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Activity of lactic acid bacteria against biofilms formed by *Staphylococcus aureus* strains involved in acute mastitis

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Staphylococcus aureus **is the leading cause of acute mastitis during lactation. This species has a remarkable ability to form biofilms and to develop antibiotic resistance, which hampers the effectiveness of current therapeutic approaches. This study aims to evaluate the therapeutic potential of three lactic acid bacteria (LAB) strains (*Limosilactobacillus fermentum* I7, *Limosilactobacillus reuteri* 7SNG3-30 and *Ligilactobacillus salivarius* 22SNG3-30) to interfere with biofilms formed by two *S. aureus* strains isolated from milk of women with acute mastitis. Both live LAB cells and their respective cell-free supernatants were able to disrupt the *S. aureus* biofilm structure and significantly reduce its cellular viability. However, the effectiveness of these treatments was dependent on the *S. aureus* strain, the LAB strain, and the type of LAB preparation (active culture or cell-free supernatant) involved in each interaction. Overall, our results suggest that the tested LAB strains have the potential to be used either as probiotics or postbiotics complementing the current therapies against acute mastitis and other staphylococcal infections.**

Keywords *Staphylococcus aureus*, Human milk, Mastitis, Biofilms, *Ligilactobacillus salivarius*, *Limosilactobacillus fermentum*, *Limosilactobacillus reuteri*, Probiotics, Postbiotics

Staphylococcus aureus is the main cause of acute mastitis in lactating women[1–3]. Once in the mammary ecosystem, this species can adhere to the mammary epithelium through cell wall-anchored proteins[4,5], a process that promotes invasive host colonization and biofilm formation[6]. Although studies on the pathogenesis of acute mastitis in the human species are very scarce, studies focused on other mammalian species indicate that the overgrowth of *S. aureus*, together with the production of a variety of extracellular toxins and their absorption in the highly vascularized lactating mammary gland, eventually leads to the local and systemic signs and symptoms that usually characterize this condition[7–10]. Persistence of *S. aureus* inside the milk ducts and the frequent recurrences of *S. aureus* mastitis seem related to different factors, including its ability to scavenge and sequester iron and other metals[11], evade the host immune system[12,13], and form biofilms[14,15]. In fact, bacterial biofilms, where microorganisms are embedded within a protective extracellular matrix composed of polysaccharides, proteins and extracellular DNA, are major contributors to staphylococcal pathogenesis[16,17]. Biofilm development is regulated by quorum sensing, specifically by the *agr* locus, which controls the expression of virulence factors essential for *S. aureus* pathogenesis[18–20].

The empirical use of antibiotics, such as amoxicillin or cloxacillin, has been, and continues to be, the most common treatment for acute mastitis. However, the widespread use of antibiotics has driven a significant rise in multi-drug resistant (MDR) *S. aureus* strains[21,22]. This resistance is not only due to the presence of transmissible antibiotic-resistance genes or antimicrobial efflux pumps but also stems from the ability of some *S. aureus* strains to develop other equally powerful drug resistance strategies, including the production of small colony variants and, most importantly, biofilms in the mammary ducts[14,23]. Biofilms hinder antibiotic penetration, reducing their effectiveness even against strains that would otherwise be susceptible[24,25]. Such mechanisms explain why a relatively high percentage of *S. aureus* strains did not respond to empiric antibiotic treatment[26]. In addition,

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exposure of lactating infants to antibiotics may alter the acquisition of the gut microbiota in a critical opportunity window, with potential long-term health consequences[27–29]. Therefore, alternative strategies for the prevention or treatment of this condition are required[30].

During the last years, the selection of some lactic acid bacteria (LAB) strains isolated from human milk and their use as probiotics has been shown to be a promising tool for the prevention or treatment of human lactational mastitis[3,31–36], through a variety of microbiological, biochemical and immunological mechanisms[37–39]. However, despite these advances, the interactions between LAB and *S. aureus* biofilms, as well as their impact on infection dynamics, are still unclear. Understanding how potential probiotic bacteria influence the formation and stability of *S. aureus* biofilms is crucial for improving or developing new therapeutic strategies. In this context, the objective of this study was to evaluate the potential of active cultures and cell-free supernatants (CFS) of selected LAB strains for the management of mastitis using in vitro-developed model biofilms of *S. aureus* strains associated with acute mastitis cases.

Materials and methods

Bacterial strains and growth conditions

Two *S. aureus* and three LAB strains were used in this study. All of them were previously isolated and identified by 16S rRNA gene sequencing. The *S. aureus* strains had been isolated from the milk of women suffering from mastitis (SA4 and SA55, originally coded as DH3a and BALD1, respectively)[1]. LAB strains were isolated from human milk (*Limosilactobacillus fermentum* I7) and the inner surface of nasogastric enteral feeding tubes used in preterm infants (*Limosilactobacillus reuteri* 7SNG3-30 and *Ligilactobacillus salivarius* 22SNG3-30)[40]. The collection of human milk samples and the experimental protocols were approved by the Ethics Committee of Hospital Clínico San Carlos of Madrid, Spain (Code: 06/41; date: 03/16/2006). Written informed consent was obtained from all participants. The study involving nasogastric enteral feeding tubes used in preterm infants was approved by the Ethics Committee on Clinical Research of La Paz University Hospital of Madrid, Spain (Code: HULP PI-3199; date: 06/27/2018), with written consent obtained from their legal guardians prior to sample and data collection. Both studies were carried out in accordance with the Declaration of Helsinki. Unless otherwise specified, *S. aureus* strains were cultured in Brain Heart Infusion (BHI) medium (Oxoid, Basingstoke, UK) and LAB strains were grown in de Man, Rogosa, and Sharpe (MRS) medium (Oxoid). All cultures were incubated aerobically at 37 °C for 24 h under static conditions, from stock cultures maintained in glycerol (1.5%, v/v) at −80 °C.

Treatment of *S. aureus* biofilms with active cultures or CFS of LAB strains

For this purpose, biofilms were developed using a carousel system described previously[41]. The system consisted of sixteen 22 × 22 mm microscope glass coverslips (Thermo Scientific, Germany) inserted vertically into narrow radial slits of a Teflon platform (6.6 cm diameter). This platform, along with its lid, was assembled using an axial stainless-steel rod for handling and placed in a 600 mL glass beaker. The entire system was sterilized at 121 °C for 15 min. For biofilm formation, 60 mL de BHI broth was inoculated with 1 mL of a bacterial suspension containing about 10^7 CFU/mL, which was then placed in the carousel system and incubated statically at 37 °C for 24 h.

