

## Antibacterial effect of postbiotics derived from *Lactobacillus acidophilus* against mastitis causing pathogens

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**Abstract:** The current study evaluated the antibacterial efficacy of postbiotics derived from probiotic *Lactobacillus acidophilus* (strain UBLA-34) against two major mastitis-causing pathogens, *Staphylococcus aureus* and *Streptococcus agalactiae*. The postbiotic was prepared by culturing *L. acidophilus* in MRS broth followed by heat inactivation, ultrasonication, centrifugation, filtration and lyophilization. Antibacterial activity was assessed using disc diffusion method. Preliminary trials were conducted to select the concentration of postbiotics. Based on the results, 600, 700 and 800 mg/mL of postbiotics were selected for studying the antibacterial effect. The results demonstrated a concentration-dependent antibacterial effect against both pathogens. For *S. aureus*, zones of inhibition (ZOI) measured were  $13.58 \pm 0.30$ ,  $17.16 \pm 0.33$  and  $19.25 \pm 0.11$  mm at postbiotic concentrations of 600, 700 and 800 mg/mL respectively. The minimum inhibitory concentration (MIC) was identified as 450 mg/mL (ZOI:  $10.17 \pm 0.25$  mm). Similarly, *S. agalactiae* exhibited ZOIs of  $14.83 \pm 0.33$ ,  $17.92 \pm 0.24$  and  $19.25 \pm 0.11$  mm for the respective concentrations. The MIC was found to be 450 mg/mL (ZOI:  $11.00 \pm 0.26$  mm). Comparative analysis indicated that *S. agalactiae* displayed slightly higher susceptibility at lower concentrations of postbiotics than *S. aureus*. The findings underscore the potential of *L. acidophilus*-derived postbiotics as natural antibacterial agents against mastitis causing pathogens. Thus postbiotics offer a promising alternative strategy for mastitis management amidst rising concerns of antibiotic resistance.

**Keywords:** Antibacterial activity, *L. acidophilus*, Minimum inhibitory concentration. Postbiotics, Zone of inhibition,

### Introduction

Mastitis is the inflammation of the udder, that leads to huge economic loss due to the reduction in milk yield and inferior quality of milk. Bacterial intra mammary infection is the predominant reason for mastitis. The symptoms of mastitis include swollen udders, fever and an elevated somatic cell count (SCC) due to immune responses. The predominant causes of mastitis include the pathogens *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae* (*S. agalactiae*), *Streptococcus dysgalactiae* and *Escherichia coli*. Bovine mastitis, primarily caused by *Staphylococcus aureus*, poses a major threat to both the dairy economy and public health because of its virulence traits and ability to develop antibiotic resistance. Overuse of antibiotics aggravates this issue by promoting resistance and leaving residues in milk. Probiotics serve as a promising and sustainable solution by suppressing *S. aureus* virulence factors, safeguarding the mammary glands, lowering the occurrence of mastitis and improving milk quality and safety (Yousif et al. 2025)

Mastitis pathogens are generally classified as contagious or environmental, depending upon their primary reservoir and method of transmission. *S. aureus* is the most representative contagious pathogen transmitted through milking equipment and teat skin. *Enterococcus faecalis* and *Pseudomonas aeruginosa* are the main environmental and opportunistic pathogens. Presently, administration of antibiotics is the most common treatment option for mastitis. Owing to the development of antibiotic resistance in mastitis causing pathogens, there is an urgent need for alternative methods to treat and prevent the disease. Postbiotics consist of inactivated microbial cells, including cell bodies, cell fissures and cellular metabolites such as short-chain fatty acids and enzymes. Postbiotics have different functional properties such as antimicrobial, antioxidant, anti-inflammatory, hypocholesterolemic and immunomodulatory activities and have various applications in food and pharmaceutical industry. The mechanism of action of postbiotics is mediated by bioactive compounds, such as peptides and

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organic acids produced during fermentation. These compounds can directly benefit health by modulating the gut microbiome. Several studies highlighted the potential use of postbiotics in the prevention and treatment of gastro-intestinal diseases. Along with their effects on the absorption of healthy substances, postbiotics play a role in the prevention of cancer, improvement of mental health and other therapeutic qualities (Isaac-Bamgboye et al. 2024). However, the available information regarding the antibacterial effect of postbiotics against mastitis causing bacteria is limited. Hence the study is designed to assess the antibacterial activity of postbiotics derived from *Lactobacillus acidophilus* against *S. aureus* and *S. agalactiae*.

