 .Materials and methods

2.1 .Sampling and Pathogen Identification

2.1.1. Sample Collection:

Milk specimens were meticulously harvested from dairy cows exhibiting overt clinical manifestations of mastitis. Prior to expression, each teat underwent thorough mechanical cleansing to remove macroscopic debris, followed by a tap water rinse to mitigate superficial bioburden. Subsequently, the teats were dried and topically disinfected using 70% ethanol-saturated cotton swabs to secure strict aseptic conditions. Following the evacuation of the initial foremilk streams, an approximate 10 mL volume of milk was aseptically drawn into sterile receptacles. Quarter-specific samples were immediately transferred into an insulated shipping container maintained at 4°C with ice packs, ensuring an uninterrupted cold chain during transportation. All diagnostic investigations were completed within four hours of collection, during which the maintained cold chain preserved sample integrity and quality, ensuring the viability and accurate identification of the causative mastitis pathogens [15].

2.1.2. Microbiological Analysis:

Upon laboratory arrival, the milk specimens were subjected to immediate microbiological profiling. Aliquots were inoculated onto both 5% defibrinated sheep blood agar and McConkey

agar (Merck, Germany), followed by an incubation period of 48 hours at 37°C. Preliminary strain identification relied on established phenotypic and biochemical taxonomies. To this end, initial colonial morphology and Gram staining characteristics were determined, complemented by oxidase and catalase reactivity profiles. Definitive differentiation was subsequently achieved through routine bacteriological screening assays, including the characterization of alpha, beta, and gamma hemolytic phenotypes alongside carbohydrate fermentation profiles [15].

2.1.3. *Lactobacillus* SPP culture:

Lactic acid bacteria (LAB) comprise a diverse group of Gram-positive, non-spore-forming, and non-motile microaerophilic or aerotolerant microorganisms characterized by a catalase-negative phenotype [16]. Lactic acid bacteria are characterized and classified using several key criteria, including their shape, the fermentation products they generate from glucose metabolism, their growth properties under different temperature conditions and salt concentrations, as well as their fatty acid profiles and motility phenotypes. Specific approaches to categorize LAB rely on assessing their ability to bring about the fermentation of various food substrates. Parameters like optimal and extreme temperatures allowing growth, salt tolerance thresholds, cell membrane lipid composition, and flagellar motility patterns serve as additional distinguishing phenotypic features [17]. The *Lactobacillus* strains investigated herein, specifically *L. acidophilus* and *L. plantarum*, were sourced from the bacterial repository of the Department of Microbiology, Faculty of Veterinary Medicine at the University of Tehran, where they had been cryopreserved at -70°C in 15% glycerol broth. For experimental reactivation, the strains were inoculated into de Man, Rogosa, and Sharpe (MRS) broth and incubated under continuous agitation for 24 hours, followed by subculturing on MRS agar for a subsequent 24-hour period. Definitive strain verification was systematically established

through an analytical matrix encompassing Gram staining, colonial morphology, catalase reactivity, endospore profiling, and specialized biochemical assays [17, 18].

2.2. Isolation of Bacteriocins and Antimicrobial Activity Assessment

2.2.1. Isolation of Bacteriocins

To isolate the bacteriocins, the designated bacterial strains were cultivated in MRS broth until achieving an optical density commensurate with the 1 McFarland turbidity standard, followed by cell separation via centrifugation at $7,000 \times g$ for 20 minutes [18]. The recovered supernatant was subsequently passed through a $0.2 \mu m$ membrane filter to guarantee the absolute exclusion of bacterial cells [18]. To inhibit proteolytic degradation, phenylmethylsulfonyl fluoride (PMSF) was introduced to the cell-free filtrate at a final concentration of 1 mM. The clarified fluid was then concentrated utilizing Amicon Ultra-4 centrifugal filters equipped with a 3 kDa molecular weight cutoff (Millipore, MA, USA) at $4,000 \times g$, with the resulting concentrates stored at $4^\circ C$ prior to subsequent analyses [18].