To prepare the treatment solutions, i.e. CFS and active cultures, an isolated colony of the respective LAB strain was suspended in 10 mL of MRS broth and incubated for 24 h at 37 °C. Then, 1 mL of this culture was added to 60 mL of MRS broth and incubated for another 24 h. The culture was then centrifuged for recovering the CFS, and the pellets were suspended and the A_{600} was measured and adjusted to 0.15 in fresh MRS broth to obtain 60 mL of the active cultures. For each strain, *S. aureus* biofilms were developed running eight carousel systems in parallel and, then, each carousel was treated according to the following set up: two carousels were used as controls (*S. aureus* biofilms were immersed whether into fresh BHI or MRS broth); three carousels were treated with the CFS of each LAB strain (*L. fermentum* I7, *L. reuteri* 7SNG3-30 and *L. salivarius* 22SNG3-30) and three were treated with the active cultures of the same microorganisms. All the carousels were incubated aerobically without shaking for 24 h at 37 °C. This experimental step up was independently replicated two times to confirm reproducibility.

For bacterial counting, three coverslips were removed from each carousel. First, each coverslip was washed with saline solution (0.85% NaCl v/v) to remove non-adherent cells. Then, the adherent cells of both sides of the coverslip were scraped using a sterile swab that was placed into 1.5 mL of sterile peptone water. After vigorous vortexing, the resulting suspension was decimally diluted, and aliquots of 20 µL of each dilution were plated onto selective medium: MRS agar for LAB counts and Baird Parker (BP, Pronadisa, Spain) agar for staphylococcal counts. Plates were incubated at 37 °C for 24 h and bacterial counts were expressed as $\log_{10}$ Colony Forming Units (CFU)/cm^2.

Visualization of biofilms by scanning electron microscopy (SEM)

For SEM analysis, biofilms formed on glass coverslips (two coverslips for condition) were first washed with 50 mL of saline solution. For fixation, coverslips were then immersed overnight into a solution containing 4% (v/v) paraformaldehyde (Sigma-Aldrich, Spain) and 3% (v/v) glutaraldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.2) at 4 °C. Following this step, samples were rinsed with Milli-Q water previously filtered through a 0.22 µm filter and dehydrated by immersion for 5 min through an increasing gradient of ethanol solutions (40%, 50%, 60%, 70%, 80%, 90%, and 100%). Dehydrated samples were further processed at the Electron Microscopy Center at the Complutense University of Madrid (Madrid, Spain), where critical point drying was performed using a Leica CPD300 (Leica, Germany). The samples were subsequently coated with a gold-palladium layer using a Leica EM ACE200 sputter coater (Leica). SEM imaging of the biofilms was carried out using a JEOL 6400 JSM scanning electron microscope.

Visualization of biofilms by confocal laser scanning microscopy (CLSM)

For CLSM analysis, biofilms formed on glass coverslips (two coverslips for condition) were first washed with 50 mL of saline solution and stained using the FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Molecular Probes, Invitrogen, Carlsbad, California). The staining solution was daily prepared by mixing 3 µL of SYTO 9 and 3 µL of propidium iodide in 1.5 mL of sterile distilled water and stored in the dark at 4 °C. Fifty µL (or more if necessary) of the staining solution were used for staining each biofilm. After 1 h incubation in the dark, coverslips were rinsed with sterile distilled water. A Nikon ECLIPSE Ti microscope (NIS Elements software, version 4.5.1.01) equipped with a Nikon C2 confocal scanning module and 488 nm and 561 nm continuous lasers was used to acquire *xy* image series with a *z*-step of 0.5 µm. An oil immersion objective lens at 100× magnification was selected for image capture. Three-dimensional projections [maximum intensity projection (MIP)] were reconstructed from *z*-stacks using IMARIS® 8.1.2 software (Bitplane AG, Zurich, Switzerland). To calculate the biovolume (μm^3), the MeasurementPro module of the same software was used. Each image was segmented into two channels, green and red, to estimate the biovolume occupied by live and dead cells.

Statistical analysis and data visualization

Statistical analysis was carried out using Statgraphics Centurion 19 software (Statistical Graphics Corporation, Rockville, MD, USA). Multifactorial analyses were conducted to determine the effect of the type of treatment (active culture or CFS) and the lactic acid bacteria strain (*L. fermentum* I7, *L. reuteri* 7SNG3-30, or *L. salivarius* 22SNG3-30) on the reduction of the attached cell population in *S. aureus* biofilms, and the biovolume and maximum thickness of the biofilms. A one-way ANOVA was applied to compare each treatment with the untreated 24-hour biofilm control, the effects of LAB active cultures and CFS within each strain, and the counts of *S. aureus* and LAB strains after the treatment with LAB active cultures. For all analyses, statistical significance was determined at the 95% confidence level ($p < 0.05$). Data visualization, including bar graphics and boxplots, was generated using *ggplot2* package[42] in RStudio version 4.3.1[43].

Ethical approval. Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements.

Results

Effect of LAB on the biofilms formed by *S. aureus* strains associated with mastitis

To explore strategies based on microbial competition to modulate the adhesion ability of *S. aureus*, two strains isolated from milk of women with acute mastitis and having good biofilm-forming ability were selected (SA4 and SA55, the latter isolated from the milk sample of a woman who had a mammary abscess). Live cultures and CFS from three LAB strains (*L. fermentum* I7, *L. reuteri* 7SNG3-30 and *L. salivarius* 22SNG3-30) were used to challenge *S. aureus* biofilms.