## Materials and Methods

**Collection of bacterial cultures:** The standard culture of *Staphylococcus aureus* (MTCC 1144) was obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh. Standard culture of *Streptococcus agalactiae* (MCC 3038) was obtained from National centre for Microbial Resource (NCMR), Pune and probiotic *Lactobacillus acidophilus* (UBLA-34) was procured from Unique Biotech, Hyderabad.

## Preparation of postbiotics from *Lactobacillus acidophilus*

*Lactobacillus* strains were cultured in MRS broth and incubated at 37±1 °C for 48 h in a CO<sub>2</sub> incubator. Then the bacterial suspension was inactivated for 121 °C for 15 min. The inactivated suspension was ultra sonicated in an ice bath at 300 W, with the cycles of 10s on/10s off for 15 min. Ultrasonication was carried out using a probe-type homogenizer, with the probe inserted approximately 1.5–2.0 cm into the liquid and positioned clear of the container walls and bottom to avoid loss of efficiency. This method ensured effective cell lysis without compromising the sample. A microscopic examination confirmed the absence of intact bacteria. The suspension was centrifuged for 10 min at 6000 rpm at 4°C to remove the bacterial fragments. The cell free supernatant was harvested and filtered through a 0.45µm and 0.22µm membrane filters and the filtered cell free supernatant was lyophilized and used as postbiotics (Moradi et al. 2019).

## Agar disc diffusion method

The antimicrobial activity of postbiotics against *S. aureus* and *S. agalactiae* was determined by agar disc diffusion method described by Tejero-Sariñena et al. (2012). Standardized suspensions of *Streptococcus agalactiae* and *S. aureus* (0.5 McFarland) were prepared and plated onto Mueller-Hinton agar for bacterial growth. Discs were loaded with three different levels of postbiotic solution. These discs were placed in Petri dishes containing the test organism, then plates were incubated for 24 h at 37°C. After incubation the zone of inhibition (ZOI) was measured. The diameters of the clear zones surrounding the discs (inhibition zones) were measured in millimetres and represented

as antibacterial activity of each postbiotic against the target pathogens. Each experiment was performed in six replications and the average inhibition zone diameter was calculated (Sevin et al. 2021).

## Minimum inhibitory concentration

Minimum Inhibition Concentration (MIC's) was determined using Inhibitory Concentrations in Diffusion (ICD) method. It was done by carrying out the diffusion test with discs of different concentration of the postbiotics. Bacterial culture (which has been adjusted to 0.5 McFarland standard), was used to lawn Muller Hinton agar plates evenly using a sterile swab. Discs treated with postbiotics were placed in Muller Hinton agar plates. The plate was then incubated at 37°C for 18 to 24 hours. After the incubation, the plates were examined for zone of inhibition. The lowest concentration that inhibits the growth of bacteria was noted and considered as the MIC value for each of the bacterial strain tested. The procedure was done as per Guerin-Faublec et al. (1996) with slight modification.

## Statistical analysis

Six replications were performed and the data collected were analysed using SPSS version 24.0. The data are reported as Mean ± Standard Error for the measurements of zone of inhibition in the antimicrobial activity tests and one-way ANOVA was used to compare the mean values.

