

RESEARCH

Open Access



In vitro characterization of probiotic potential of *Lactobacillus plantarum* CM49 against selected cattle mastitogens

Muhammad Zeeshan Izhar¹, Muhammad Nawaz^{1*}, Tahir Yaqub¹, Muhammad Avais² and Aftab Ahmad Anjum¹

Abstract

Background Bovine mastitis results in significant economic losses for the dairy industry globally due to milk production losses and decreased herd efficiency. This research aimed to isolate, select, and characterize indigenous lactobacilli with probiotic properties. A total of 40 lactobacilli were isolated from healthy milk samples of cattle and identified at the species level through 16S rDNA sequencing. All isolates were initially screened for antimicrobial activity, and selected isolates underwent in vitro assessment of probiotic properties.

Results Among the lactobacilli isolates, varying levels of activity (9 to 19 mm) against cattle mastitogens; *Staphylococcus aureus* (*Staph. aureus*), *Escherichia coli* (*E. coli*) and *Streptococcus dysgalactiae* (*Strep. dysgalactiae*) were observed in the well diffusion assay. These isolates demonstrated auto-aggregation (ranging from $14.29 \pm 0.96\%$ to $62.11 \pm 1.09\%$) and co-aggregate (ranging from $9.21 \pm 0.14\%$ to $55.74 \pm 0.74\%$) with mastitogens after 2 h. *Lactobacillus* (*Lb.*) *plantarum* CM49 showed sensitivity to most antibiotics tested and exhibited strong inhibitory effects, with mean log₁₀ reductions of 3.46 for *Staph. aureus*, 2.82 for *E. coli*, and 1.45 for *Strep. dysgalactiae* in co-culture experiments. Furthermore, *Lb. plantarum* CM49 significantly decreased the adhesion rate of mastitogens on the bovine mammary cell line and mouse model, demonstrating its potential effectiveness in preventing mastitis.

Conclusion It is concluded that *Lb. plantarum* CM49 has remarkable probiotic potential with activity against cattle mastitogens in the laboratory and cell culture and competitively excludes mastitogens from bovine mammary cells and ameliorates *Staph. aureus*-induced mastitis in mice.

Keywords Mastitis, Probiotics, *Lactobacillus plantarum* CM49, Mastitogens

*Correspondence:

Muhammad Nawaz
muhammad.nawaz@uvas.edu.pk

¹Institute of Microbiology, University of Veterinary and Animal Sciences,
Lahore 54000, Pakistan

²Department of Medicine, University of Veterinary and Animal Sciences,
Lahore 54000, Pakistan



© The Author(s) 2024. **Open Access** This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

Background

Livestock drives Pakistan's socioeconomic advancement, contributing approximately 62.68% to agricultural merit and 14.36% to the overall GDP. With a cattle population of 55.5 million and a buffalo population of 45 million, Pakistan is the fourth-largest milk-producing country globally, trailing only China, India, and the USA, with an annual gross production of 67,873 thousand tons [1]. This sector provides livelihoods for over 8 million rural families, constituting around 35 to 40% of their income [2]. Despite its significance, milk production in Pakistan faces various challenges, including infectious diseases and management issues [3].

Mastitis, a prevalent disease in the dairy sector, imposes severe clinical and economic burdens, resulting in significant economic losses [4]. Mastitis, characterized by mammary gland inflammation, is primarily caused by microorganisms such as *Staph. spp.*, *Strep. spp.*, and *E. coli* [5]. The mastitis caused by *Staph. aureus* has the highest prevalence (30%) [6]. The mastitis manifests in two forms: subclinical, characterized by no visible symptoms but decreased milk quantity and altered milk composition, and clinical, presenting with swollen and painful udders, clots, and sometimes blood in the milk [7].

Traditional control methods for bovine mastitis include diagnostics, animal isolation, enhanced hygiene, and antibiotic therapy [8]. However, the emergence of antibiotic resistance underscores the urgent need for alternative treatment strategies [9]. In this context, plant extracts, essential oils of medicinal plants [10], vaccines [11] and bacteriophage therapies have emerged as promising alternatives to antibiotics [12, 13]. Probiotics are another alternative approach that has gained reasonable success in controlling and treating various microbial diseases in livestock and Poultry [14, 15].

Probiotic lactobacilli have gained particular attention as a preventive measure against bovine mastitis. A recent study in Pakistan demonstrated that *Lb. rhamnosus* GG has the potential to be a therapeutic agent in the management of sub-clinical mastitis by reducing somatic cell count and inhibiting the growth of mastitis-causing bacteria [16]. Lactobacilli, known to be safe for consumption, provide various benefits such as adhesion to bovine mammary epithelial cells, colonization, and bacteriocin production, which helps suppress pathogen growth [17]. Other than mitigation of mastitis, probiotics can provide a wide range of additional benefits [14, 18, 19].

Our previous studies using the 16 S rDNA metagenomic approach revealed that the milk microbiota of mastitic and healthy cattle and buffalo varies, indicating a possible role of udder microbial communities in preventing mastitis. This highlights the possible importance of exploring and enhancing the udder microbiome using probiotic candidates [20, 21]. Therefore, this study was

designed to isolate and characterize lactobacilli strains from the udder microbiota of cattle, evaluate their antibacterial properties against selected mastitogens, and assess other probiotic traits. Through comprehensive analysis and experimentation, our goal is to develop indigenous probiotics tailored for controlling and treating bovine mastitis, addressing a critical need in Pakistan's dairy industry.

Methods

Place of study and milk sampling

This study was conducted at the Probiotics Research Laboratory, Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan. The milk samples were collected from healthy Holstein Friesian ($n=50$) and Sahiwal cattle ($n=20$) from different dairy farms in Lahore after confirming the animals' health (absence of mastitis) using the California mastitis test [22]. The teats and udder surfaces were washed with tap water and dried. They were then swabbed with a cotton swab soaked in 70% alcohol. After discarding the first three milk strips, approximately 10 ml of milk sample was collected. The samples were then placed on racks for easy handling and transferred to the laboratory in a transport container with ice packs to maintain a cold chain.

Isolation and identification of lactobacilli

For the isolation of lactobacilli, milk samples underwent 10-fold serial dilutions in normal saline, and 100 μ l aliquots were spread on MRS (De man, Rogosa, and Sharpe agar) media plus nystatin (100 μ g/100 ml) to prevent fungal growth. The media plates were incubated at 37 °C for 24–48 h in an anaerobic environment. Colonies with distinct morphologic characteristics were selected, purified, and subjected to subsequent Gram staining and catalase testing. Once confirmed, the selected colonies were preserved in MRS broth supplemented with 20% glycerol. For molecular identification, DNA from isolates identified as Gram-positive, rods and catalase-negative was extracted using a Thermo Scientific Genomic DNA purification kit and confirmed using a *Lactobacillus* genus-specific PCR. Species-level identification was achieved by amplifying the partial 16 S rRNA gene using previously described primers and conditions [23]. On 1.5% agarose gel electrophoresis, the genus-specific (250 bp) and 16 S rRNA gene (1400 bp) amplicons were resolved and sent for sequencing from Macrogen, Seoul, South Korea. After analyzing the sequences with BioEdit, the species were determined by BLAST comparison of the recovered sequences with the NCBI GenBank database. Additionally, the sequences were submitted to NCBI GenBank to obtain accession numbers.

In vitro characterization of lactobacilli for their probiotic properties

Screening of lactobacilli for anti-mastitogen activity

Lactobacilli isolates were screened for activity against mastitogens (*Staph. aureus*, *E. coli* and *Strep. dysgalactiae*) of bovine origin using the well diffusion assay. The lactobacilli were cultured in MRS broth anaerobically for 24 h, and their cell-free supernatants (CFS) were obtained by centrifugation for 5 min. Mastitogens were swabbed onto Muller-Hinton agar plates, wells were made, and CFSs of lactobacilli were added to wells. The media plates were then incubated for 24 h at 37 °C, and the diameter of the zone of inhibition was measured in millimeters around the wells [24]. Mastitic milk samples from various dairy farms in a separate study in our lab were processed for the isolation of bovine mastitogens (*Staph. aureus*, *E. coli*, and *Strep. dysgalactiae*), confirmed by PCR followed by sequencing and used as indicator organisms in this study.