Both strains of *S. aureus* had a similar ability to form dense biofilms after 24 h with counts of 4.93 ± 0.12 $\log_{10}$ CFU/cm^2 for SA4 and 4.84 ± 0.07 $\log_{10}$ CFU/cm^2 for SA55 (Fig. 1a). Before evaluating the treatments, 24 h *S. aureus* biofilms were immersed in fresh BHI and MRS as controls. After 24 h of incubation in BHI, the bacterial counts for SA4 and SA55 increased to 5.73 ± 0.18 $\log_{10}$ CFU/cm^2 and 5.44 ± 0.12 $\log_{10}$ CFU/cm^2, respectively. Similarly, incubation in MRS resulted in counts of 5.31 ± 0.14 $\log_{10}$ CFU/cm^2 for SA4 and 6.24 ± 0.20 $\log_{10}$ CFU/cm^2 for SA55. These controls confirmed that neither culture medium affected negatively the viability of *S. aureus* biofilms. Then, treatments with active cultures and CFS from the three LAB strains were evaluated. The effect of these treatments was assessed based on the reduction in the attached cell population of staphylococcal biofilms. A multifactorial analysis was performed for each *S. aureus* strain to evaluate two factors: the effect of the treatment type (active culture or CFS) and that of the LAB strain (*L. fermentum* I7, *L. reuteri* 7SNG3-30 or *L. salivarius* 22SNG3-30). The efficiency of the treatments against SA4 biofilms varied significantly depending on both factors, the LAB strain used ($F = 54.57$; $p < 0.0001$) and, more notably, the treatment type ($F = 803.83$; $p < 0.0001$), which had a greater influence on treatment efficacy. Moreover, there was also significant interaction between these two variables ($F = 54.57$; $p < 0.0001$). Similar findings were observed when challenging SA55 biofilms. Again, the efficacy of the treatment was strongly dependent on the type of treatment ($F = 123.45$; $p < 0.0001$) and the LAB strain ($F = 78.12$; $p < 0.0001$), with significant interaction between the two factors ($F = 65.32$; $p < 0.0001$).

The treatment of SA4-biofilms with CFS from any of the three LAB strains resulted in an average of 5 $\log_{10}$ reduction of the initially attached population (Fig. 1a; left panel). On the other hand, when LAB active cultures were added to the already formed SA4-biofilms, the efficiency of the treatment was strain-dependent and significantly lower ($p < 0.001$) than that achieved with the corresponding CFS. Whereas *S. aureus* SA4 attached population was slightly reduced in the presence of *L. fermentum* I7 active cultures (1.76-$\log_{10}$ reduction), *L. reuteri* 7SNG3-30 yielded an approximately 4-$\log_{10}$ reduction in bacterial counts.

Viable attached cell population in SA55-biofilms (Fig. 1a; right panel) was drastically reduced when treated with both *L. reuteri* 7SNG3-30 and *L. salivarius* 22SNG3-30 active cultures and CFS, being the two treatments equally effective ($p > 0.05$). On the contrary, active cultures of *L. fermentum* I7 were significantly more efficient than its CFS against biofilm-associated SA55 cells (4.84-$\log_{10}$ vs. 3.24-$\log_{10}$ reduction) ($p < 0.001$).

Changes in the composition of *S. aureus* biofilms after treatment with active cultures of LAB strains

To demonstrate if the LAB active cultures added to challenge biofilms were able to integrate into already formed structures, we measured changes in the composition of the LAB-treated *S. aureus* biofilms (Fig. 1b). The SA4-biofilm composition shifted significantly after treatment with any of the three LAB strains (Fig. 1b; left panel). Treatment of SA4-biofilms with *L. fermentum* I7 resulted in a biofilm integrated by both species, having counts of 3.17 $\log_{10}$ units (35.82%) of *S. aureus* SA4 and 5.68 $\log_{10}$ units (64.18%) of *L. fermentum* I7 cells. Similarly, after