## Results and discussion Antibacterial activity of postbiotics derived from *L. acidophilus*

Antibacterial activity of postbiotics derived from *L. acidophilus* against *S. aureus* and *S. agalactiae* was determined using disc diffusion method. Preliminary trials were conducted to select the effective concentration of postbiotic solution based on the zone of inhibition against *S. aureus* and *S. agalactiae*. Postbiotic solutions were evaluated for their antibacterial activity against test organisms at concentrations of 100, 200, 300, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900 mg/mL. Diameter of Zone of inhibition increased progressively with the increase in concentration of postbiotics. Based on the preliminary trials, four concentrations of postbiotics (450, 600, 700 and 800mg/mL) were selected for further study. At these concentrations, the growth of *S. aureus* and *S. agalactiae* were effectively inhibited, as indicated by significant zones of inhibition. The results demonstrated that *L. acidophilus* postbiotics exhibited potent antibacterial activity against these organisms at tested concentrations. Coconnier et al. (1997) confirmed the inhibitory capacity of *L. acidophilus* against both Gram-positive and Gram-negative bacteria, such as *Salmonella typhimurium*, *E. coli* and *S. aureus*. More recently, the study by Khani et al. (2025) reported concentration-dependent antibacterial activity of postbiotics derived from *L. acidophilus* against *S. aureus*, aligning closely with the trends observed in the present investigation. The

similarity between current and previous findings strengthens the evidence for the potential application of *L. acidophilus* postbiotics as effective antibacterial agents, particularly against common mastitis causing pathogens. The zone of inhibitions obtained against *S. aureus* and *S. agalactiae* in preliminary trials are summarized in Table 1.

**Antibacterial activity of *L. acidophilus* against *S. aureus***

The antibacterial activity of postbiotics was assessed using disc diffusion method. Discs were loaded with selected concentrations of postbiotic solution such as 450, 600, 700 and 800 mg/mL based on the preliminary trials. The discs were placed on Petri dishes inoculated with *S. aureus* and incubated for 24 hours at 37°C. After incubation, zones of inhibitions (ZOI) were measured. Postbiotics at all the four concentrations inhibited the growth of *S. aureus* with significant zone diameters. The zone of inhibition at 450 mg/mL was 10.17 ± 0.25 mm which indicated weakest antibacterial effect. The zone of inhibition at 600 and 700 mg/mL were 13.58 ± 0.30 mm and 17.16 ± 0.33 mm, respectively, indicating

moderate antibacterial activity. The highest antibacterial effect was observed at 800 mg/mL, with a zone diameter of 18.92 ± 0.24 mm. These findings align with several previous studies that highlight the antimicrobial potential of *L. acidophilus* and other probiotic-derived postbiotics. For instance, Kareem et al. (2014) reported potential enhancement of postbiotic activity of *L. plantarum* through the incorporation of inulin, resulting in inhibition zones ranging from 7.66 to 12.16 mm against various pathogens. The present study revealed that postbiotics derived from *L. acidophilus* exhibited superior antibacterial activity against *S. aureus* with greater zones of inhibition. Georgieva et al. (2015) also demonstrated the effectiveness of antibacterial substances produced by *Lactobacillus* and *Bifidobacterium* spp., with inhibition zones of >10 mm against *S. aureus*, *E. coli* and *B. cereus*, supporting the antibacterial efficacy shown in our findings. Noori et al. (2023) found that postbiotics from *Lactobacillus casei* produced inhibition zones up to 22 mm against *Salmonella enterica* and 19 mm against *S. aureus*. Although these values are slightly higher than those observed in the current study, they fall within a similar range and strengthen

**Table 1:** Zone of inhibition of postbiotics of *L. acidophilus* against *S. aureus* and *S. agalactiae* (Mean ± SE)

<i>L. acidophilus</i> Postbiotic concentration (mg/mL)	Zone of inhibition(mm) <i>S. aureus</i> (Mean ± SE)	Zone of inhibition(mm) <i>S.</i> <i>agalactiae</i> (Mean ± SE)
100	6.50 ± 0.29	6.66 ± 0.29
200	7.16 ± 0.17	7.83 ± 0.16
300	7.50 ± 0.29	8.00 ± 0.29
400	9.33 ± 0.17	9.50 ± 0.29
450	10.00 ± 0.29	11.17 ± 0.17
500	11.33 ± 0.33	11.83 ± 0.44
550	11.67 ± 0.17	12.50 ± 0.29
600	13.58 ± 0.30	14.00 ± 0.29
650	14.50 ± 0.29	15.00 ± 0.29
700	17.00 ± 0.57	17.33 ± 0.33
750	18.16 ± 0.17	18.33 ± 0.29
800	18.33 ± 0.17	19.00 ± 0.29
850	18.83 ± 0.17	19.16 ± 0.17
900	19.16 ± 0.33	19.33 ± 0.17

Means are averages of three replications.