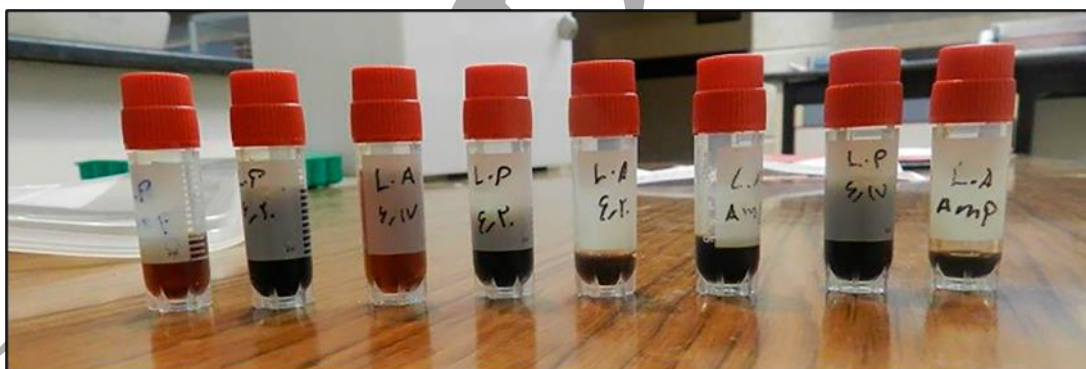


Figure 1. Bacteriocins extracted from *Lactobacillus acidophilus* and *Lactobacillus plantarum*.

2.2.2. Antimicrobial Activity

The antimicrobial activity of these bacteriocins against mastitis-causing bacterial agents was determined through well-established agar-based methods, specifically the agar well diffusion, disk diffusion and macro dilution broth assays [18].

2.2.3. Preparation of Bacterial Suspension

The bacterial strains of interest were cultivated in a physiologic serum, and their turbidity was adjusted to 0.5 McFarland for precise and standardized comparisons.

2.3. Agar Well Diffusion Method

For this assay, the isolated pathogenic strains were initially propagated in Nutrient Broth (Merck, Germany) at 37°C for 24 hours, after which the resulting bacterial suspensions were adjusted to an optical density matching the 0.5 McFarland turbidity standard. Seeded agar layers were then prepared by homogenizing 200 µL of each standardized broth culture with molten Nutrient Agar and soft agar, followed by uniform distribution across the surface of base agar plates [18]. Once solidified, equidistant wells with an approximate diameter of 5 mM were mechanically punched into the agar matrix utilizing sterile stainless-steel borers. Ultimately, each well was inoculated with a 100 µL aliquot of the respective bacteriocin solution [18].

2.4. Disk Diffusion Method

The susceptibility profiles of the isolated bacterial strains to standard antimicrobial agents were determined utilizing the standardized Kirby-Bauer disk diffusion methodology. Briefly, the isolates were initially propagated on tryptic soy agar (Merck, USA) for a 24-hour incubation period [19], after which the resulting colonies were suspended in sterile physiological saline to achieve an optical density equivalent to the 0.5 McFarland turbidity standard [19]. This standardized inoculum was uniformly spread across blood agar matrices using sterile cotton swabs. Subsequently, commercial antibiotic disks (Padtan Teb, Iran) were systematically applied to the agar surface at equidistant intervals. Following plate incubation at 37°C for 24 to 48 hours, the diameters of the zones of inhibition were meticulously quantified in millimeters [19]. The phenotypic categorization of strains as susceptible, intermediate, or resistant was interpreted in strict accordance with clinical standard guidelines. The comprehensive panel of evaluated antimicrobials comprised ciprofloxacin (5 µg), amikacin (30 µg), ceftriaxone (30

μg), cefotaxime (30 μg), ampicillin (10 μg), cefepime (30 μg), cotrimoxazole (25 μg), gentamicin (10 μg), kanamycin (30 μg), methicillin (5 μg), tetracycline (30 μg), enrofloxacin (10 μg), penicillin (10 μg), nalidixic acid (30 μg), erythromycin (15 μg), vancomycin (30 μg), norfloxacin (10 μg), chloramphenicol (30 μg), and doxycycline (30 μg) [19].