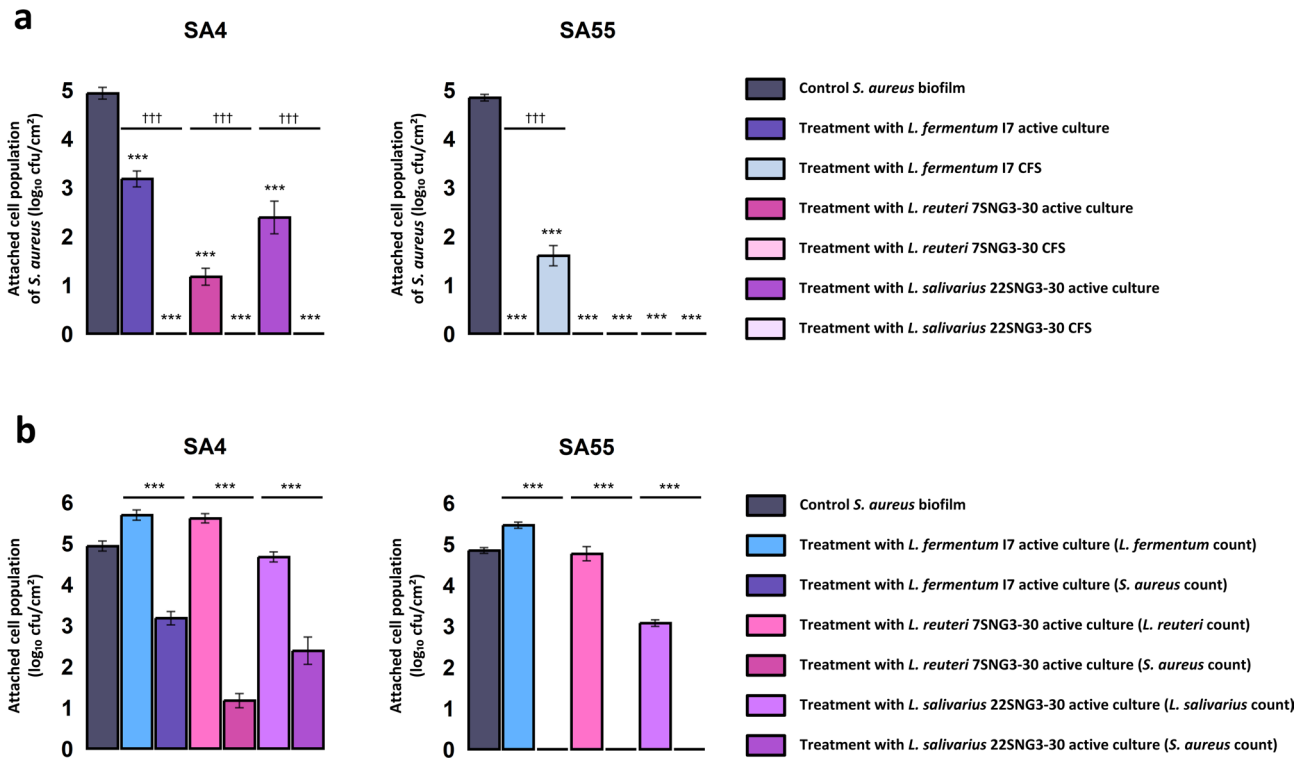

Fig. 1. Impact of lactic acid bacteria (LAB) strains on *S. aureus* biofilms associated with mastitis. **a** *S. aureus* attached cell population (log_{10} CFU/cm^2) in SA4 (left) and SA55 (right) biofilms before and after treatment with active cultures and CFS of *L. fermentum* I7, *L. reuteri* 7SNG3-30 and *L. salivarius* 22SNG3-30. All counts were performed in triplicate, and the bars represents the mean *S. aureus* counts with error bars showing the standard deviation (SD). Comparisons between the effect of each treatment and the 24-hour biofilm control were made using one-way ANOVA (*** = p-value < 0.001), and comparisons between the effects of LAB active cultures and CFS within each strain were made using one-way ANOVA (††† = p-value < 0.001). **b** Changes in the composition (log_{10} CFU/cm^2) of *S. aureus* SA4 (left) and SA55 (right) biofilms after treatment with active cultures of *L. fermentum* I7, *L. reuteri* 7SNG3-30 and *L. salivarius* 22SNG3-30. All counts were performed in triplicate, and data are shown as mean values ± SD for the counts of *S. aureus* and each LAB strain. Comparisons between the counts of *S. aureus* and those of each LAB strain were made using one-way ANOVA (*** = p-value < 0.001).

treatment of SA4-biofilms with *L. reuteri* 7SNG3-30, the resulting community was dominated by this LAB strain, reaching a cell density of 5.60 log_{10} units (82.72% of the total biofilm cellular composition). Again, treatment with *L. salivarius* 22SNG3-30 led to a new dual biofilm composed of 2.38 log_{10} units (33.81%) of *S. aureus* SA4 and 4.66 log_{10} units (66.19%) of *L. salivarius* 22SNG3-30.

In contrast, the composition of the SA55-biofilms changed significantly following treatment with any of the three LAB strains, resulting in undetectable levels of viable *S. aureus* SA55 cells, which were totally displaced by the corresponding LAB strain (Fig. 1b; right panel).

Image analysis of structural changes in *S. aureus* biofilms after treatment with LAB strains

To gain deeper insight into how LAB active cultures might modify the original structure and the cellular viability of *S. aureus* SA4 and SA55 biofilms, treated biofilms were visualized using laser scanning confocal microscopy (CLSM) and scanning electron microscopy (SEM) (Figs. 2 and 3). As expected, in CLSM images, most cells in control SA4 and SA55 biofilms showed green fluorescence after staining with Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit, indicating that most of the cells were alive. Interestingly, spatial distribution pattern by biofilm cells was strain-dependent. CLSM images of SA4 biofilms showed scattered spherical aggregates whereas SA55 biofilms appeared as a monolayer of cells homogeneously distributed all over the support surface. SEM images revealed dense cellular aggregates characteristic of *S. aureus* biofilms surrounded by more empty areas where cells had a diplococcal pattern.

Biofilm composition changed drastically after treatment with any of the three LAB active cultures (Figs. 2 and 3). CLSM images of LAB-treated *S. aureus* biofilms had a higher proportion of red-stained cells, i.e. dead or damaged cells, especially *S. aureus* cells as revealed by morphological distinction between both bacterial species. Interestingly, LAB cells appeared mostly in green and fully integrated into the biofilm structure, as demonstrated by their widespread distribution, especially when SA4 biofilms were treated with *L. fermentum* I7 and *L. reuteri* 7SNG3-30. Indeed, the presence of SA4 biofilms seems to stimulate the growth and spread of these LAB strains. SEM images confirmed these findings by clearly differenciating the cellular morphologies of LAB and *S. aureus*.