Antibiogram of selected lactobacilli

Probiotic bacteria must not exhibit transferable antibiotic resistance [25]. The antibiogram of selected lactobacilli was determined using a method outlined in previous studies [26]. Lactobacilli were cultured on MRS media, and a 1 McFarland ($\sim 3 \times 10^8$ cfu) suspension was prepared. This suspension was then swabbed onto MRS media plates, and the most commonly used antibiotic discs, including penicillin, amoxicillin, cefixime, polymyxin B, bacitracin, vancomycin, tetracycline, erythromycin, chloramphenicol, and fusidic acid were placed on the plates. The plates were incubated anaerobically at 37 °C for 24 h. After incubation, the isolates were classified as sensitive (S) or resistant (R) based on the zone of inhibition (mm) and breakpoints adopted by the Clinical Laboratory Standards Institute (CLSI) or European Food Safety Authority (EFSA) as described previously [23].

Auto-aggregation, co-aggregation of selected lactobacilli isolates with selected mastitogens

The evaluation of auto-aggregation of lactobacilli was conducted according to a previously established protocol [24]. Fresh lactobacilli cultures were centrifuged, washed with PBS, and re-suspended in PBS. The lactobacilli suspensions were then incubated at 37 °C, and optical density (O.D.) was measured after 1 and 2 h at 600 nm using a spectrophotometer. Additionally, the co-aggregation of lactobacilli with mastitogens (*Staph. aureus*, *Strep. dysgalactiae* and *E. coli*) was determined following the method described previously [24]. Co-aggregation was quantified as a percentage, indicating the potential of lactobacilli to inhibit the indicator organisms (*Staph. aureus*, *Strep. dysgalactiae* and *E. coli*).

$$\text{Coaggregation\%} = 1 - A_{\text{mix}} (A_{\text{isolate}} + A_{\text{pathogen}} / 2) \times 100.$$

Where A_{isolate} , A_{pathogen} and A_{mix} , respectively, stand for the test strains, pathogenic strains, and their mixing following a two-hour incubation period.

Inhibition of mastitogens in broth culture

Equal amounts of 10^6 cfu per mL of freshly grown cultures of selected lactobacilli (100 μ l) and mastitogens (*Staph. aureus*, *Strep. dysgalactiae* and *E. coli*) (100 μ l) isolated from cattle were mixed in 10 ml nutrient broth in separate tubes and then incubated at 37 °C. Mastitogens were enumerated on Mannitol salt agar, Edward medium, and MacConkey agar, respectively, from this mixture after different time intervals (0, 8, 16, and 24 h) to determine the growth kinetics in terms of log₁₀ reduction of mastitogens with potential lactobacilli [27].

Effect of *Lactobacillus plantarum* CM49 against mastitogens on bovine mammary cell line

Mammary epithelial cells

Primary cell culture was utilized to establish the mammary cells. Bovine mammary tissue was obtained [28] Under sterile conditions, transported to the lab within 1 h and cut into small pieces. Tissue digestion was achieved by incubation in Dulbecco's Modified Eagle Medium (DMEM), with 100 μ g/mL penicillin G. The cells were separated through centrifugation at 300 \times g for 10 min, washed twice, transferred into T25 flasks, and maintained at 37 °C with 5% CO₂. These cells were then cultured in DMEM, having 10% heat-inactivated fetal calf serum. The cells were kept in humidified conditions at 37 °C, 5% CO₂ until they reached confluence [29].

Before the adhesion assay, bacteria grown in nutrient broth were centrifuged at 6000 rpm for 5 min, and pellets were obtained. The pellets were washed thrice with PBS and resuspended in antibiotic-free DMEM to get the suspension of *Lb. plantarum* CM49 to (10^8 cfu/mL) and mastitogens (10^7 cfu/mL). Mammary cell monolayers at a 2×10^5 density per well in 6-well plates were seeded with *Lb. plantarum* and mastitogens for the adhesion assay.

Adhesion assay

Adhesion assays were conducted following the protocol established previously [30], modified to include Competition, Displacement and Inhibition assays: Competition assay: *Lb. plantarum* CM49 (10^8 cfu/mL) and *Staph. aureus* (10^7 cfu/mL) suspension in DMEM was added to the cells and incubated for 2 h at 37 °C in a 5% CO₂ environment. Following the incubation phase, MEC monolayers underwent three PBS washes, and 1 ml of 0.2% Triton-X100 in sterile water was used to lyse the cells. The adhered bacteria was quantified using a total viable count, followed by percentage inhibition by comparing with their controls. Displacement assay: cells were

incubated with 4 ml of *Staph. aureus* suspension for two hours to achieve infection and then washed thrice with PBS, followed by incubation with 4 ml of *Lb. plantarum* CM49 suspension for two hours. Inhibition assay: cells were initially exposed to *Lb. plantarum* CM49 for 2 h, washed thrice with PBS and infected with *Staph. aureus* for 2 h. The adhesion assay involved using *Staph. aureus* alone, and *Lb. plantarum* CM49 alone was used as a control. The same protocol was followed for each *E. coli* and *Strep. dysgalactiae* separately, along with *Lb. plantarum* CM49.

In vivo evaluation of the probiotic potential of *Lactobacillus plantarum* CM49 against *Staphylococcus aureus*-induced mastitis in mice

Based on its superior in vitro activity, the isolated strain *Lb. plantarum* CM49 was selected to evaluate its effects on preventing and controlling experimentally induced *Staph. aureus* mastitis in mice, following a modified protocol [31]. Lactating mice (approximately 35–40 g), 12–14 days post the birth of their offspring, were used for this experiment. The pups were separated 1–2 h before the inoculation of *Staph. aureus* into the mammary gland. The fourth mammary gland (L4), the largest one from head to tail, was disinfected with 70% ethanol before intramammary (IMAM) infection with *Staph. aureus*.

A total of 25 lactating mice were divided into five groups. Mice in Group A were given intramammary injection of *Staph. aureus* (1×10^5 cfu, 100 μ l) only, and group B (treatment model) was infected with *Staph. aureus* (1×10^5 cfu, 100 μ l) followed by inoculation of *Lb. plantarum* CM49 (3×10^7 cfu, 100 μ l) for 24 h. In Group C (prevention model), the *Lb. plantarum* CM49 was inoculated 24 h before the infection with *Staph. aureus* for 12 h. Group D was the PBS control group, while Group E was inoculated with *Lb. plantarum* CM49 alone. Following that, the mice were anesthetized with ketamine/xylazine at doses of 87 and 13 mg/kg, respectively. Mammary glands were collected, and *Lb. plantarum* CM49 and *Staph. aureus* were enumerated on respective selective media. The enumeration data was converted to the reduction in log₁₀ cfu/gm.

Statistical analysis

Enumeration data were presented as the mean \pm standard deviation to describe the variability of the data. A one-way ANOVA (Analysis of Variance) was performed to evaluate significant differences among the isolates and determine any significant differences between the means of the groups. Following the ANOVA, Tukey's Multiple Comparison Test was used to show significant differences in their means. These analyses help to understand where the isolates' performance differences exist.

Results

Isolation and identification of lactobacilli isolates

Culturing milk samples on MRS agar plates resulted in isolating 55 probable lactobacilli, characterized as catalase-negative and gram-positive rods. Among 55 isolates, 40 were confirmed to be lactobacilli through genus-specific PCR. Out of the 40 confirmed lactobacilli, five isolates underwent further species identification by sequencing a partial 16 S rRNA gene. Sequencing analysis of the selected lactobacilli strains revealed their species identification as *Lb. plantarum* (03) and *Lb. pentosus* (02). The Gene Bank accession numbers assigned to these lactobacilli are OR230148, OR230149, OR230150, OR230130 and OR230152. An evolutionary tree based on 16 S rRNA gene sequences, is presented in Fig. 1.

