EFFECT OF POSTBIOTICS OF LACTICASEIBACILLUS PARACASEI SD1 AND LACTICASEIBACILLUS RHAMNOSUS SD4 AGAINST METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS SUBSP. AUREUS (ATCC 43300)

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ARTICLE HISTORY
Received: 1 September 2025 **Revised:** 22 September 2025 **Published:** 7 October 2025

ABSTRACT
This study investigated the antimicrobial activity of *Lacticaseibacillus paracasei* SD1 (SD1) and *Lacticaseibacillus rhamnosus* SD4 (SD4), as well as their postbiotics, against methicillin-resistant *Staphylococcus aureus* ATCC 43300. Antimicrobial activity was performed using the agar overlay method for probiotics and the agar well diffusion method for postbiotics, with inhibition zones measured in millimeters (mm). The mean inhibition zones of probiotic cells were 12.60±1.64 mm for SD1 and 16.07±1.98 mm for SD4, whereas postbiotics exhibited significantly larger inhibition zones of 14.27±0.55 mm for SD1 and 17.08±0.65 mm for SD4. Minimum inhibitory concentration (MIC) values for postbiotics were 62.50±0.00 µg/ml for SD1 and 31.25±0.00 µg/ml for SD4, while both demonstrated the same minimum bactericidal concentration (MBC) of 250±0.00 µg/ml. These findings indicated that postbiotics of both strains exhibit stronger antimicrobial potency against methicillin-resistant *S. aureus* compared with live probiotic cells.
Keywords: Probiotics, Postbiotics, Antimicrobial activity, MIC, MBC

CITATION INFORMATION: Ubonsutvanit, N., Pahumunto, N., & Teerakanok, S. (2025). Effect of Postbiotics of *Lacticaseibacillus paracasei* SD1 and *Lacticaseibacillus rhamnosus* SD4 against Methicillin-Resistant *Staphylococcus aureus* Subsp. *aureus* (ATCC 43300). *Procedia of Multidisciplinary Research, 3*(10), 32.

INTRODUCTION

Antimicrobial resistance (AMR) is a significant global public health issue with impact on healthcare systems and economies (Organization, 2014; Phumart et al., 2012). Thailand has concerns about the severity of this health issue and developed the Strategic Plan for Antimicrobial Resistance Management (2017-2022). This strategic plan focuses on the prevention and control of drug-resistant infections, responsible usage, public awareness, and knowledge promotion, to ensure the safe and effective use of these antibiotics (Division of Epidemiology, 2022).

Recent studies have suggested that probiotics become an alternative to combating drug-resistant pathogens. Probiotics and their postbiotics can inhibit the growth and virulence of pathogenic bacteria through various mechanisms, such as inhibiting growth, anti-biofilm, and immunomodulation. Previous studies have reported that *Lactobacillus* species inhibit the growth of pathogens such as *Acinetobacter baumannii, Escherichia coli, Pseudomonas aeruginosa,* and methicillin-resistant *Staphylococcus aureus* (MRSA) (Ahn et al., 2018; Kumar et al., 2017; Onbas et al., 2019; Sambanthamoorthy et al., 2014). These findings supported the potential use of probiotics and postbiotics as an adjunctive therapy against antibiotic resistance. However, their mechanisms and therapeutic applications still require further study to be elucidated. The study aims to investigate the abilities of probiotics and postbiotics of *Lacticaseibacillus paracasei* SD1 and *Lacticaseibacillus rhamnosus* SD4 in inhibiting the growth of antimicobial-resistant bacteria.