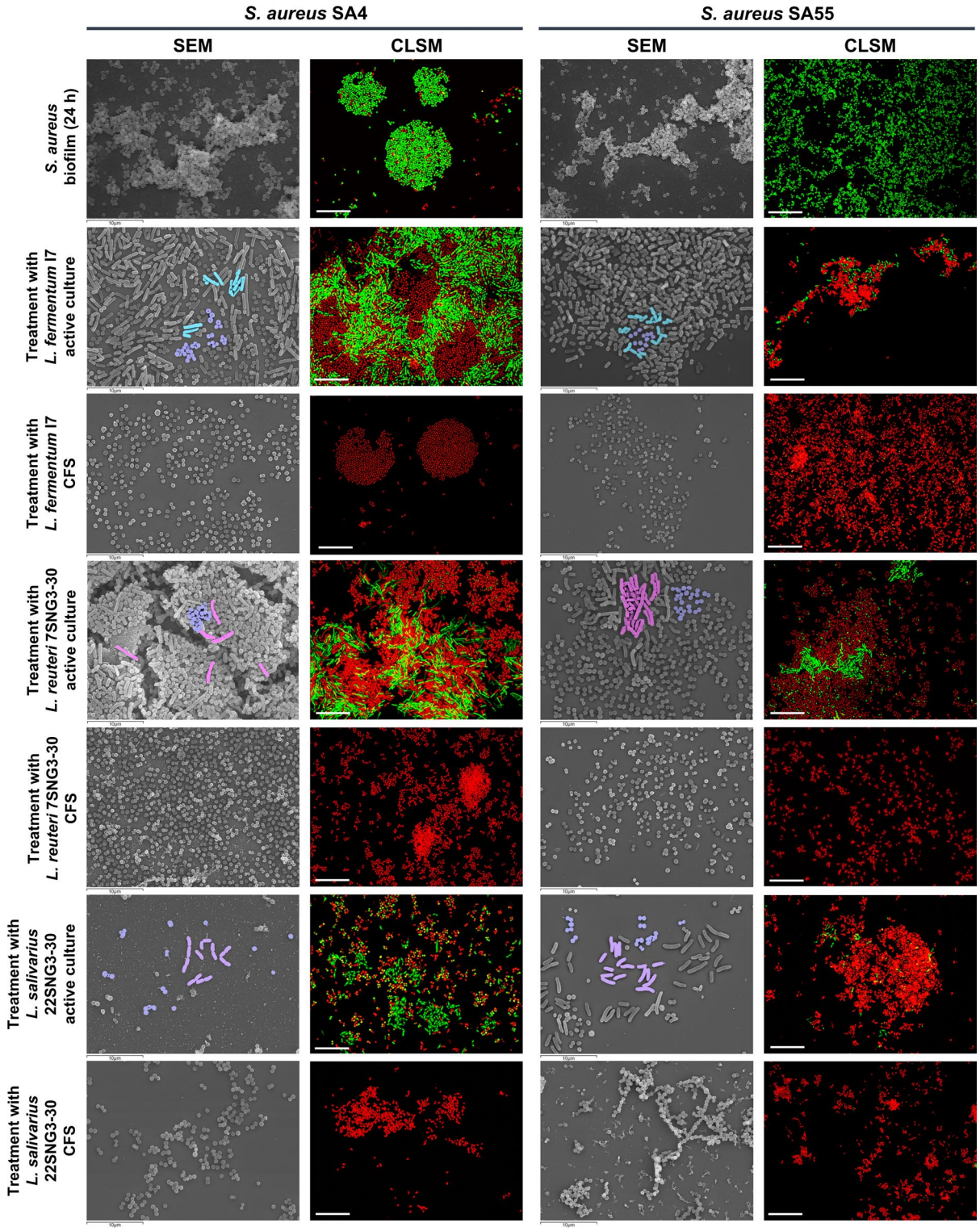

Fig. 2. Representative SEM (3000×) and CLSM images (stained using the Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit) of *S. aureus* SA4 and SA55 biofilms at 24 h and following treatment with active cultures and CFS of *L. fermentum* I7, *L. reuteri* 7SNG3-30 and *L. salivarius* 22SNG3-30. In the SEM images of treatments with active lactic acid bacteria cultures, some representative cells were pseudocolored light blue (*L. fermentum* I7), pink (*L. reuteri* 7SNG3-30), violet (*L. salivarius* 22SNG3-30), and dark blue (*S. aureus*). In the CLSM images, live cells are stained green and dead cells are stained red. SEM scale bar: 10 µm; CLSM scale bar: 20 µm.

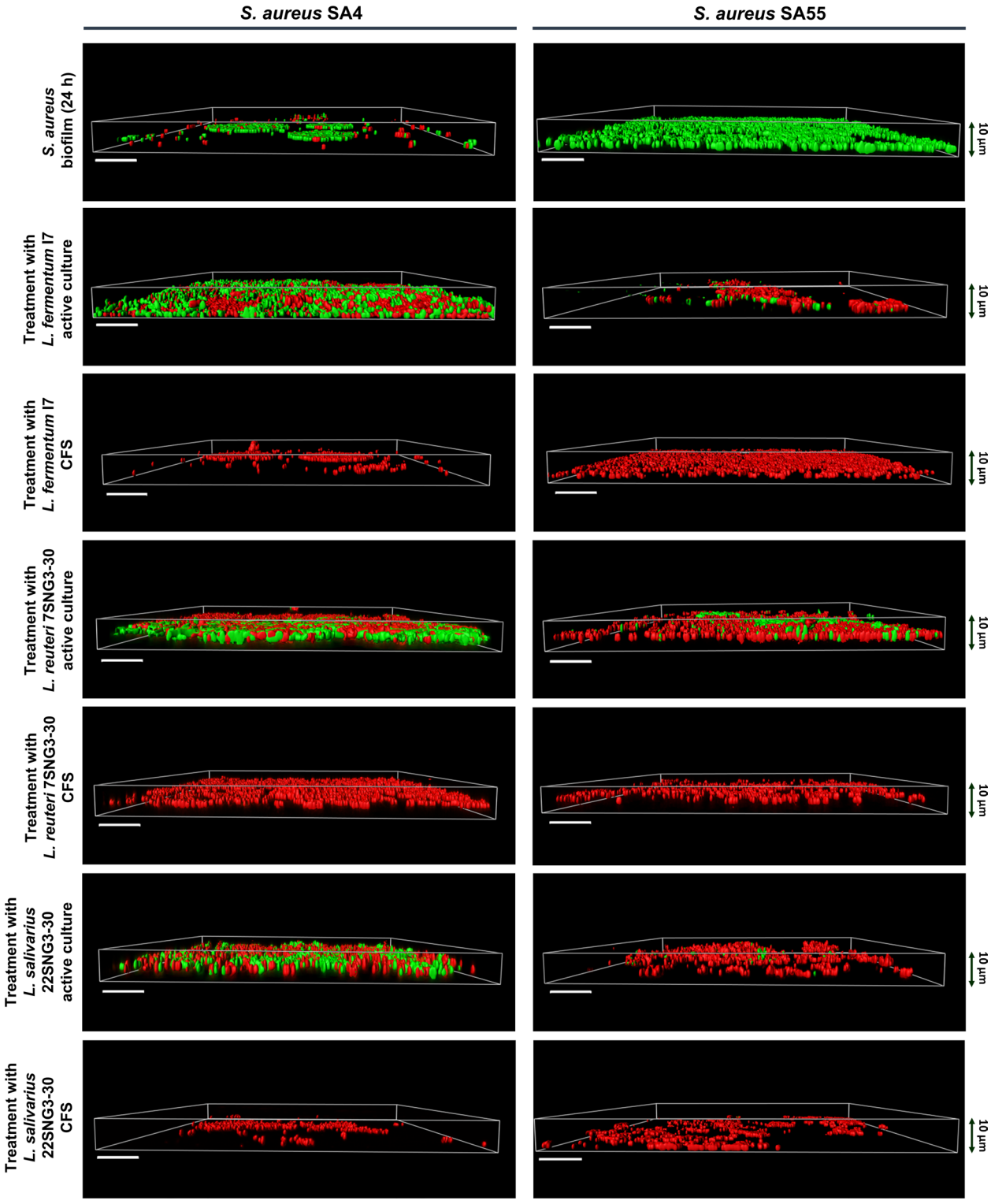

Fig. 3. 2D Cross-sections (10 µm wide) of CLSM images (stained using the Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit) of *S. aureus* SA4 (left) and SA55 (right) biofilms at 24 h and after treatment with active cultures and CFS of *L. fermentum* I7, *L. reuteri* 7SNG3-30 and *L. salivarius* 22SNG3-30. Scale bar: 20 µm.