Anti-mastitogens activity of cell-free supernatants of lactobacilli

All lactobacilli isolate cell-free supernatants (CFS) were used directly after being adjusted to pH=7. The CFS of 32 out of 40 lactobacilli isolates showed anti-*Staph. aureus* activity (32/40, 80%) while 20 lactobacilli isolates exhibited anti-*Staph. aureus* and anti-*E. coli* activity (20/40, 50%). Additionally, 5 lactobacilli isolates (CM12, CM22, CM28, CM41, CM49) demonstrated activity against all three selected mastitogens (*Staph. aureus*, *E. coli*, and *Strep. dysgalactiae*), with no zone of inhibition observed at pH7 in all cases. The highest anti-mastitogens activity, indicated by the zone of inhibition in millimeters (mm), was shown by the CFS of lactobacillus isolates CM49 (19 ± 1 mm, 15 ± 1 mm and 12 ± 0.57 mm), CM28 (16 ± 0.57 mm, 15 ± 0.57 mm and 12 ± 1 mm), followed by CM22 (15 ± 0.57 mm, 13 ± 0.57 mm and 11 ± 1 mm) against *Staph. aureus*, *E. coli*, and *Strep. dysgalactiae*, respectively, compared to other isolates. Figures 2 and 3, and 4 illustrate the activity of CFSs derived from various isolates against mastitogens.

Auto-aggregation and co-aggregation pattern of lactobacilli

Optical density (OD) values were measured using a spectrophotometer at 1 and 2 h in the auto-aggregation and co-aggregation experiments. The auto-aggregation activity of the isolates after 2 h ranged from $14.29 \pm 0.96\%$ to $62.11 \pm 1.09\%$. The highest percentage of auto-aggregation was observed in lactobacilli isolates CM49 ($62.11 \pm 1.09\%$), followed by CM22 ($58.08 \pm 0.86\%$) and CM41 ($56.80 \pm 0.75\%$) after 2 h, showing a significant difference from other lactobacilli isolates. In contrast, CM9 ($15.62 \pm 0.68\%$) and CM14 ($14.29 \pm 0.96\%$) exhibited poor auto-aggregation activity after the 2-hour time interval compared to the control LGG isolate ($38.58 \pm 0.20\%$), as detailed in Table 1.

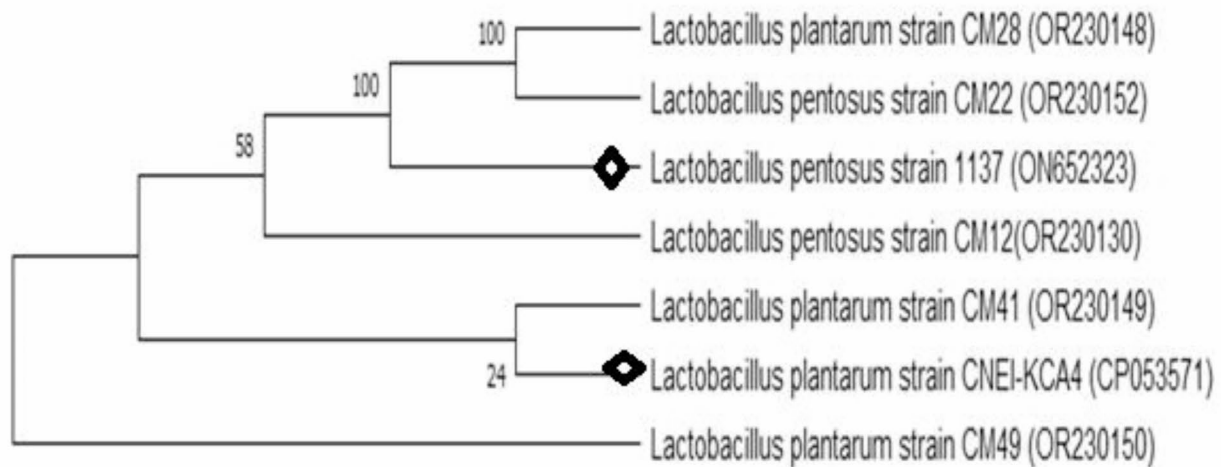


Fig. 1 Phylogenetic tree generated using the neighbor-joining method by MEGA software

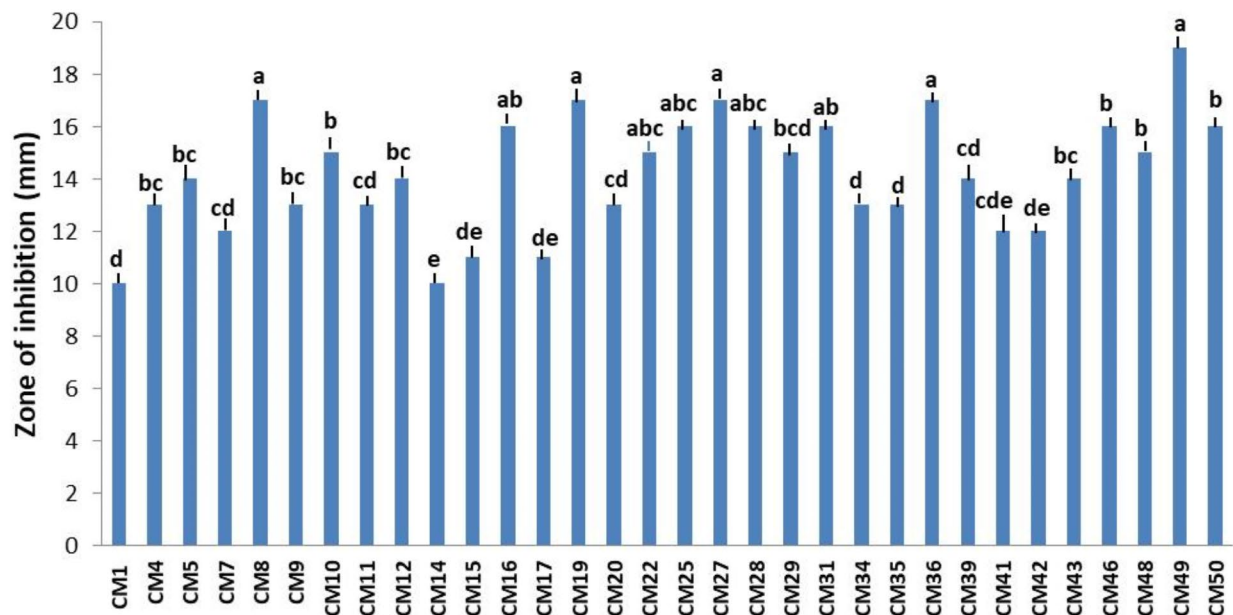


Fig. 2 Anti-*Staph. aureus* activity of CFS of lactobacilli. The non-significant ($p > 0.05$) difference between isolates is indicated by a-e bars with the same alphabet, and the significant difference ($p < 0.05$) between isolates is indicated by bars with distinct alphabets

The co-aggregation activity of the isolates after 2 h ranged from $15.43 \pm 0.17\%$ to $55.74 \pm 0.74\%$. Isolate CM49 exhibited the highest percentage of co-aggregation activity ($55.74 \pm 0.74\%$, $51.99 \pm 0.41\%$ and $49.41 \pm 0.57\%$) after the 2-hour time interval, followed by isolate CM22 ($52.84 \pm 0.57\%$, $45.32 \pm 0.45\%$ and $44.45 \pm 0.71\%$) against bovine mastitogens (*Staph. aureus*, *E. coli*, and *Strep. dysgalactiae*) respectively. Isolates CM4 and CM9 displayed poor co-aggregation activity after the 2-hour interval compared to the control LGG isolate activity

($46.07 \pm 0.70\%$, $40.07 \pm 0.70\%$ and $39.07 \pm 0.70\%$) against *Staph. aureus*, *E. coli* and *Strep. dysgalactiae*, as detailed in Supplementary Table S1.

Significant differences ($p < 0.05$) exist between means that do not share a letter in the same column.