LITERATURE REVIEWS

Antimicrobial resistance (AMR) is a global health concern in hospitals throughout Thailand. According to a report from the Ministry of Public Health, in collaboration with the National Health Security Office, there was an increase of 3.24 million days in hospital stays and 38,481 deaths annually (Phumart et al., 2012). National surveillance has listed nine antimicrobial-resistant bacteria as national-level warning, including *Acinetobacter baumannii* (resistant to carbapenems and colistin), *Pseudomonas aeruginosa* (resistant to carbapenems and colistin), *Klebsiella pneumoniae* (resistant to carbapenems, colistin, and third-generation cephalosporins), *Enterococcus spp.* (resistant to vancomycin), *Staphylococcus aureus* (resistant to methicillin, vancomycin), *Streptococcus pneumoniae* (to penicillin, ceftriaxone, cefotaxime), *Escherichia coli* (resistant to carbapenems, colistin, fluoroquinolones, cephalosporins), *Salmonella spp.* (resistant to colistin, fluoroquinolones, cephalosporins), and *Neisseria gonorrhoeae* (resistant to cefixime) (Division of Epidemiology, 2022).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most clinically significant findings. In previous studies, *S. aureus* has been the cause of several diseases, such as skin and soft tissue infections (Mempel et al., 2003; Mills et al., 2023), pneumonia, endocarditis, sepsis, osteomyelitis (Tong et al., 2015), prosthetic joint infections (Holder et al., 2024), glomerulonephritis, and chronic granulomatous disease (Parzen-Johnson et al., 2022). In oral cavity, *S. aureus* acts as an opportunistic pathogen associated with dentoalveolar infections, oral mucosal lesions (Al-Akwa et al., 2020), oral Crohn's disease (Gibson et al., 2000), periodontitis, peri-implantitis (Cuesta et al., 2010; Murdoch et al., 2004; O'Connor et al., 2018), and recurrent tonsillitis (Zautner et al., 2010). *S. aureus* resistant strains acquired penicillin-binding protein 2a (PBP2a), which prevents antibiotics from inhibiting bacterial cell wall synthesis. This mechanism makes MRSA infections more difficult to treat and limits the effectiveness of beta-lactam antibiotics drugs, including penicillins, cephalosporins, and carbapenems (Pantosti & Venditti, 2009). MRSA can cause chronic inflammation and tissue damage by activating toll-like receptors (TLR1, TLR2, TLR6), NF-κB signaling, and proinflammatory cytokines (IL-6, IL-8, TNF-α) (Maiti & Jiranek, 2014). Enterotoxins and other toxins from *S. aureus* exacerbate disease progression by activating the immune cells, such

as keratinocytes, Langerhans cells (LCs), mast cells, macrophages, CD4+ T cells, Th1 cells, and microglia. (Chen et al., 2022).

Probiotics were considered as potential alternatives for inhibiting MRSA and other drug resistant pathogens. *Lactobacillus*, *Enterococcus*, *Bacillus*, *Streptomyces*, *Saccharomyces cerevisiae*, *Corynebacterium accolens*, and *Lactococcus lactis* (nisin-producing strains), exhibited inhibitory activity against MRSA biofilms (Jalalifar et al., 2022). Among these, *Lactobacillus* species demonstrated antimicrobial activity against *A. baumannii*, *E. coli*, *P. aeruginosa*, and MRSA (Sambanthamoorthy et al., 2014). *Lactobacillus species* produced organic acids, bacteriocins, hydrogen peroxide, and other surfactants, which lower the pH and inhibit pathogen growth (Ahn et al., 2018; Kumar et al., 2017; Onbas et al., 2019). Lipoteichoic acid from *Lactiplantibacillus plantarum* has been shown to inhibit biofilm development by suppressing biofilm-related gene expression (ica operon) in *S. aureus* (Ahn et al., 2018).

In-vitro and animal studies further supported the therapeutic potential of probiotics. Diets supplemented with *L. plantarum* and *L. brevis* increased the lifespan of *Caenorhabditis elegans (C. elegans)* and conferred resistance to MRSA. The mechanism was linked to the DBL-ligand of the transforming growth factor-β (TGF-β) signaling pathway (Møller et al., 2022; Mørch et al., 2021). The bioactive compounds, such as the methanolic extract from *Streptomyces spp*. and *Bacillus paralicheniformis*, have also demonstrated anti-MRSA, antibiofilm, and antioxidant activities (Ahire et al., 2020; Mangzira Kemung et al., 2020). The protective mechanisms of probiotics include 1) increased strength of epithelial barrier integrity, 2) excluded pathogens and inhibited mucosal adhesion, 3) produced antimicrobial peptides such as bacteriocins, and 4) modulated host immunity through cytokine regulation (Bermudez-Brito et al., 2012).