On the contrary, when SA55 biofilms were treated with the same LAB strains a partial integration of the LAB cells was observed although the LAB stimulation stated before in SA4 biofilms was lacking (Figs. 2 and 3).

CLSM images revealed that when *S. aureus* biofilms were treated with CFS, most of the cells were stained red, indicating a potent effect of CFS upon biofilms of both *S. aureus* strains. SEM images corroborated these

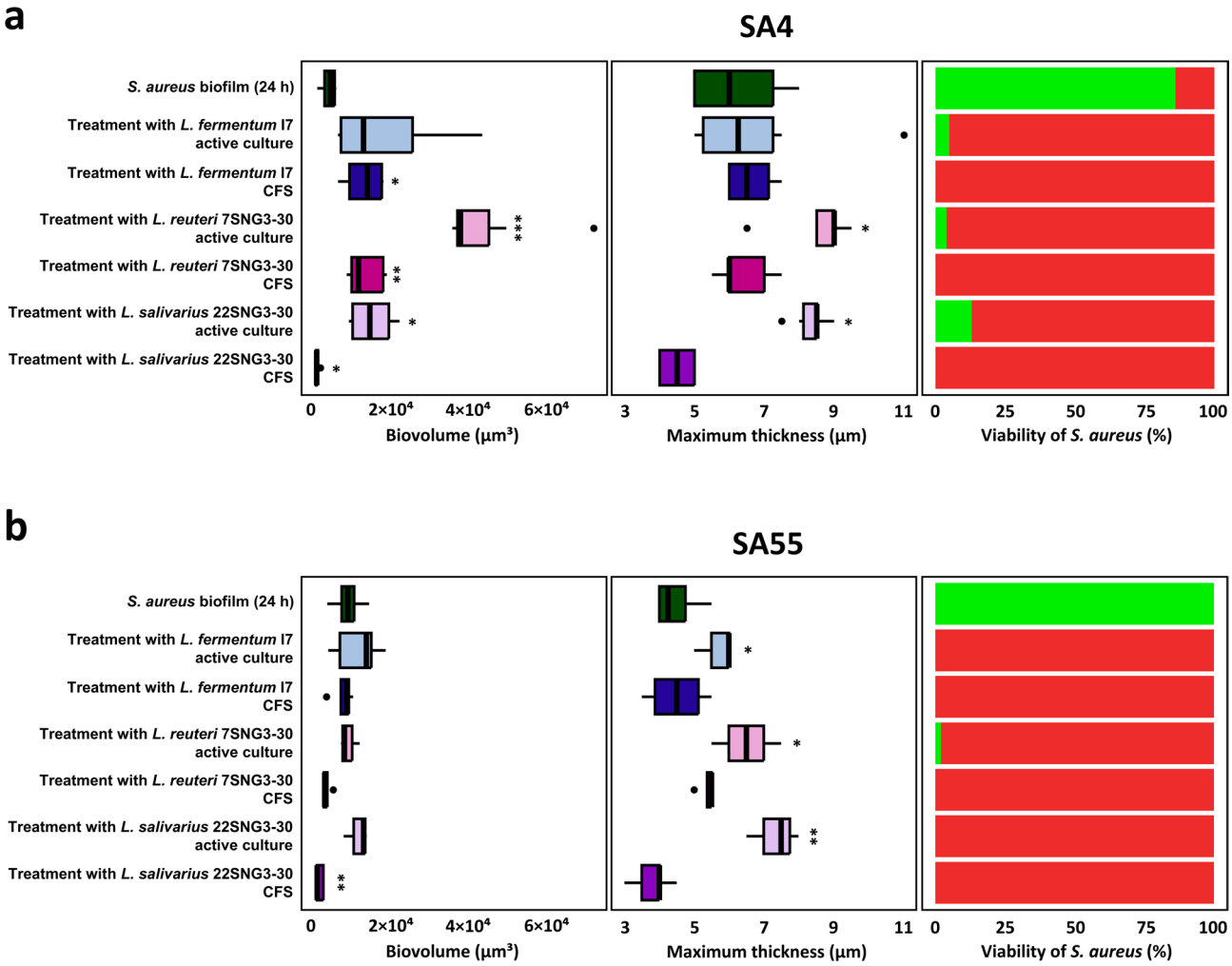

Fig. 4. Changes in biofilm characteristics of *S. aureus* strains associated with mastitis after treatment with lactic acid bacteria strains. This figure illustrates the changes in three key biofilm parameters: biovolume (μm^3) (left), maximum thickness (µm) (middle), and cellular viability (%) (right) in *S. aureus* SA4 **a** and SA55 **b** biofilms after treatment with active cultures and CFS of *L. fermentum* I7, *L. reuteri* 7SNG3-30 and *L. salivarius* 22SNG3-30. Biovolume, maximum thickness, and cellular viability data were analyzed using IMARIS software. The biovolume and maximum thickness were assessed through the processing of confocal laser scanning microscopy (CLSM) images, while cellular viability was calculated based on the segmentation of live and dead cell populations, with the MeasurementPro module of IMARIS used for quantification (assisted by the Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit). The boxplots display the biovolume and maximum thickness, with the center line representing the median, box limits indicating the upper and lower quartiles, whiskers showing extreme values, and circles denoting outliers. Comparisons between biovolume and maximum thickness for treated and untreated 24-hour control biofilms were performed using one-way ANOVA. Statistical significance is marked as *($p < 0.05$), **($p < 0.01$), and ***($p < 0.001$). For each condition, the following number of samples were analyzed: SA4: $n = 4$ and SA55: $n = 4$ for *S. aureus* biofilm 24 h; SA4: $n = 6$ and SA55: $n = 5$ for treatment with *L. fermentum* I7 active culture; SA4: $n = 4$ and SA55: $n = 4$ for treatment with *L. fermentum* I7 CFS; SA4: $n = 7$ and SA55: $n = 3$ for treatment with *L. reuteri* 7SNG3-30 active culture; SA4: $n = 5$ and SA55: $n = 4$ for treatment with *L. reuteri* CFS; SA4: $n = 6$ and SA55: $n = 3$ for treatment with *L. salivarius* 22SNG3-30 active culture; and, SA4: $n = 4$ and SA55: $n = 5$ for treatment with *L. salivarius* 22SNG3-30 CFS.

observations, showing cellular debris and disintegration of the biofilm structure. Notably, *L. salivarius* 22SNG3-30 CFS, apart from cellular death, seemed to stimulate biofilm dispersal (Figs. 2 and 3).