Antibiogram of selected lactobacilli

The study results indicated that lactobacilli exhibited high levels of resistance to vancomycin (32/32, 100%) and polymyxin B (32/32, 100%). On the other hand, they

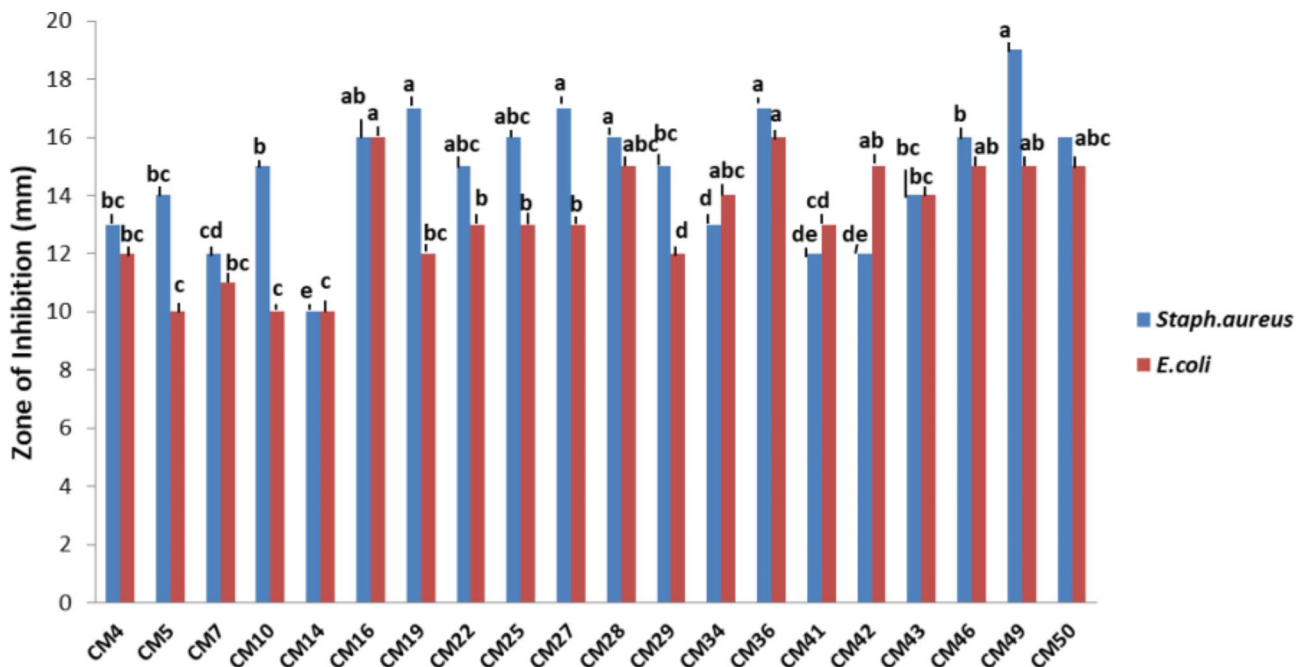


Fig. 3 Anti-*Staph. aureus* and anti-*E. coli* activity of CFS of lactobacilli. The non-significant ($p > 0.05$) difference between isolates is indicated by a-e bars with the same alphabet and the significant difference ($p < 0.05$) between isolates is indicated by bars with distinct alphabets

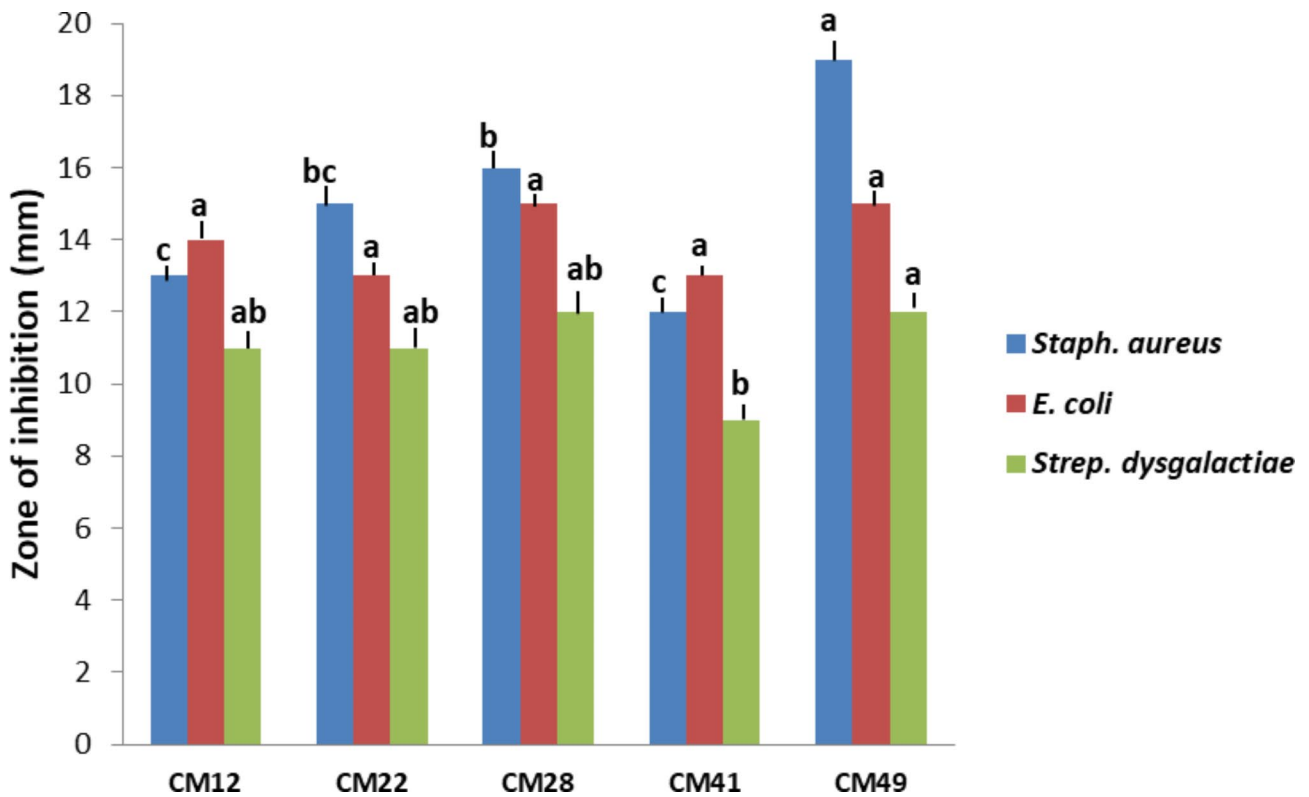


Fig. 4 Anti-mitogens activity of CFS of lactobacilli against *Staph. aureus*, *E. coli* and *Strep. dysgalactiae*. The non-significant difference between isolates is indicated by a-c bars with the same alphabet and the significant difference between isolates is indicated by bars with distinct alphabets

Table 1 Auto-aggregation activity of lactobacilli after 1 and 2 h

Isolates	Percentage Auto-aggregation (Mean ± SD)	
	1 h	2 h
CM1	24.85 ± 0.55 ^m	29.37 ± 0.67 ⁿ
CM4	15.38 ± 0.32 ^m	19.23 ± 0.14 ^p
CM5	28.77 ± 0.44 ^{gh}	41.03 ± 0.46 ^{hij}
CM7	11.51 ± 0.43 ^{nop}	21.58 ± 0.5 ^{op}
CM8	10.1 ± 0.41 ^p	15.33 ± 0.71 ^q
CM9	12.5 ± 0.82 ^{no}	15.62 ± 0.68 ^q
CM10	35.13 ± 0.92 ^{abc}	44.87 ± 1.12 ^g
CM11	12.41 ± 0.69 ^{no}	21.57 ± 0.54 ^{op}
CM12	24.47 ± 0.38 ^k	38.54 ± 1.01 ^{ijklm}
CM14	10.71 ± 0.80 ^{op}	14.29 ± 0.96 ^q
CM15	31.89 ± 0.58 ^{de}	40.74 ± 0.51 ^{hijk}
CM16	15.43 ± 0.44 ^m	30.86 ± 0.59 ⁿ
CM17	27.08 ± 0.66 ^{hij}	42.18 ± 0.47 ^{ghi}
CM19	31.31 ± 0.53 ^{ef}	42.30 ± 0.58 ^{gh}
CM20	18.31 ± 0.62 ^l	30.69 ± 0.54 ⁿ
CM22	30.43 ± 0.82 ^{efg}	58.08 ± 0.86 ^{bc}
CM25	9.69 ± 0.36 ^p	23.03 ± 1.08 ^o
CM27	34.67 ± 0.39 ^{bc}	39.19 ± 1.01 ^{ijkl}
CM28	14.97 ± 0.61 ^m	20.29 ± 0.56 ^{op}
CM29	30.34 ± 0.21 ^{efg}	37.81 ± 0.99 ^{klm}
CM31	13.16 ± 0.64 ⁿ	21.54 ± 0.33 ^{op}
CM34	28.69 ± 0.52 ^{ghi}	54.26 ± 0.80 ^{de}
CM35	26.02 ± 0.38 ^{jk}	36.22 ± 1.02 ^{lm}
CM36	36.59 ± 0.54 ^a	44.89 ± 1.44 ^g
CM39	26.81 ± 0.36 ^{ij}	35.75 ± 1.13 ^m
CM41	33.29 ± 0.99 ^{cd}	56.80 ± 0.75 ^{cd}
CM42	28.91 ± 0.77 ^{gh}	39.75 ± 0.66 ^{hijk}
CM43	33.29 ± 0.25 ^{cd}	50.80 ± 1.24 ^f
CM46	36.96 ± 0.39 ^a	60.58 ± 1.54 ^{ab}
CM48	29.58 ± 0.59 ^{fg}	52.08 ± 1.33 ^{ef}
CM49	35.71 ± 0.70 ^{ab}	62.11 ± 1.09 ^a
CM50	33.60 ± 0.52 ^{cd}	57.26 ± 1.06 ^{cd}
LGG	18.40 ± 0.30 ^l	38.58 ± 0.20 ^{klm}