2.5. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of bacteriocins derived from *Lactobacillus plantarum* and *Lactobacillus acidophilus* were determined utilizing the macro broth dilution technique [19]. For each assay, a series of eleven tubes was prepared containing 5 mL of trypticase soy broth (Merck, Germany), incorporating dedicated positive (medium supplemented with bacterial inoculum) and negative (sterile medium only) controls [19]. Following the addition of 500 μL of Brain Heart Infusion (BHI) broth to each tube, a 500 μL aliquot of the respective cell-free *Lactobacillus* supernatant was introduced into the first experimental tube [19]. Serial two-fold dilutions were subsequently performed across the designated treatment gradient, establishing a concentration spectrum of 8270 μ/mL to 32 μ/mL for the *L. acidophilus* bacteriocin and 8540 μg/mL to 33 μg/mL for the *L. plantarum* bacteriocin [19]. Each tube, excluding the negative control, was then inoculated with 500 μL of the standardized target mastitis pathogen and incubated under continuous agitation at 37°C for 48 hours; all trials were executed in triplicate to guarantee experimental reproducibility [19]. To determine the MBC, 10 μL aliquots drawn from the concentrations immediately bounding the determined MIC were inoculated onto blood agar plates using sterile micropipettes [19]. Following a 24-hour incubation period at 37°C, the MBC was established as the lowest bacteriocin concentration demonstrating a 99.9% reduction in the initial bacterial population [19].

2.6. Statistical Analysis

Quantitative datasets were systematically processed utilizing SPSS software (version 19.0). To evaluate the comparative antimicrobial dynamics of the two bacteriocins against the designated mastitis etiologies, statistical disparities were determined employing Chi-square and Kruskal-Wallis tests, with statistical significance rigorously defined at a threshold of 0.05 [19].

3. Results

3.1. Antimicrobial activity of the bacteriocins

3.1.1. Agar well diffusion method

In the well plates, growth of mastitis-causing bacteria was observed, as well as zones of inhibition or reduced growth in some bacteria. The results are presented in Table 1, indicating that, based on the data analyzed by SPSS software, *L. acidophilus* bacteriocins displayed the largest zone of inhibition against *S. uberis*, while *S. aureus* exhibited the smallest zone of inhibition. Against *L. plantarum*, the *C. ulcerans* and *S. agalactiae* strains displayed the largest zones of inhibition. In general, when comparing the two *Lactobacillus* species, *L. plantarum* exhibited significantly higher antibacterial effectiveness.

As illustrated in the table 1, the antimicrobial effect exerted by *L. plantarum* was notably larger than that of *L. acidophilus*. Notably, differences in sensitivity *K. pneumoniae* were observed, with *L. acidophilus* exhibiting higher sensitivity to its produced bacteriocin than *L. plantarum*. The lowest level of sensitivity was observed in *S. aureus* when challenged with *L. acidophilus* bacteriocin.