**Table 2:** Zone of inhibition

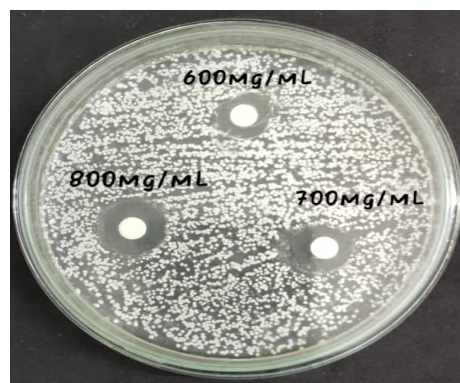
Postbiotic concentration (mg/mL)	Zone diameter (mm) against <i>S.</i> <i>aureus</i> (Mean ± SE)	Zone diameter (mm) against <i>S.</i> <i>agalactiae</i> (Mean ± SE)
450	10.17 <sup>a</sup> ± 0.25	11.00 a ± 0.26
600	13.58 <sup>b</sup> ± 0.30	14.83 b ± 0.33
700	17.16 <sup>c</sup> ± 0.33	17.92 c ± 0.24
800	18.92 <sup>d</sup> ± 0.24	19.25 d ± 0.11

Means are averages of six replications.

Means having different superscript differ significantly (p<0.05) with in a column.



**Fig. 1** Zone of inhibition against *S. aureus*



**Fig. 2** Zone of inhibition against *S. agalactiae*

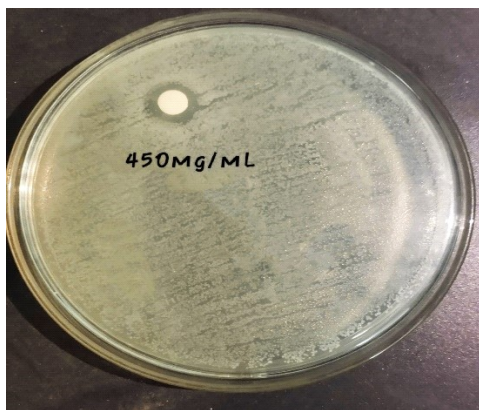
the concept that postbiotics from different *Lactobacillus* species have promising antimicrobial properties against pathogens. Tumbarski et al. (2025) assessed the antimicrobial activity of the four *Lactobacillus* strains using agar well diffusion method against *S. aureus*, *Bacillus subtilis*, *E. coli* and *Pseudomonas aeruginosa*. Inhibition zones were measured at 24 and 48 hours. Antimicrobial activity was classified based on zone size:  $\geq 18$  mm was considered as high antimicrobial activity, 12–18 mm was indicated as moderate antimicrobial activity and  $< 12$  mm was indicated low antimicrobial activity. This classification provided a useful framework for interpreting the present results. In the present study, the zone of inhibition observed at 800 mg/mL could be considered as high antimicrobial activity, while the zones at 600 and 700 mg/mL represented moderate antibacterial activity. The results of the present study confirm the previous reports. The mean diameters of zone of inhibition against *Staphylococcus aureus* are presented in Table 2 and shown in figure 1.

#### Antibacterial activity of *L. acidophilus* against *S. agalactiae*

The disc diffusion method was used to evaluate the antibacterial activity of postbiotics. Based on preliminary trials, discs were loaded with postbiotic solutions at concentrations of 450, 600, 700 and 800 mg/mL. These discs were placed onto Petri dishes containing *S. agalactiae* and incubated for 24 hours at 37°C. Following incubation, the diameters of the zone of inhibition were recorded. All the four concentrations of postbiotics demonstrated effective inhibition of *S. agalactiae*. The zone of inhibition obtained at 450 mg/mL was  $11.00 \pm 0.26$  mm. The zone diameter at 600 mg/mL was  $14.83 \pm 0.33$  mm and 700 mg/mL was  $17.92 \pm 0.24$  mm which indicates moderate antibacterial activity. The strongest inhibitory effect was observed at 800 mg/mL, with zone diameter of  $18.92 \pm 0.24$  mm. The result obtained indicates that the postbiotic showed higher antibacterial activity at 800mg/mL concentration against *S. agalactiae*. Al-Dohail et al. (2011) evaluated the use of *L. acidophilus* against *Staphylococcus* spp, *Aeromonas hydrophila* and *S. agalactiae* in African catfish. *L. acidophilus* exhibited the strongest inhibitory effect against *S.*