To quantify the observed changes on *S. aureus* biofilms due to the treatment with LAB, CLSM images were processed to calculate key parameters such as biovolume, maximum thickness and cellular viability (Fig. 4). First, a multifactorial analysis was performed on both biovolume and maximum thickness to assess the impact of the LAB strain (*L. fermentum* I7, *L. reuteri* 7SNG3-30 or *L. salivarius* 22SNG3-30) and the applied treatment (active culture or CFS). In the case of SA4 biofilms, biovolume varied significantly depending on the LAB strain ($F = 12.43$; $p = 0.0002$) and the treatment type ($F = 22.49$; $p = 0.0001$), whereas maximum thickness was only

affected by the treatment type ($F = 22.59$; $p = 0.0001$). For SA55 biofilms, the treatment type had a significant effect on maximum thickness ($F = 49.57$; $p < 0.0001$) and biovolume ($F = 20.07$; $p = 0.0003$), whereas the impact in both variables was independent of the LAB strain ($F = 3.13$; $p = 0.0681$ and $F = 2.41$; $p = 0.1181$, for maximum thickness and biovolume, respectively). Overall, the use of LAB active cultures or their CFS had a significant influence on both parameters, biovolume and maximum thickness.

Then, specific changes in the biofilm structure were analyzed for each strain. Control SA4 biofilms had an average biovolume of 3.84×10^3 µm³ and an average maximum thickness of 6.25 µm (Fig. 4a). Biovolume values significantly increased when these biofilms were treated with CFS from *L. fermentum* I7 ($p < 0.05$) and *L. reuteri* 7SNG3-30 ($p < 0.01$), whereas a reduction in biovolume was observed with CFS from *L. salivarius* 22SNG3-30, confirming the previously observed biofilm dispersal (Figs. 2 and 3). Both parameters increased significantly after treatment with active cultures of *L. salivarius* 22SNG3-30 ($p < 0.05$) and especially with *L. reuteri* 7SNG3-30 ($p < 0.001$), supporting the observed colonization of *S. aureus* biofilms by these species (Figs. 2 and 3).

Control SA55 biofilms had an average biovolume of 8.65×10^3 µm³ and an average maximum thickness of 4.50 µm (Fig. 4b). The treatment with CFS from *L. salivarius* 22SNG3-30 also led to a significant decrease in biovolume to nearly one-sixth of the original ($p < 0.01$), supporting the dispersal effect previously stated (Figs. 2 and 3). As observed in SA4 biofilms, maximum thickness increased significantly when SA55 biofilms were treated with active cultures of *L. fermentum* I7 ($p < 0.05$), *L. reuteri* 7SNG3-30 ($p < 0.05$) and *L. salivarius* 22SNG3-30 ($p < 0.01$).

Discussion

Lactational mastitis causes significant pain and discomfort for many breastfeeding women, negatively affecting the quality of life and emotional well-being of the mother-infant pair[3]. This condition threatens the WHO recommendations, which advocate exclusive breastfeeding for the first six months of life and continued breastfeeding, alongside complementary foods, as long as mutually desired by mother and child for 2 years or beyond, because of the recognized benefits that it provides for the mother-infant dyad[44,45]. The treatment of acute mastitis often relies on empiric antibiotherapy, but the increasing antimicrobial resistance in *S. aureus* complicates therapeutic success. It has been shown that 78.5% of the *S. aureus* strains isolated from human milk were methicillin-resistant *S. aureus* (MRSA) displaying high antibiotic resistance to several beta-lactam antibiotics, the group of antibiotics that are usually prescribed for treating acute mastitis[26]. Therefore, it is crucial to explore alternatives to traditional antibiotic treatments that enable mothers to overcome mastitis while continuing to breastfeed.

In recent years, the use of probiotics and postbiotics has gained significant attention for their ability to disrupt biofilm formation, positioning them as promising alternatives in mastitis management. In this study, both live cultures and CFS from three LAB strains (*L. fermentum* I7, *L. reuteri* 7SNG3-30 and *L. salivarius* 22SNG3-30) effectively reduced the viability and adherence of *S. aureus* cells within preformed biofilms of mastitis-associated *S. aureus* strains.

Treatment with LAB cultures not only diminished the number of viable *S. aureus* cells but also modified significantly biofilm composition, increasing the proportion of LAB while displacing *S. aureus*. When integrated into pathogenic biofilms, LAB appears to compete more effectively for nutrients and modify the microbial environment by producing antimicrobial metabolites[46–49]. These mechanisms could play a key role in *S. aureus* decolonization and may explain, at least partly, the efficacy demonstrated by some LAB strains from human milk in preventing or treating human staphylococcal mastitis[32,35,50]. Natural presence of LAB and coagulase-negative staphylococci in the milk of healthy women may also explain why oral breast milk application is beneficial for preventing MRSA colonization in neonates undergoing surgery[51,52].

It has been shown that a *Bacillus subtilis* strain produces lipopeptides (fengycins) that are able to eliminate *S. aureus* by competing with the Agr autoinducing peptides and blocking AgrC-dependent quorum sensing in this species[53], interfering with its biofilm-forming ability, reducing the expression of its virulence factors and leading to its nasal and intestinal decolonization, without affecting other members of the host microbiota[54]. Similarly, it has been described that another *B. subtilis* strain inhibits the formation of *S. aureus* biofilms in the mammary gland of mice, preventing the development of mastitis in mice[55].

The incorporation of probiotic LAB into biofilms may also contribute to restoring and maintaining a balanced microbiota in the mammary gland, offering lasting protection by strengthening the integrity of the mammary epithelium, modulating local immunity, and increasing resistance to future pathogenic challenges[37–39]. A relevant advantage of LAB as mastitis-targeting probiotics is that, upon oral administration, they can colonize the mammary gland through the entero-mammary pathway[32,56–60].