showed lower levels of resistance to cefixime (16/32, 50%) and fusidic acid (10/32, 31%), while no antibiotic resistance was seen against all other six antibiotics, penicillin, amoxicillin, bacitracin, tetracycline, erythromycin and chloramphenicol. The resistance profiles of each isolate are detailed in Supplementary Table S2. Isolates CM1, CM10, CM12, CM25, CM28, CM35, CM42 and CM49, displayed susceptibility to most antibiotics tested.

Inhibition of mastitogens in broth culture by *Lactobacillus plantarum* CM49

The *Lb. plantarum* CM49 was co-cultured with bovine mastitogens (*Staph. aureus*, *E. coli* and *Strep. dysgalactiae*) in nutrient broth. The counts of these microorganisms were measured at four different time points: 0, 8, 16, and 24 h using their respective selective media. The

kinetics in terms of the mean log₁₀ cfu/gm for mastitogens are shown in Fig. 5.

Lactobacillus plantarum CM49 demonstrated the ability to reduce the viable counts of mastitogens at various time points in the experiment. The mean log₁₀ cfu/mL for *Staph. aureus* was 4.90 ± 0.11, 3.30 ± 0.21, and 2.20 ± 0.13 at 8, 16, and 24 h, respectively, compared to the initial count of 5.66 ± 0.10 cfu/mL. Similarly, the mean log₁₀ cfu/mL for *E. coli* was 5.01 ± 0.20, 3.54 ± 0.21 and 2.80 ± 0.16 at 8, 16 and 24 h, respectively compared to the initial count of 5.62 ± 0.23 cfu/mL. For *Strep. dysgalactiae*, the mean log₁₀ cfu/mL was 5.07 ± 0.16, 4.17 ± 0.14 and 3.5 ± 0.18 at 8, 16, and 24 h respectively, compared to the initial count of 5.62 ± 0.23 cfu/mL. The findings suggest that after 8 h, *Lb. plantarum* CM49 resulted in a non-significant decrease in bovine mastitogens count ($p > 0.05$), while a significant reduction ($p < 0.05$) was observed after 16 and 24 h, respectively, compared to the initial counts.

Inhibition of mastitogens adhesion on bovine mammary cell line by *Lactobacillus plantarum* CM49.

Various models, including Competition, Inhibition and Displacement, were tested to assess the ability of *Lb. plantarum* CM49 to inhibit the adhesion of mastitogens (*Staph. aureus*, *Strep. dysgalactiae* and *E. coli*) on the bovine mammary cell line. *Lb. plantarum* CM49 significantly inhibits the adhesion rates of mastitogens on the bovine mammary cell line in different experimental conditions, as shown in Table 2.

The results of the Competition showed that the adhesion values of mastitogens decreased when *Lb. plantarum* CM49 and mastitogens were added simultaneously. *Staph. aureus* exhibited a more substantial reduction in adhesion (69%), followed by *Strep. dysgalactiae* (64%) and *E. coli* (61%). In the Inhibition assay, the *Lb. plantarum* CM49 was first allowed to attach to the cells. Afterward, mastitogens were added, and the reduction in the attachment rate was measured as 77% for *Staph. aureus*, followed by *Strep. dysgalactiae* (69%) and *E. coli* (65%). The Inhibition assay showed that *Lb. plantarum* CM49 significantly ($P < 0.05$) inhibited *Staph. aureus* attachment, whereas *Staph. aureus* did not inhibit the *Lb. plantarum* CM49. Similarly, in Displacement, where mastitogens were first allowed to attach to cells, followed by *Lb. plantarum* CM49, the percentage inhibition was measured 61%, 54% and 50% for *Staph. aureus*, *E. coli* and *Strep. dysgalactiae* respectively.

Inhibition of *Staphylococcus aureus*-induced mastitis in a mouse model by *Lactobacillus plantarum* CM49

The results of this study indicated a decrease in the *Staph. aureus* count in both the treatment and preventive models as compared to a positive control group where the *Staph. aureus* count (cfu/gm) remained higher. The

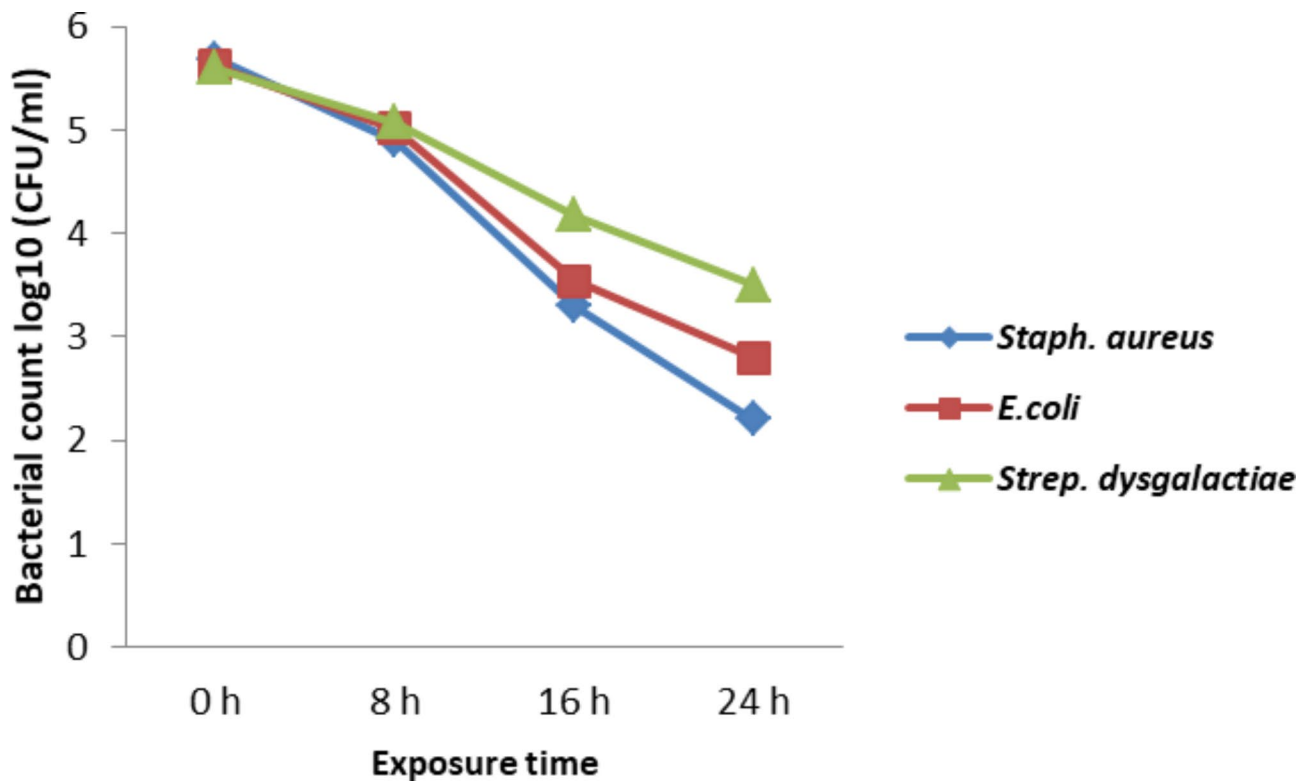


Fig. 5 Enumeration of mastitogens after different time intervals in co-culture assay

Table 2 Percentage inhibition in adhesion of mastitogens by *Lb. Plantarum* CM49

Mastitogens	Competition (M+L)	Inhibition (L/M)	Displacement (M/L)
<i>Staph. aureus</i>	69.49 ± 0.6 ^a	77.8 ± 0.75 ^a	61.09 ± 0.45 ^a
<i>Strep. dysgalactiae</i>	64.4 ± 0.56 ^b	69.63 ± 0.41 ^b	54.29 ± 0.3 ^b
<i>E. coli</i>	61.14 ± 0.48 ^b	65.07 ± 0.65 ^c	50.93 ± 0.44 ^b

Significant differences ($p < 0.05$) exist between means that do not share a letter in the same column. Adhesion inhibition of the control mastitogens was considered as 0%.

