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Unlocking the potential of lactic acid bacteria mature biofilm extracts as antibiofilm agents

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Abstract
The continuous growth of biofilm infections and their resilience to conventional cleaning methods and antimicrobial agents pose a worldwide challenge across diverse sectors. This persistent medical, industrial, and environmental issue contributes to treatment challenges and chronic diseases. Lactic acid bacteria have garnered global attention for their substantial antimicrobial effects against pathogens and established beneficial roles. Notably, their biofilms are also predicted to show a promising control strategy against pathogenic biofilm formation. The prevalence of biofilm-related problems underscores the need for extensive research and innovative solutions to tackle this global challenge. This novel study investigates the effect of different extracts (external, internal, and mixed extracts) obtained from *Lactobacillus rhamnosus* GG biofilm on pathogenic-formed biofilms. Subsequently, external extracts presented an important eradication effectiveness. Furthermore, a 6-fold concentration of these extracts led to eradication percentages of 57%, 67%, and 76% for *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* biofilms, respectively, and around 99.9% bactericidal effect of biofilm cells was observed for the three strains. The results of this research could mark a significant breakthrough in the field of anti-biofilm and antimicrobial strategies. Further studies and molecular research will be necessary to detect the molecules secreted by the biofilm, and their mechanisms of action engaged in new anti-biofilm strategies.

Key points
• Using *L. rhamnosus* GG biofilm extracts to eradicate pathogenic biofilms. Antimicrobial effect of *L. rhamnosus* GG biofilm external extracts against biofilm-associated bacteria. Increasing biofilm eradication by combining with a mixture of antimicrobial agents.

Keywords Biofilm, Lactic acid bacteria, *L. rhamnosus* GG, Antibiofilm, Antimicrobial

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Introduction

Invisible to the naked eye yet pervasive throughout the natural world, biofilms are complex, dynamic communities of various microorganisms (bacteria, fungi, algae, and others), embedded within a self-produced extracellular matrix (Vestby et al. 2020; Karygianni et al. 2020). The matrix is composed of extracellular polymeric substances (EPS) typically consisting of polysaccharides, proteins or peptides, lipids, as well as deoxyribonucleic acids (DNA) (Samrot et al. 2021). These complex structures facilitate the coherence of cells and cell surface attachment (Hindieh et al. 2022), and play a pivotal role in the microbial universe and human health (Jo et al. 2022). These microbial conglomerates are widespread and can thrive on various surfaces, from submerged rocks in rivers and oceans to the nooks and crannies of medical devices, water pipelines, and even our bodies (Lenhart et al. 2014).

The process of bacterial biofilm formation initiates with the initial attachment of bacteria to a surface, facilitated by reversible adhesive interactions. Once firmly attached, bacteria release extracellular polymeric substances (EPS), creating a sticky matrix that encases the cells and provides structural integrity for the biofilm (Tolker-nielsen 2015). Bacteria divide and form microcolonies within this matrix, establishing a three-dimensional structure. As the biofilm matures, cells differentiate into specialized roles, and communication pathways enhance coordination and resistance to external threats (Wilking et al. 2013). Ultimately, some biofilm cells detach, return to their planktonic lifestyle, and disperse to new surfaces, perpetuating the cycle of biofilm formation and colonization (Chua et al. 2014). In agreement with the fact that bacteria are closely associated in biofilms, bacterial cells within biofilms communicate and synchronize their behavior using signaling molecules, constituting a system known as Quorum Sensing (QS), which extends beyond bacterial cell density (Prazdnova et al. 2022). These molecules regulate specific gene expression in reaction to high cell density (Rutherford and Bassler 2012). Primarily, the roles of QS are categorized into maintaining and dividing cells (production of exoenzymes and siderophores), facilitating horizontal gene transfer, influencing host–pathogen interactions (production of antibiotics and bioluminescence), and modulating behavior (such as movement and biofilm formation and dispersion) (Preda and Săndulescu 2019). Disruption of QS signals can occur through various mechanisms, including inhibiting autoinducer synthesis, blocking binding to receptors, degrading autoinducers, competitive inhibition, and blocking target genes triggered by the QS signal (Srinivasan et al. 2021).

On the negative side, biofilms can cause significant economic losses in industrial settings by blocking pipelines, fouling equipment, and decreasing energy efficiency (de Carvalho 2018). Furthermore, biofilms can impact agriculture by contaminating irrigation systems and reducing crop yields (Butucel et al. 2022). In healthcare, they are a persistent source of infections associated with medical devices, leading to extended hospital stays and increased healthcare costs (Hassett et al. 2014). In the United States, the annual incidence of biofilm-related infections is 1.96 million cases, causing an estimated 268,000 deaths, with more than $US18 billion in direct costs spent on the treatment of these infections (Amin Omar et al. 2017). Biofilm-forming microorganisms have developed various strategies to evade the immune system and antibiotics. Dormant bacteria within a biofilm can cause tissue damage and trigger acute infections (Stewart and Costerton 2001). To cope with oxygen scarcity and nutrient limitations, biofilm bacteria modify their metabolism, gene expression, and protein production, resulting in slower growth and reduced susceptibility to antimicrobials (Costerton et al. 1999). Bacterial biofilm serves as a crucial mechanism for antibiotic resistance, with biofilm-enclosed bacteria exhibiting up to 1000 times greater resistance to antibiotics (Srinivasan et al. 2021). Biofilm bacteria also exhibit increased horizontal gene transfer, facilitating the spread of resistance genes to susceptible bacteria (Madsen et al. 2012). Consequently, biofilm-associated diseases are typically persistent, slow-developing infections that are extremely difficult to prevent and cure. Many studies showed that *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* are some of the most important biofilm-forming pathogens with a wide variety of complications as well as life-threatening infections (Katongole et al. 2020; Hoteit et al. 2022; Tuon et al. 2022; Bevers et al. 2022). In a literature review from 2020 focusing on biofilms in periprosthetic infections, Shoji and Chen 2020 found that *S. aureus* biofilms were present in 21–43.6% of these infections. Furthermore, *P. aeruginosa* biofilms is one of the most prevalent pathogens in hospital environments, causing more than 50% of healthcare-acquired infections (Tuon et al. 2022). In addition, *E. coli* accounts for 70–95% of urinary tract infections (UTIs) worldwide (Maione et al. 2023). In a publication from 2017, the World Health Organization (WHO) also listed these bacteria as among the highest priority for new antibiofilm therapy development (Tacconelli et al. 2018). Hence, it is critically important to design or screen novel antibiofilm agents that can effectively prevent biofilm formation or eradicate existing biofilm. While often regarded for their detrimental effects in various industries and healthcare, biofilms also have some positive aspects that should be acknowledged. Biofilms contribute positively to natural ecosystems by playing crucial roles in nutrient cycling and organic matter decomposition (Rumbaugh and Sauer 2020). They