Interestingly, CFS derived from the LAB cultures exhibited a stronger antimicrobial effect, particularly against SA4 strain, where complete biofilm elimination was achieved. The ability of CFS to penetrate and disrupt the biofilm matrix is critical, as it facilitates access to bacteria protected within these structures, leading to greater pathogen elimination. The antimicrobial efficacy of LAB-derived CFS against *S. aureus* strains involved in mastitis and other infections in humans and other mammalian species has been described in previous studies[61–66]. LAB-derived CFS contain metabolites, including well-known antimicrobial compounds, such as organic acids (e.g., lactic and acetic acid), ethanol, diacetyl, carbon dioxide, hydrogen peroxide, bacteriocins, short- and long-chain fatty acids, biosurfactants and other bioactive molecules that contribute to the antimicrobial activity and create unfavorable environments for pathogens like *S. aureus*[48,50,67]. Other compounds present in LAB-derived CFS may also help in the prevention or treatment of mastitis by exerting antioxidant, anti-inflammatory, and immunomodulatory activities[67–69].

The use of CFS offers the advantage that their efficacy does not rely on bacterial viability, simplifying storage and administration, and eliminating the need for special preservation conditions typically required for live cultures[70]. But this strategy may also have major disadvantages over live bacteria. In the case of dairy ruminants,

CFS could be applied inside the udder through the teat canal but this is not possible in women, impeding their direct application to prevent or treat human mastitis. In contrast to live LAB strains, oral administration of bacterial CFS faces the problem that many, if not all, of the antimicrobial compounds present in CFS may not resist the transit through the digestive tract.

Microscopic analysis using CLSM and SEM provided visual evidence of the effects of LAB active cultures and CFS on biofilm viability and structure. Biofilms treated with active LAB cultures showed a high proportion of live LAB cells, suggesting that these bacteria not only effectively kill *S. aureus* but also colonize biofilms. This colonization ability is particularly relevant for preventing infection recurrence, as LAB cells can occupy ecological niches left vacant by pathogenic bacteria, thereby reducing the likelihood of recolonization by pathogens[47,71,72]. On the other hand, biofilms treated with CFS showed nearly complete *S. aureus* cell death, revealing the high antimicrobial efficacy of CFS. Specifically, SEM images showed significant biofilm disintegration following CFS treatment, supporting the postulate that CFS directly disrupt the extracellular matrix while inducing massive *S. aureus* cell death. These findings confirm the results of previous studies documenting the biofilm-disrupting effectiveness of LAB CFS[64–66].

CLSM imaging assessment of the biovolume and thickness of the staphylococcal biofilms after treatment with LAB active cultures and their CFS showed distinct responses depending on the type of treatment. Biofilm biovolume and thickness increased in some instances following treatment with active cultures, likely due to LAB displacing *S. aureus*, occupying its ecological niche, and forming their own beneficial biofilms. This probiotic colonization is favorable for host protection, as previously indicated. However, changes in biofilm biovolume and thickness after treatment with CFS varied depending on the strain. Treatment with *L. fermentum* I7 and *L. reuteri* 7SNG3-30 CFS resulted in stable or slightly increased biofilm thickness, suggesting early inhibition of *S. aureus* growth followed by eventual cell death, while a clear decrease in thickness was observed with *L. salivarius* 22SNG3-30 CFS indicating accelerated biofilm cell dispersion. The overall reduction in maximum biofilm thickness across treatments suggests effective penetration of LAB and/or their metabolites into the biofilm matrix, facilitating access to bacterial cells that would otherwise be protected. This finding is particularly relevant as biofilm size and density are directly related to their ability to resist conventional antimicrobial treatments and confirm other studies that highlight the ability of LAB to reduce both the size and viability of pathogenic biofilms[73].

The variability in the response of different *S. aureus* strains to treatments with LAB emphasizes the importance of carefully considering both the probiotic and pathogenic strains when selecting a therapeutic strategy[50]. While all three LAB strains demonstrated notable antimicrobial activity, their effectiveness varied depending on the type of treatment (active culture or CFS) and the specific *S. aureus* strain. For instance, LAB active cultures were less effective than CFS in eliminating SA4 biofilms, suggesting that metabolites produced by LAB play a crucial role in this action. In contrast, treatment with *L. fermentum* I7 active culture was more effective than CFS for SA55 biofilms, indicating that live LAB cells may contribute significantly through mechanisms such as adhesion and competition for ecological niches. These findings are consistent with studies that emphasize the strain-specific nature of probiotic interactions, where factors such as antimicrobial metabolite production or adherence capacity vary according to the strains involved[50,62].

Work is in progress to overcome some of the limitations of this study, by increasing the number of interacting *S. aureus* and LAB strains and introducing transcriptomic and metabolomic analyses of such interactions. The results obtained with the CFS from the studied LAB strains have shown significant antimicrobial efficacy although further studies are needed to identify and characterize the bioactive compounds responsible for this activity. Nevertheless, we cannot discard that the observed activity towards *S. aureus* biofilms depends on a synergistic action of the metabolic by-products present in the CFS. In this case, they could be used as postbiotics in their present form[74]. The final goal is to select the best strains to perform, first, in vitro assays using breast epithelial lines with secretory potential to approximate the in vivo environment, and then, in vivo assays in animal models and human clinical trials.

Conclusions

This study highlights the promising potential of LAB probiotics and postbiotics for treating infections associated with *S. aureus* biofilms, such as mastitis. Both LAB active cultures and CFS demonstrated effectiveness in reducing biofilm viability and disrupting its structure, emphasizing their value as alternative or complementary therapeutic strategies to traditional antibiotics. However, the efficacy of these treatments seems to be somehow influenced by the *S. aureus* and LAB strains involved, emphasizing the importance of identifying the compounds responsible for the antimicrobial activity against this pathogen[53,75,76]. Furthermore, these treatments should be evaluated in animal and clinical models to optimize interventions for various *S. aureus* strains.

Data availability

The authors declare that the data supporting the findings of this work are available within the paper. Should any raw data files be needed, they are available from the corresponding author upon request.

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Author contributions

RJ conducted the main experiments, performed the data analysis and the figure layout and wrote the original draft; JJ performed some of the experiments and contributed to the data analysis; AA and NH contributed to the data analysis; JMR wrote and reviewed the manuscript; LF and BO designed and supervised the work and wrote and reviewed the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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