Competition (M+L): Mastitogen and *Lactobacillus* added simultaneously.

Inhibition (L/M): Mastitogen added after *Lactobacillus*.

Displacement (M/L): *Lactobacillus* added after mastitogen.

mean log₁₀ reduction of 1.25 and 1.9 in the *Staph. aureus* count in treatment and preventive models was noted. Results also revealed an increase of 3.01 mean log₁₀ and 1.73 mean log₁₀ for lactobacilli in the prophylactic and treatment models, respectively, as shown in Fig. 6 and Supplementary Table S3.

Discussion

The treatment of mastitis in cows typically involves using antibiotics for both therapeutic and prophylactic purposes during the drying-off period. However, due to the excessive and improper use of antibiotics, there is increasing concern over antibiotic resistance in bacteria, which has posed a significant challenge in this approach [12]. Hence, there is an immediate need to explore alternative methods such as using probiotics to manage mastitis in dairy cattle effectively. *Lactobacillus* species are widely used as dairy probiotics as they offer several benefits. These bacteria are considered safe [32] for the host and have been categorized as Generally Recognized As Safe (GRAS). *Lactobacillus* species are naturally found in various environments, including fermented foods and the GIT of both humans and animals [33, 34]. The bovine udder microbiota comprises diverse microorganisms, making it a valuable source for identifying effective probiotic strains [35].

From this research, 40 lactobacilli were isolated from the milk of healthy cattle. Among the 40 lactobacilli isolates, 32 showed anti-*Staph. aureus* activity, while 20 isolates exhibited activity against *Staph. aureus* and anti-*E. coli* activity. Five lactobacilli isolates (CM12, CM22, CM28, CM41, and CM49) demonstrated activity against all three selected mastitogens (*Staph. aureus*, *E. coli*, and *Strep. dysgalactiae*). Previous studies have successfully

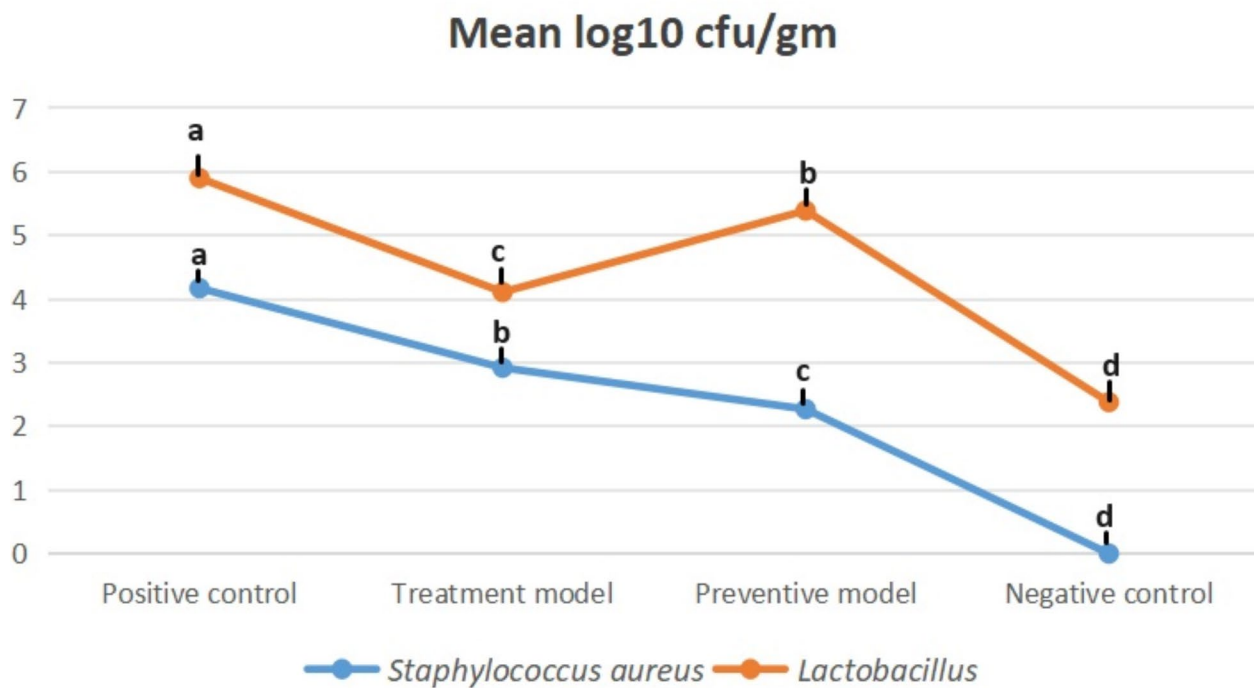


Fig. 6 Column graph showing mean log₁₀ cfu/gm of *Staphylococcus aureus* and lactobacilli. Significant differences ($p < 0.05$) exist between means that do not share a letter in the same group

isolated lactic acid bacteria from the raw milk of healthy cattle and examined their antimicrobial properties against mastitogens [36]. The species-level identification of the five selected lactobacilli revealed their classification as *Lb. plantarum* and *Lb. pentosus*. Our research findings align with previous studies that have similarly identified lactobacilli, such as *Lb. plantarum*, *Lb. fermentum*, and *Lb. acidophilus* [37]. Additionally [38], identified lactic acid bacteria from cow milk as a potential probiotic strain to prevent mastitis. Jimenez's study also identified *lactobacillus* strains, *Lb. salivarius* CECT5713 and *Lb. gasseri* CECT5714, from breast milk and found them to be an effective treatment for lactational infectious mastitis during lactation after oral administration [39]. Similarly, a recent study isolated 55 lactobacilli from healthy chickens and observed a significant reduction in *Salmonella gallinarum* in broilers [40].

The antimicrobial activity demonstrated by lactobacilli strains against three key mastitis pathogens is an attractive research area with two significant roles. Firstly, it aims to impede the invasion of bacterial strains identified as disease-causing agents. Secondly, it seeks to prevent new infections caused by multidrug-resistant bacterial strains [41]. The inhibitory impact of probiotics against mastitis pathogens is recognized as a crucial characteristic in selecting effective probiotic strains [42].

Lactobacillus plantarum CM49 used in this study was selected based on in vitro probiotic criteria, including antimicrobial activity against mastitogens, auto-aggregation, co-aggregation, antibiotic resistance profile and in vitro inhibition in broth culture. The mechanism of action of probiotics is still an area of ongoing research, and factors such as their ability to produce hydrogen peroxide, lactic acid, acetic acid, organic acids, and bacteriocins are believed to contribute to their inhibitory effects. Probiotics can also compete for nutrients, secrete antibacterial substances, modulate immune responses and cytokine patterns, competitively exclude pathogens, colonize mammary epithelial cells and promote the establishment of a healthy udder flora [43]. Kober further suggests that immunobiotics play a beneficial role in mammary gland immunobiology offering a potential preventive strategy for managing bovine mastitis and addressing antimicrobial resistance [44].

Autoaggregation and coaggregation are crucial characteristics to consider when selecting probiotics. Autoaggregation refers to the strain's ability to cluster together, indicating its tendency to adhere to epithelial cells. This property is unique to each microorganism and can predict its adhesion capability [45]. On the other hand, coaggregation plays a role in creating a barrier effect against the colonization of pathogens [46]. The study demonstrated a range of auto-aggregation among the lactobacilli

isolates, with values varying from 14 to 62% after 2 h. Similarly, co-aggregation values ranged from 25 to 56% for *Staph. aureus*, 18–51% for *E. coli*, and 15–49% for *Strep. dysgalactiae* after 2 h, with reduced co-aggregation activity observed after 16 h. This co-aggregation pattern was also noted between four strains of *Lb.*, specifically *Lb. acidophilus*, *Lb. plantarum*, *Lb. casei* and *Lb. reuteri*, and two strains of *Staph. aureus* [47]. Boris proposed that this inhibitory effect would impede the colonization of epithelial cells by pathogenic bacteria, thereby facilitating their elimination from the system [48].