Table 1. Agar well diffusion results. The statistically difference were specified by *. ($P > 0.05$)

Mastitis isolated bacteria	Zone of inhibition diameter (cm)	
	<i>L. acidophilus</i> extract	<i>L. plantarum</i> extract
<i>Corynebacterium ulcerans</i>	1.6	2.2

<i>Streptococcus uberis</i>	1.9	2.1
<i>Streptococcus agalactiae</i>	1.6	2.2
<i>Klebsiella pneumonia</i>	1.1	0.7
<i>Escherchial coli</i>	1	1.6
<i>Staphylococcus aureus</i>	0.7	0.8

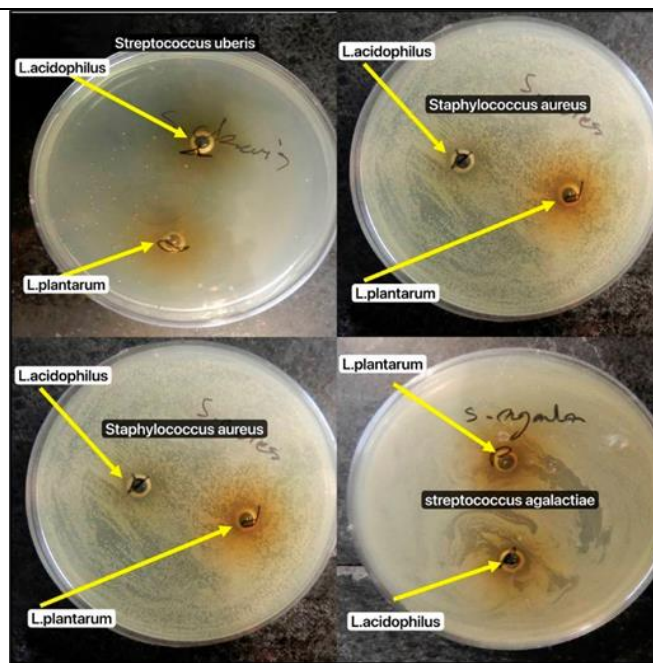


Figure 2. Mastitis causing bacteria Sensitivity to *L. plantarum* and *L. acidophilus*.

3.1.2. Disk Diffusion Sensitivity Method:

Comparison between the two Lactobacillus species extracts indicates that *S. uberis* showed the widest inhibition zone against both *L. acidophilus* bacteriocins and *L. plantarum* bacteriocins. Conversely the narrowest zone of inhibition was observed for *E. coli* against *L. acidophilus* disks and for *K. pneumonia* and *C. ulcerans* against *L. plantarum* disks. In overall *L. acidophilus* disks made wider inhibition zones in comparison to *L. acidophilus* disks.

Mastitis causing bacteria	Diameter of zone of inhibition against <i>L. acidophilus</i> disks (mM)	Diameter of zone of inhibition against <i>L. plantarum</i> disks (mM)
<i>C. ulcerans</i>	16	17
<i>S. uberis</i>	24	26

<i>S. agalactiae</i>	22	25
<i>K. pneumonia</i>	16	18
<i>E. coli</i>	17	16
<i>S. aureus</i>	18	20

Table 2. Inhibition zones of *L. acidophilus* and *L. plantarum* against mastitis pathogens.

3.1.3. MIC and MBC results

The lowest MIC was observed against *S. agalactiae* and *S. uberis*. The MIC and MBC values were identical for bacteriocins derived from both *Lactobacillus* strains, with no significant difference between them ($P > 0.05$). Overall, bacteriocin derived from *L. plantarum* exhibited greater antibacterial activity than that derived from *L. acidophilus*. The effective concentration for the tested bacteria was higher than 2000 micrograms per milliliter, which applied to *S. aureus*, *E. coli*, and *C. ulcerans*. Values of MIC and MBC for both *L. acidophilus* and *L. plantarum* are shown in Tables 3.

Based on the statistical analysis using Kruskal-Wallis and one-way ANOVA tests derived from the mean MBC values, there was no noticeable distinction in the MBC values of bacteriocins produced by *L. plantarum* in comparison to *Lactobacillus acidophilus* for all tested bacteria ($p < 0.05$). The minimum bactericidal concentration of bacteriocins from both *Lactobacillus* strains against mastitis-causing factors was equal for all bacteria except *S. agalactiae* and *S. aureus*, which displayed the highest antibacterial activity for bacteriocins produced by *L. acidophilus* and *L. plantarum*, respectively.