*agalactiae* and the weakest against *Aeromonas hydrophila*. These findings confirmed the effectiveness of *L. acidophilus* against *S. agalactiae*. The current study's results agreed with the findings reported by Sevin et al. (2021). They evaluated the antibacterial effects of postbiotics derived from *Lactobacillus sakei* on mastitis-causing pathogens such as *S. agalactiae*, *E. coli* and *S. aureus* in dairy cows. The postbiotics exhibited strong antibacterial activity primarily due to lactic acid (21.42 mg/mL), with a minimum inhibitory concentration of 40–50 mg/L. The reports of Lin et al. (2022) further support the relevance of strain selection in postbiotic efficacy. Their study showed that a multi-strain postbiotic extract containing *L. acidophilus* exhibited effective inhibition against *S. agalactiae*, among other pathogens. In the present study, even though a single strain of *L. acidophilus* was used, it exhibited significant antibacterial action. Moreover, Noori et al. (2023) reported strong antibacterial activity of *Lactobacillus casei* postbiotics against various pathogens. Inhibition zones reported were 20 mm for *L. monocytogenes* and 22 mm for *S. enterica*. Additionally, strong antibacterial activity was observed against *S. aureus* (19 mm) and *Bacillus cereus* (18 mm). The results of this investigation closely aligned with those of the research conducted by Tumbarski et al. (2025), which used the agar well diffusion method to evaluate the antibacterial activity of four *Lactobacillus* strains against *S. aureus*, *Bacillus subtilis*, *E. coli* and *Pseudomonas aeruginosa*. In the present study high antimicrobial activity was observed at a postbiotic concentration of 800 mg/mL with inhibition zone diameter of 19.25 mm. The results are comparable with the trend observed in other studies and confirms the effectiveness of *L. acidophilus* derived postbiotics against *S. agalactiae*. The results of the present research study are in agreement with previous research and underscore the promising antibacterial properties of *L. acidophilus* postbiotics, particularly at higher concentrations against *S. agalactiae*. The mean diameter of zones of inhibition against *Streptococcus agalactiae* are presented in Table 2. and depicted in Figure 2.

#### Minimum inhibitory concentration of *S. aureus* and *S. agalactiae*



**Fig. 3** The minimum zone of inhibition against *S. agalactiae*

The preliminary trials were conducted to assess the antibacterial activity of postbiotics at different concentrations, against *S. aureus* and *S. agalactiae*. Among the tested concentrations, 450 mg/mL demonstrated the weakest antibacterial effect against both bacterial strains. The minimum zone of inhibition against *S. agalactiae* and *S. aureus* are depicted in figure 3 and 4. For *S. aureus*, the inhibition zone measured was  $10.17 \pm 0.25$  mm and it showed lowest antibacterial activity. Similarly, for *S. agalactiae*, the same concentration produced a smaller inhibition zone of  $11.00 \pm 0.26$  mm. These values fall within the low antimicrobial activity category as proposed by Tumbarski et al. (2025). Hence 450 mg/mL was identified as the minimum inhibitory concentration (MIC) for both pathogens. These results suggested that 450 mg/mL concentration was least effective in preventing bacterial growth. Kim et al. (2005) compared the antibacterial activity of natural extracts, derived from *Curcuma longa* and found that 2 mg/mL of ethanolic extract showed minimum antibacterial effect against MRSA, while methanol and water extracts exhibited less potent activity. Though these MIC values are less than the values observed in the current investigation, the comparison underscores variability of antimicrobial activity based on the source and chemical nature. Islam et al. (2008) evaluated the minimum inhibitory concentration of cloxacillin against ten Methicillin resistant *S. aureus* (MRSA) strains. The MIC values were found to be  $e^{-32}$  mg/mL for five strains,  $e^{-128}$  mg/mL for one strain and above  $e^{-128}$  mg/mL for four strains. Wiegand et al. (2008), stated that MIC represents the lowest concentration of an antimicrobial agent that visibly inhibits bacterial growth. Georgieva et al. (2015) produced antibacterial substances by cultivating *Lactobacillus* and *Bifidobacterium* spp. in MRS broth with 10 per cent skim milk. They concentrated and stored the cell-free supernatants (CFS) following lyophilization, centrifugation, pH correction, filtration and incubation. The inhibition zone diameter of  $e^{-10}$  mm against test microorganisms such as *S. aureus* and *Bacillus cereus* was considered as MIC. The MIC value was more than 256 mg/mL. Similarly, Khosravi et al. (2023) demonstrated that postbiotics produced by various lactic acid bacteria, including *L. acidophilus*, exhibited minimum