Recent studies emphasized probiotics such as *L. paracasei* SD1, *L. rhamnosus* SD4, *L. rhamnosus* SD11, and *L. rhamnosus* GG, which exhibited significant antifungal and antibacterial properties, inhibited proinflammatory cytokine production, and enhanced host immune defenses (Chantanawilas et al., 2024; Thananimit et al., 2022). Postbiotics of these probiotics also reported antimicrobial activity. In comparison, postbiotics present lower potential to inhibit *P. gingivalis, F. nucleatum, S. enterica*, and ETEC compared to probiotic live cells (Pahumunto & Teanpaisan, 2023). Clinical studies demonstrated that probiotic supplementation reduced levels of pathogenic oral bacteria (e.g., *Streptococcus mutans*), improved gut microbiota composition, and attenuated systemic inflammation in colorectal cancer patients (Rungsri et al., 2017; Wanitsuwan et al., 2024). These findings suggested that probiotics utilized *Lactobacillus* species may serve as adjunctive strategies for preventing and managing MRSA and other antimicrobial-resistant infections.

RESEARCH METHODOLOGY

Research hypothesis

H_0: There is no difference between *L. paracasei* SD1 and *L. rhamnosus* SD4 in inhibiting the growth of *S. aureus* ATCC 43300.

H_1: There are differences between *L. paracasei* SD1 and *L. rhamnosus* SD4 in inhibiting the growth of *S. aureus* ATCC 43300.

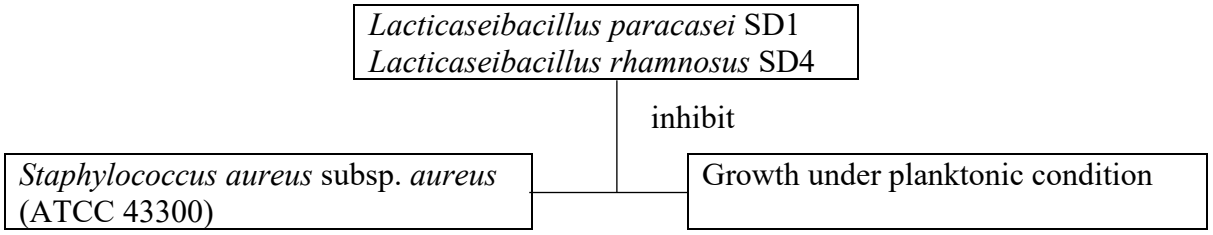

Figure 1 Conceptual framework

Cultivation of probiotics and pathogens (Piwat & Teanpaisan, 2013; Teanpaisan & Dahlén, 2006)

Two probiotic strains, *Lacticaseibacillus paracasei* SD1 and *Lacticaseibacillus rhamnosus* SD4, were used in this study. Strains were stored at -80°C in the Microbiology Laboratory, Faculty of Dentistry, Prince of Songkla University. Probiotics were cultured on de Man, Rogosa, and Sharpe (MRS) agar and incubated at 37°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) for 24-48 hours. Identification was based on colony morphology, Gram-positive staining, and a negative catalase reaction and confirmed by 16S rRNA gene sequencing using PCR.

The antibiotic-resistant pathogen used was methicillin-resistant *Staphylococcus aureus* subsp. *aureus* (ATCC 43300). The pathogen was cultured on blood agar containing 5% blood and incubated at 37°C for 24 hours. One to two colonies were then transferred to brain heart infusion (BHI) broth and incubated at 37°C for 24 hours. These cultures were later utilized for growth inhibition assays involving probiotics and their postbiotics.

Antimicrobial activity of probiotics and postbiotics against *S. aureus* ATCC 43300 strains

Agar overlay method (Pahumunto & Teanpaisan, 2023)

Probiotics were cultured in MRS broth at 37°C for 48 hours under anaerobic conditions. Cultures were centrifuged at 5,000 rpm for 10 min, and supernatants were collected for the agar well diffusion test. Pellets were washed twice with phosphate-buffered saline (PBS, pH 7.0) and adjusted to an optical density at 600 nm (OD_{600}) of 0.2, corresponding to ~10^8 CFU/ml.