Antibiotic resistance patterns of the isolates were also examined due to safety concerns regarding probiotics. The study's results indicated that some lactobacilli resisted vancomycin and polymyxin B only, while others showed resistance to vancomycin, polymyxin B, cefixime, and fusidic acid. However, no antibiotic resistance was observed against the six major antibiotics: penicillin, amoxicillin, bacitracin, tetracycline, erythromycin and chloramphenicol. Isolates that displayed acquired resistance to more than two antibiotics were considered unsuitable for probiotic use and excluded from further consideration. *Lactobacillus plantarum* CM49 was susceptible to this study's most commonly used antibiotics. It did not possess acquired resistance, indicating its safety for inclusion in the food chain without the risk of antibiotic resistance transfer. A previous study by Danielsen and Wind reported that lactobacilli isolated from milk exhibited chromosomal resistance to bacitracin, ciprofloxacin, kanamycin, gentamicin, metronidazole, streptomycin, and vancomycin [49, 50].

Another approach for determining the antibacterial action of probiotic lactobacilli is the co-culture assay, where two organisms are cultured together in broth to measure how one organism affects the growth of the other [51, 52]. *Lactobacillus plantarum* CM49 was selected for the co-culture experiment in this study due to its probiotic potential, antimicrobial efficacy against mastitogens in the well-diffusion assay, auto-aggregation and co-aggregation capabilities and lack of resistance to the majority of antibiotics studied. The outcomes showed that *Lb. plantarum* CM49 significantly reduced (>3.4, >2.5 and >1.4 log reduction) the growth of mastitogens (*Staph. aureus*, *E. coli*, and *Strep. dysgalactiae*) by more than 3.4, 2.5, and 1.4 log reductions, respectively. These findings align with those of Bilge et al., who found that lactobacilli-producing antimicrobial substances have a high potential for preventing the growth of pathogenic bacteria [53].

Afterward, *Lb. plantarum* CM49 was observed to inhibit the adhesion of mastitogens on the bovine mammary cell line. Specifically, in the Inhibition assay, *Lb. plantarum* CM49 significantly reduced the adhesion rate of mastitogens on the bMEC line, in the order of *Staph.*

aureus (77%)>*Strep. dysgalactiae* (69%)>*E. coli* (65%) indicating the higher adhesion capability of *Lb. plantarum* CM49 on bMEC when competing with mastitogens. After that, reduction in inhibition of mastitogens was also noted in the Competition and Displacement assays by *Lb. plantarum* CM49 on bMEC. Bouchard et al. reported that the capacity of *Lb. plantarum* to compete with pathogens for colonizing bMEC varies based on species and specific strains [30]. Previous findings also suggest that various strains of *Lb. casei*, among lactic acid bacteria (LAB) can prevent the colonization of bMECs by *Staph. aureus* RF122 up to 40% [42] while *Lb. gasseri* LA806 resulted in a five-fold decrease in the adhesion and internalization of *Staph. aureus* NB305 into bMECs [54].

We also assessed the preventive and therapeutic impacts of *Lb. plantarum* CM49 on *Staph. aureus*-induced mastitis in mice. In the treatment and preventive models, *Lactobacillus plantarum* CM49 led to a mean log₁₀ reduction of 1.25 and 1.9 in mastitogen counts, respectively. This indicates the ability of *Lactobacillus* to effectively compete with the mastitogen and inhibit their colonization in the mammary gland tissue of mice. A previous study have demonstrated strong anti-inflammatory effects of *Lb. plantarum* mice with *E. coli*-induced mastitis [55]. Furthermore, another recent study revealed that *Lb. casei* possesses diverse bioactivities, including anti-inflammatory and antioxidant properties, showing its preventive effectiveness against *E. coli*-induced mastitis in mice [56]. The novelty of this work lies in isolating lactobacilli from the milk of healthy cattle from different dairy farms experiencing a mastitis outbreak. The rationale behind choosing these particular dairy farms was based on the hypothesis: cattle that remain unaffected during mastitis outbreak may possess superior microbiota. By exploring the microbiota of these healthy cattle, it was anticipated that potential probiotic strains could be identified and selected for further investigation. To the best of our knowledge, this is the first study conducted in Pakistan to examine the probiotic capabilities of *Lb. plantarum* CM49 against bovine mastitogens both in vitro and in vivo.

Conclusions

From the results of the current study, it is concluded that *Lb. plantarum* CM49, an indigenous strain isolated from healthy cattle milk, has remarkable autoaggregation and co-aggregation ability and is safe regarding no acquired antibiotic resistance. Furthermore, *Lb. plantarum* CM49 can inhibit cattle mastitogens in the laboratory, competitively exclude mastitogens from bovine mammary cells, and ameliorate *Staph. aureus*-induced mastitis in mice. It is recommended that anti-mastitis activity

of *Lb. plantarum* CM49 may further be investigated in cattle before developing probiotic products based on this strain.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12866-024-03464-5>.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Acknowledgements

The authors acknowledge the Punjab Higher Education Commission for funding this work under project number PHEC/ARA/PIRCA/20211/9.

Author contributions

Conceptualization, Muhammad Zeeshan Izhar; Methodology, Muhammad Nawaz; Validation, Muhammad Avais; Formal analysis, Aftab Ahmad Anjum; Writing, original draft preparation, Muhammad Zeeshan Izhar; Investigation, Muhammad Zeeshan Izhar; Resources, Muhammad Nawaz and Tahir Yaqub; Writing, review and editing, Muhammad Zeeshan Izhar; Visualization, Muhammad Nawaz; Supervision, Muhammad Nawaz; project administration, Muhammad Nawaz and Tahir Yaqub; funding acquisition, Muhammad Nawaz; All authors have read and agreed to the published version of the manuscript.

Funding

The Punjab Higher Education Commission provided funding for this study under Project # PHEC/ARA/PIRCA/20211/9.

Data availability

The data sets generated and/or analyzed during the current study are available in the NCBI under Gene Bank accession numbers OR230148, OR230149, OR230150, OR230130 and OR230152.

Declarations

Consent for publication

Not Applicable.

Competing interests

The authors declare no competing interests.

Ethical approval

This research protocol was approved by the Institutional Guidelines of the Ethical Review Committee at the University of Veterinary and Animal Sciences Lahore, Pakistan.

Declaration of competing interest

The authors declare no conflict of interest.