Bacteria	<i>L. acidophilus</i> MIC	<i>L. acidophilus</i> MBC	<i>L. plantarum</i> MIC	<i>L. plantarum</i> MBC
<i>Corynebacterium ulcerans</i>	2067.5	2067.5	2135	2135
<i>Streptococcus uberis</i>	1033.75	1033.75	1067.5	1067.5

<i>Streptococcus agalactiae</i>	1033.75	1033.75	1067.5	1067.5
<i>Klebsiella pneumonia</i>	2067.5	2067.5	2135	2135
<i>Escherichia coli</i>	2067.5	2067.5	2135	2135
<i>Staphylococcus aureus</i>	2067.5	2067.5	2135	2135

Table 3. MIC and MBC values of *L. acidophilus* and *L. plantarum*-derived bacteriocins against mastitis-related bacterial strains (µg/mL).

4. Discussion

While bovine mastitis universally afflicts mammalian species, it inflicts severe multi-dimensional setbacks on the dairy and food industries by triggering substantial economic deficits, compromising milk quality traits, and posing tangible hazards to veterinary and public health sectors [5]. The pervasive, often indiscriminate administration of conventional antibiotics for infection management, alongside their routine incorporation as growth promoters in livestock feed, has accelerated the evolutionary pressure driving the emergence and dissemination of multi-drug resistant bacterial phenotypes, thereby leading to pervasive clinical treatment failures [20].

In this study, the biotherapeutic efficacy of bacteriocins synthesized by *L. plantarum* and *L. acidophilus* was rigorously quantified against a diverse panel of field isolates recovered from clinical mastitis cases. Our findings reveal that *L. plantarum* possessed robust and uniform antagonistic dynamics across both agar well diffusion and disk diffusion platforms. Notably, *S. agalactiae* and *C. ulcerans* manifested the most pronounced susceptibility profiles, whereas discrete inter-species fluctuations in resistance kinetics were documented among the remaining target pathogens. Concurrently, the inhibition zones generated via the agar well diffusion method systematically exceeded those produced by the disk diffusion assay, a phenomenon pointing to superior diffusive and inhibitory bioperformance of *L. plantarum* bacteriocins

within the agar matrix under well-loaded conditions. Extant literature has increasingly substantiated the strategic deployment of bacteriocins for the prophylaxis and metaphylaxis of bovine intramammary infections. For instance, lactacin has been shown to exert protective barriers against staphylococcal and streptococcal mastitis, notably without any documented emergence of resistance phenotypes to date [20]. In a similar vein, nisin-based antimicrobial formulations have demonstrated rapid bactericidal kinetics, significantly depleting *S. aureus* and *E. coli* bioburdens following brief therapeutic exposures in cattle [21]. Furthermore, when bacteriocins derived from five distinct *S. aureus* strains were screened against 65 mastitis-associated staphylococcal isolates, narrow-spectrum inhibition was observed for most strains; however, universal susceptibility was achieved exclusively against bacteriocins encoded by a bacterial artificial chromosome (BAC) plasmid system [6].

The therapeutic potential of lactic acid bacteria (LAB) bacteriocins has been heavily validated across alternative infectious paradigms. Specifically, *Lactocaseibacillus rhamnosus* supernatants have been reported to compromise *Helicobacter pylori* viability within 24 hours, achieving total eradication by 72 hours, which reinforces their utility as adjunct regimens against recalcitrant infections [22]. Moreover, bacteriocins harvested from vaginal *L. acidophilus* consortia have shown potent antagonistic efficacy against *Gardnerella vaginalis* in reproductive tract infection models [23]. Mechanistically, it has been widely posited that bacteriocins exhibit preferential potency against Gram-positive over Gram-negative bacteria due to structural disparities in cell wall architecture, particularly the absence of an outer membrane protective barrier in the former. Aligning with this paradigm, the current study observed overall superior zones of inhibition among Gram-positive cohorts, with the notable exception of *S. aureus*. Contrarily, certain investigations have reported no significant divergence in the bioactivity of *L. plantarum* bacteriocins across Gram-positive and Gram-negative boundaries [24]. To circumvent this outer membrane barrier, contemporary