A 10 µl of probiotic cells were inoculated in small spots (about 1 cm in diameter) on MRS and brain heart infusion agar (BHA) plates and incubated anaerobically at 37°C for 24 hours. After that, the pathogenic strain (~10^8 CFU/ml) was added to melted BHA and overlaid onto the plates. The plates were incubated for 24 hours under anaerobic conditions. Antimicrobial activity was indicated by a clear inhibition zone surrounding the probiotic colonies. Inhibition zone diameters (mm) were measured with a vernier caliper and presented as mean ± SD. Experiments were performed in triplicate, with PBS serving as the negative control.

Agar well diffusion method (Pahumunto & Teanpaisan, 2023)

Cell-free supernatants (postbiotics) were filtered through a 0.45 µm pore-size membrane, frozen at −80°C overnight, and lyophilized for 8 hours. The dried samples were reconstituted in distilled water at a 10× concentration, and pH was measured. Postbiotics were streaked on MRS agar and incubated at 37°C for 24 hours to confirm sterility. Postbiotics were prepared at a concentration of 1 g/ml for antimicrobial testing.

For testing, 1 ml of a pathogenic suspension (~10^8 CFU/ml) was mixed with 20 ml of melted BHA medium at approximately 50°C and poured into plates containing 6 mm diameter wells. Each well was filled with 90 µl of postbiotic. Plates were incubated under anaerobic conditions at 37°C for 18-24 hours. Antibacterial activity was determined by measuring inhibition zone diameters (mm) and presented as mean ± SD. All experiments were performed in triplicate, with PBS as the negative control.

Determination of Minimal Inhibitory Concentration (MIC) (CLSI, 2023)

The minimal inhibitory concentration (MIC) of the postbiotics was determined by the broth microdilution method following CLSI guidelines (CLSI, 2023). 100 µl of BHI broth was added to each well of a 96-well round-bottomed microtiter plate, followed by 100 µl of postbiotics. Two-fold serial dilutions were prepared to yield decreasing concentrations. Bacterial suspensions adjusted to 10^8 CFU/ml were added (100 µl per well), and plates were incubated at 37°C under suitable conditions. The BHI broth was used as the negative control, and the BHI broth with bacterial suspensions was used as the positive control. The MIC was defined as the lowest postbiotic concentration that completely inhibited visible growth.

Determination of Minimum Bactericidal Concentration (MBC) (CLSI, 2023)

The minimum bactericidal concentration (MBC) was determined by subculturing samples from the wells in the previous experiment showing no visible growth onto blood agar (BA) plates, followed by incubation at 37°C under anaerobic conditions for 24 hours. The MBC was defined as having the lowest concentration, producing no bacterial colonies. All experiments were performed in triplicate.

Statistical Analysis.

Inhibition zones, MIC, and MBC were expressed as mean ± standard deviation (SD). Statistical analyses were performed using SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA). For parametric data, multiple comparisons were evaluated using one-way analysis of variance (ANOVA). For non-parametric data, multiple comparisons were assessed using the Kruskal-Wallis test, while differences between two groups were analyzed using the Mann-Whitney U test. A p-value < 0.05 was considered statistically significant.

RESEARCH RESULTS

Antimicrobial activity of probiotics and postbiotics against *S. aureus* ATCC 43300 strains

Table 1 showed the antimicrobial effects of probiotic cells and postbiotics on *S. aureus* ATCC 43300. The inhibition zones were 12.60±1.64 mm for *L. paracasei* SD1 (SD1) and 16.07±1.98 mm for *L. rhamnosus* SD4 (SD4). In comparison, postbiotics demonstrated significantly more vigorous antimicrobial activity, with inhibition zones of 14.27±0.55 mm for SD1 and 17.08±0.65 mm for SD4. These results show that postbiotics are more effective at inhibiting *S. aureus ATCC 43300* than probiotic cells.

Table 1 Antimicrobial activity of probiotics and postbiotics against *S. aureus* ATCC 43300 strains

Type of samples	Inhibition zone (mm), (mean±SD)
Probiotic cells	
L. paracasei SD1	$12.60\pm1.64^{b,B}$
L. rhamnosus SD4	$16.07\pm1.98^{a,B}$
Postbiotics	
L. paracasei SD1	$14.27\pm0.55^{b,A}$
L. rhamnosus SD4	$17.08\pm0.65^{a,A}$

Capital letters showed a significant difference between probiotics and postbiotics against *S. aureus* ATCC 43330. ($p < 0.05$)