Received: 2 June 2024 / Accepted: 16 August 2024

Published online: 22 August 2024

References

- Lakhani MO, Tauseef S, Chattha WA. Assessing the financial sustainability of a rural livestock practice: a case of Pakistan. *Agric Financ Rev*. 2023;83(2):286–98.
- Riaz M et al. Livestock integrated farming in rural area of Pakistan. in International Conference on Improving Tropical Animal Production for Food Security (ITAPS 2021). 2022. Atlantis Press.
- Sharun K, et al. Advances in therapeutic and management approaches of bovine mastitis: a comprehensive review. *Vet Q*. 2021;41(1):107–36.
- Azooz M, El-Wakeel SA, Yousef H. Financial and economic analyses of the impact of cattle mastitis on the profitability of Egyptian dairy farms. *Vet World*. 2020;13(9):1750.
- Raspanti CG, et al. Prevalencia Y sensibilidad a antibióticos de especies de estafilococos coagulasa negativos provenientes de mastitis subclínica en bovinos de tambos de la región central de Argentina. *Rev Argent Microbiol*. 2016;48(1):50–6.
- Liu J et al. Epidemiological investigation of *Staphylococcus aureus* infection in dairy cattle in Anhui, China. *Pak Vet J*. 2022.
- Bangar YC, et al. A systematic review and meta-analysis of prevalence of sub-clinical mastitis in dairy cows in India. *Trop Anim Health Prod*. 2015;47:291–7.
- Vissio C, et al. Accuracy of the composite somatic cell count to detect intramammary infection in dairy cows using latent class analysis. *Prev Vet Med*. 2014;113(4):547–55.
- Lin J, et al. Mechanisms of antibiotic resistance. *Front Microbiol*. 2015;6:123658.
- Sipahi N, Kekeç Al, Halaç B. In vitro effect of some essential oils against multiple antibiotic-resistant bacteria from cats and dogs. *Pak Vet J*. 2022;42:561–5.
- Dad RK et al. Evaluating the effectiveness of Multidrug Resistant *Staphylococcus aureus* Mastitis vaccines in dairy cattle. *Pak Vet J*. 2022; 42(3).
- Rajoka MSR, et al. Isolation and evaluation of probiotic potential of lactic acid bacteria isolated from poultry intestine. *Microbiol*. 2018;87:116–26.
- Chouhan S, Sharma K, Guleria S. Antimicrobial activity of some essential oils—present status and future perspectives. *Med*. 2017;4(3):58.
- Murtaza N, et al. Impact of *Limosilactobacillus fermentum* probiotic treatment on gut microbiota composition in sahiwal calves with rotavirus diarrhea: a 16S metagenomic analysis study. *BMC Microbiol*. 2024;24(1):1–13.
- Rashid S et al. *Bacillus*-based Probiotics: an Antibiotic Alternative for the treatment of salmonellosis in Poultry. *Pak Vet J*. 2023; 43(1).
- Ullah Q et al. Efficacy prediction of *Lactobacillus rhamnosus* GG and platelet-rich plasma (PRP) against Sub-clinical Bubaline Mastitis. *Pak. Vet J*. 2023; 43(4).
- Pokorná A, Maňáková T, Čížek A. Properties of potentially probiotic *Lactobacillus* isolates from poultry intestines. *Acta Vet Brno*. 2019;88(1):73–84.
- Saeed A, et al. *Streptococcus lactarius* MB622 and *Streptococcus salivarius* MB620 isolated from human milk reduce chemokine IL-8 production in response to TNF- α in Caco-2 cell line, an exploratory study. *Cytokine*. 2023;168:156232.
- Malik SS, et al. Anticarcinogenicity of microbiota and probiotics in breast cancer. *Int J Food Prop*. 2018;21(1):655–66.
- Salman MM, et al. Exploring the milk microbiota of healthy and Mastitic Nili Ravi Buffalo using 16S rRNA gene base metagenomic analysis. *J Anim*. 2023;13(14):2298.
- Salman MM, et al. Investigation of milk microbiota of healthy and mastitic Sahiwal cattle. *BMC Microbio*. 2023;23(1):304.
- McFadden M, et al. California mastitis test and milk quality. *Mich Dairy Rev*. 2011;16(2):1–3.
- Nawaz M, et al. Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. *Curr Microbiol*. 2011;62:1081–9.
- Bao Y, et al. Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. *Food Control*. 2010;21(5):695–701.
- Saeed A, et al. Unveiling the antibiotic susceptibility and antimicrobial potential of bacteria from human breast milk of Pakistani women: an exploratory study. *Biomed Res Int*. 2023;2023(1):6399699.
- Saleem N et al. Phenotypic and Molecular Analysis of Antibiotic Resistance in Lactobacilli of Poultry Origin from Lahore, Pakistan. *Pak Vet J*. 2018; 38(4).
- Khan I et al. Isolation and in vitro characterization of Anti-*Salmonella Enteritidis* Probiotic potential of indigenous lactobacilli from Poultry. *Pak Vet J*. 2019; 39(4).
- Sattari Z, et al. Bovine mammary epithelial cells can grow and express milk protein synthesis genes at reduced fetal bovine serum concentration. *Cell Biol Int*. 2024;48(4):473–82.
- Vachkova E et al. Culturing of primary bovine mammary epithelial cells and validation of biotransformation capacity in experiments with enrofloxacin. *Bulg J Vet Med*. 2021; 24(1).
- Bouchard DS, et al. Inhibition of *Staphylococcus aureus* invasion into bovine mammary epithelial cells by contact with live *Lactobacillus casei*. *Appl Environ Microbiol*. 2013;79(3):877–85.
- Breyne K, et al. A pilot study using a mouse mastitis model to study differences between bovine associated coagulase-negative staphylococci. *JDS*. 2015;98(2):1090–100.

32. Gul ST, Alsayeqh AF. Probiotics as an Alternative Approach to Antibiotics for Safe Poultry Meat production. *Pak Vet J.* 2022. 42(3).
33. Sornplang P, et al. Antibiotic resistance of lactic acid bacteria isolated from a fermented fish product, Pla-Chom. *Res J Microbiol.* 2011;6(12):898.
34. Kerry RG, et al. Benefaction of probiotics for human health: a review. *JFDA.* 2018;26(3):927–39.
35. Kable ME, et al. The core and seasonal microbiota of raw bovine milk in tanker trucks and the impact of transfer to a milk processing facility. *MBio.* 2016;7(4). <https://doi.org/10.1128/mbio.00836-16>.
36. Mithun S, Dipak V, Sheela S. Isolation and identification of lactobacilli from raw milk samples obtained from Aarey Milk Colony. *IJSRP.* 2015;5(4):1–5.
37. Espeche MC, et al. Screening of surface properties and antagonistic substances production by lactic acid bacteria isolated from the mammary gland of healthy and mastitic cows. *Vet Microbiol.* 2009;135(3–4):346–57.
38. Pellegrino MS, et al. In vitro characterization of lactic acid bacteria isolated from bovine milk as potential probiotic strains to prevent bovine mastitis. *Probiotics Antimicro.* 2019;11:74–84.
39. Jiménez E et al. Oral administration of *Lactobacillus* strains isolated from breast milk as an alternative for the treatment of infectious mastitis during lactation. *Am Soc Microbiol.* 2008.
40. Vélez MP, et al. Identification and characterization of starter lactic acid bacteria and probiotics from colombian dairy products. *J Appl Microbiol.* 2007;103(3):666–74.
41. Bouchard DS, et al. Lactic acid bacteria isolated from bovine mammary microbiota: potential allies against bovine mastitis. *PLoS ONE.* 2015;10(12):e0144831.
42. Wang Y, Gu Q. Effect of probiotic on growth performance and digestive enzyme activity of Arbor Acres broilers. *Res Vet Sci.* 2010;89(2):163–7.
43. Kober AH, et al. Immunomodulatory effects of probiotics: a novel preventive approach for the control of bovine mastitis. *Microorganisms.* 2022;10(11):2255.
44. Gueimonde M, Salminen S. New methods for selecting and evaluating probiotics. *Dig Liver Dis.* 2006;38:S242–7.
45. Soleimani NA, et al. Antagonistic activity of probiotic lactobacilli against *Staphylococcus aureus* isolated from bovine mastitis. *Afr J Microbiol Res.* 2010;4(20):2169–73.
46. Bogni C et al. War against mastitis: current concepts on controlling bovine mastitis pathogens. *Sci against Microb Pathogens: Communicafing Curr Res Technological Adv.* 2011; p. 483–94.
47. Boris S, et al. Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. *Infect Immune.* 1998;66(5):1985–9.
48. Danielsen M, Wind A. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *Int J Food Microbiol.* 2003;82(1):1–11.
49. Temmerman R, et al. Identification and antibiotic susceptibility of bacterial isolates from probiotic products. *Int J Food Microbiol.* 2003;81(1):1–10.
50. Lim SM, Im DS. Screening and characterization of probiotic lactic acid bacteria isolated from Korean fermented foods. *J Microbiol Biotechnol.* 2009;19(2):178–86.
51. Dowarah R, et al. Selection and characterization of probiotic lactic acid bacteria and its impact on growth, nutrient digestibility, health and antioxidant status in weaned piglets. *PLoS ONE.* 2018;13(3):e0192978.
52. Çadirci BH, Çitak S. A comparison of two methods used for measuring antagonistic activity of lactic acid bacteria. *Pak J Nutr.* 2005;4(4):237–41.
53. Blanchet F, et al. Heat inactivation partially preserved barrier and immunomodulatory effects of *Lactobacillus gasserii* LA806 in an in vitro model of bovine mastitis. *Benef Microbes.* 2021;12(1):95–106.
54. Li K, et al. The Prevention Effect of *Lactobacillus plantarum* 17–5 on *Escherichia coli*-Induced mastitis in mice. *Probiotics Antimicrob.* 2023;15(6):1644–52.
55. Li K, et al. The preventive effects of *Lactobacillus casei* 03 on *Escherichia coli*-induced mastitis in vitro and in vivo. *J Inflamm Res.* 2024;21(1):1–9.

Publisher's note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.