**Fig. 4** The minimum zone of inhibition against *S. aureus*

inhibitory concentrations ranging from 125 to 500 mg/mL against multiple Gram-positive bacterial species. The MIC observed in the current study also comes under this range. Noori et al. (2023) also demonstrated that antimicrobial activity of postbiotics derived from *Lactobacillus casei* showed the most effective results against Gram-positive and Gram-negative bacteria, including *Listeria monocytogenes* and *Salmonella enterica*. Inhibition zones reported were 20 mm for *L. monocytogenes* and 22 mm for *S. enterica*. Additionally, strong activity was observed against *S. aureus* (19 mm) and *Bacillus cereus* (18 mm). Fakharian et al. (2024) reported minimum inhibitory concentration values ranging from 250 to 500 mg/mL for postbiotics synthesised using cost effective substrates, including cheese whey and skim milk, which are in close agreement with the findings of the current study. This finding aligns partially with the results of Khani et al. (2025), who investigated the antibacterial, antibiofilm, antioxidant and cytotoxic properties of postbiotics derived from *L. acidophilus*. They reported a concentration-dependent antibacterial effect, where higher doses of postbiotics resulted in greater bacterial inhibition. Notably, their MIC was lower (100 mg/mL) when compared to the MIC observed in the present study, suggesting that the postbiotic composition, bacterial strain and experimental conditions significantly influence antimicrobial efficacy. The findings of this study are in agreement with the results reported by different researchers. The MIC value of 450 mg/mL identified in this study indicates the lowest concentration capable of inhibiting visible growth of *S. agalactiae* and *S. aureus*. However, the relatively small inhibition zones observed at this concentration reflect limited antimicrobial potency. In the present study, postbiotics derived from *L. acidophilus* exhibited significant antibacterial activity against mastitis-causing pathogens such as *S. aureus* and *S. agalactiae*. These findings underscore the potential of such bioactive compounds as alternative or complementary strategies for the prevention and management of mastitis. In view of the increasing challenge of antimicrobial resistance, the application of postbiotics offers a promising direction for future advancements in veterinary therapeutics.

## Conclusion

The study demonstrated that postbiotics derived from *Lactobacillus acidophilus* (strain UBLA-34) possess significant antibacterial activity against *S. aureus* and *S. agalactiae*, two major pathogens responsible for bovine mastitis. A clear dose-dependent inhibition pattern was observed, with higher concentrations yielding larger zones of inhibition. The minimum inhibitory concentration for both pathogens was found to be 450 mg/mL. These findings highlight the potential of *L. acidophilus* derived postbiotics as a promising alternative or complementary strategy to conventional antibiotics in mastitis management. A major limitation of the present study is its *in vitro* nature, as the stability, safety and efficacy of the *L. acidophilus*-derived postbiotics have not been validated under field conditions. Nevertheless, the results indicate that these postbiotics hold considerable promise as alternative or adjunct therapeutic agents to conventional antibiotics for the control of bovine mastitis. From a practical standpoint, such postbiotic formulations may be incorporated into teat dips, udder hygiene sprays to support udder health and mitigate the incidence of mastitis in dairy herds.

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