Lower letters showed a significant difference between probiotics or postbiotics against *S. aureus* ATCC 43330. ($p < 0.05$)

MIC and MBC of postbiotics against *S. aureus* ATCC 43300 strains

The MIC and MBC values of postbiotics against *S. aureus* ATCC 43300 were shown in Table 2. The MIC of postbiotics from *L. paracasei* SD1 was 62.50 µg/ml, whereas *L. rhamnosus* SD4 exhibited a significantly lower MIC of 31.25 ± 0.00 µg/ml. For MBC, both postbiotics demonstrated the same concentration of 250 ± 0.00 µg/ml. These findings showed that postbiotics from SD4 possess more potent antimicrobial activity, as reflected by the lower MIC compared with SD1.

Table 2 MIC and MBC of postbiotics against *S. aureus* ATCC 43300 strains

Postbiotics	SD1	SD4
MIC (µg/ml)	62.50 ± 0.00^{A}	31.25 ± 0.00^{B}
MBC (µg/ml)	250 ± 0.00^{A}	250 ± 0.00^{A}

Capital letters showed a significant difference between postbiotics against *S. aureus* ATCC 43330. ($p < 0.05$)

DISCUSSION & CONCLUSION

Probiotics are widely studied for their potential, which inhibits the growth of pathogens, inhibits biofilm formation, modulates host immune response, and facilitates competitive exclusion of pathogens (Ahn et al., 2018; Kumar et al., 2017; Plaza-Diaz et al., 2019). Among these, *L. paracasei* and *L. rhamnosus* have been investigated in previous studies, demonstrating the capacity to suppress *S. aureus* (Hill et al., 2014*). L. paracasei* SD1 *isolated from the oral cavity suppressed wild-type S. aureus by producing bacteriocins, like paracasin A* (Surachat et al., 2017). *L. rhamnosus* exhibited antimicrobial activity against several pathogens, including *Escherichia coli*, *Bacillus cereus*, *S. aureus*, *Listeria monocytogenes*, and *Salmonella spp.* (Oliveira et al., 2017). *Type III polyketide synthase (T3PKS) genes have been found in several Lactobacillus spp., such as L. plantarum and L. rhamnosus. This gene have reported to produced many secondary* metabolism*, relate to antimicrobial activity and signaling* (Wonglapsuwan et al., 2024). These findings suggest that SD1 and SD4 have potential for combating antimicrobial-resistant pathogens.

In this study, both *L. paracasei* SD1 and *L. rhamnosus* SD4 showed antimicrobial activity against *S. aureus* ATCC 43300. Postbiotics demonstrate significantly stronger antimicrobial activity than live probiotic cells. The inhibition zones of probiotic cells were 12.60±1.64 mm for SD1 and 16.07±1.98 mm for SD4, while their postbiotics showed larger inhibition zones (14.27±0.55 mm for SD1 and 17.08±0.65 mm for SD4).

Postbiotics are increasingly recognized for their stability, safety, and effectiveness in inhibiting pathogens. Unlike live probiotics, postbiotics avoid the risks, including systemic infections in immunocompromised individuals and loss of function during storage (Tsilingiri & Rescigno, 2013). The present findings in this study suggest that the antimicrobial capacity of postbiotics is more potent than live probiotic cells.

The MIC of SD4 postbiotics (31.25 µg/ml) was half that of SD1 (62.50 µg/ml), suggesting that SD4 has more potent antimicrobial activity. Nevertheless, both postbiotics demonstrated the same MBC of 250 µg/ml, indicating comparable bactericidal capacity at higher concentrations. Sambanthamoorthy et al. reported that *L. rhamnosus* achieved 80-93% killing activity against *S. aureus* UAMS-1 and MRSA at 50 mg/ml, significantly inhibited biofilm formation at 25 mg/ml, and caused structural damage to bacterial cell walls (Sambanthamoorthy et al., 2014).

In conclusion, this study demonstrated that postbiotics from *L. paracasei* SD1 and *L. rhamnosus* SD4 presented significant antimicrobial activity against *S. aureus* ATCC 43300, surpassing that of the corresponding probiotic cells. Among the tested strains, *L. rhamnosus* SD4 exhibited greater antimicrobial activity than *L. paracasei* SD1, as reflected by its larger inhibition zone and lower MIC value.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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