efforts have focused on expanding the antimicrobial spectrum of *L. plantarum* peptides against Gram-negative pathogens by co-administering them with membrane-permeabilizing agents such as chelators [25]. Interestingly, *S. aureus* in our study exhibited relatively restricted zones of inhibition compared to other tested microflora, despite preceding reports validating the high sensitivity of *Staphylococcus* species to *L. plantarum* metabolites [24]. Nevertheless, antimicrobial fractions from *L. acidophilus* have been heavily documented to both suppress and eradicate methicillin-resistant *S. aureus* (MRSA) biofilms [22]. Given that bacteriocins operate via highly targeted, niche-specific modes of action, their intrinsically narrow spectrum underscores the clinical necessity of designing multi-bacteriocin cocktails to achieve comprehensive antimicrobial coverage and concurrently neutralize polymicrobial etiologies [24].

From an epidemiological perspective, the phenotypic susceptibility testing unveiled that the highest rates of resistance were directed against trimethoprim-sulfamethoxazole and tetracycline across all isolates. This widespread resistance is tightly coupled with the empirical, unregulated, and irrational misuse of frontline antibiotics without prior pathogen isolation or antibiogram guidance. Such diagnostic negligence not only amplifies the risk of immediate therapeutic failure but also facilitates the horizontal transfer of resistance determinants across microbial communities, threatening the efficacy of previously potent antimicrobials [22]. In our broth macrodilution trials, no statistically significant variations ($P > 0.05$) were observed in the minimum inhibitory concentrations (MIC) of *L. plantarum* and *L. acidophilus* bacteriocins against the tested mastitis pathogens. Crucially, the MIC and minimum bactericidal concentration (MBC) values aligned identically, with *Streptococcus agalactiae* and *Streptococcus uberis* emerging as the most sensitive targets in both quantitative models. Collectively, these insights substantiate that the bacteriocins engineered by these specific LAB

strains represent highly promising, next-generation antimicrobial peptides viable for the prevention and therapeutic management of bovine mastitis.

5. Conclusion

In summary, bacteriocins produced by *L. plantarum* and *L. acidophilus* exhibited inhibitory activity against all tested mastitis-associated bacterial pathogens. Bacteriocin derived from *L. plantarum* demonstrated greater antibacterial activity than that of *L. acidophilus*, while *S. agalactiae* and *S. uberis* showed the highest susceptibility. These findings suggest that bacteriocins, particularly those produced by *L. plantarum*, may serve as promising alternative or complementary antimicrobial agents for the control of bovine mastitis. However, further in vivo studies and optimization of large-scale production and purification processes are required before clinical application.

Authors contributions

Nima Abdi Khorsand: Methodology; investigation; formal analysis.

Farid Heydari: Project administration and supervisor; conceptualization; review and editing; scrub data and maintain research data.

Bahar Nayeri Fasaee: Project administration and supervisor; conceptualization; review and editing; scrub data and maintain research data.

Sepideh Asadi: Project administration and supervisor; conceptualization; review and editing; scrub data and maintain research data.

Nima Nafisi: Methodology; investigation; formal analysis.

Acknowledgements

The authors received no financial support for this article's research, authorship, and/or publication

Data Availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval

The manuscript does not contain any studies with human participants or animals performed by any authors.

Conflict of interest

All the authors have declared no conflict of interest.

Funding

The authors declare they have no financial interests.

Use of Generative AI and AI-assisted Technologies Statement

The authors declare that no generative AI or AI-assisted technologies were used in the preparation of this manuscript.

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