also serve as indicators for monitoring ecological health and water quality (Highmore et al. 2022). Moreover, in recent years, more and more attention has been paid to biofilms of lactic acid bacteria (LAB) including species of *Lactobacillus* that serve as agents of food fermentation and potentially impart health benefits (Nahle et al. 2022a, b). Thus, due to their health benefits and ability to defeat intestinal pathogens, regulate intestinal flora balance, and maintain the intestinal barrier, more importance is given nowadays to these LAB strains (Matsubara et al. 2016; Zhang et al. 2020; Nahle et al. 2023). In addition, these strains exerted antimicrobial effects through the production of compounds such as bacteriocins and many others such as *E. coli* (Li et al. 2020), and *S. aureus* (Li et al. 2020), and *P. aeruginosa* (Mohammed Aman et al. 2021). Furthermore, a certain type of Lactic Acid Bacteria biofilm could be used as a protective biofilm against pathogens and their associated biofilms (Mgomi et al. 2023). Additionally, scientists worldwide have embraced the LAB biofilm-based techniques instead of the conventional planktonic phase alone (Assaf et al. 2019; Speranza et al. 2020). *L. rhamnosus* GG was selected for this investigation due to its Generally Recognized as Safe (GRAS) status granted by the Food and Drug Administration (FDA) (Nahle et al. 2022a, b). Additionally, it is extensively used as a probiotic, with publicly available genome (Marques et al. 2023).

This novel study aims to investigate the potential of *L. rhamnosus* GG biofilm extracts in disrupting, disassembling, and inhibiting pathogenic microbial biofilms. Hence, different extracts from *L. rhamnosus GG* biofilm (external, internal, and mixed extracts) are extracted, and their effect are assessed against different pathogenic bacteria including *E. coli*, *S. aureus* and *P. aeruginosa* formed biofilms. Moreover, the bactericidal effect against biofilm-associated bacteria is also assessed.

Materials and methods

Bacterial strain and culture conditions

Lactobacillus rhamnosus GG (ATCC 53103), *Escherichia coli* (ATCC 10536), *Staphylococcus aureus* (ATCC 49619), and *Pseudomonas aeruginosa* (ATCC 27853) were obtained in lyophilized tablet form from Microbiologics (St. Cloud, MN, USA). For the biofilm eradication experiments, these strains were introduced into various growth media that are specific to their requirements. Consequently, *L. rhamnosus* GG was cultured in MRS broth (de Man-Rogosa-Sharpe) (Scharlab S.L., Spain), while *E. coli* and *S. aureus* in TSB (Tryptic Soy broth) (Liofilchem, Italy), and *P. aeruginosa* in LB broth (Luria–Bertani) (HiMedia Laboratories Pvt.Ltd., India). These strains were then cultivated overnight at 37℃ under aerobic conditions, as detailed by Parvekar et al. (2020). The turbidimetric method was used to determine the bacterial cell concentration in MRS broth, TSB, and LB (Begot et al. 1996). Thus, the absorbance was measured at 600 nm (OD600) using a spectrophotometer (Thermo Fisher Scientific, MA, USA), and the bacterial growth curves were constructed over a 24-h incubation period (Figs. S1, S2, S3, S4). The logarithmic value of bacterial concentration was also obtained by using the solid media counting method. Equations for the calculation of the bacterial concentration for each bacterial strain were then generated with a compliant coefficient of determination (R^2=0.999).

Preparation of the in vitro *L. rhamnosus* GG biofilm

L. rhamnosus GG biofilm was formed using 6-wells flat bottom treated microplates (Techno Plastic Products AG, Switzerland). Several sets of microplates have been prepared to specifically track the progression of biofilm formation over a 7 day-duration, thus allowing for the daily collection of various extracts from the biofilm. To initiate the experiments, a bacterial suspension with an adjusted volume was inoculated into the MRS broth, resulting in a final volume of 3 mL within each well and a bacterial concentration of 10^7 bacteria/mL. The microplates were then incubated, during a suitable period, under aerobic conditions without shaking at 37°C. Day 1 of biofilm formation is considered after 3 days of incubation.

Preparation of the in vitro *E. coli*, *S. aureus*, and *P. aeruginosa* biofilms assay

In this study, *E. coli*, *P. aeruginosa*, and *S. aureus* biofilms were formed using 96-well flat-bottom microplates (Techno Plastic Products AG, Switzerland). An adjusted volume of bacterial suspension was introduced into the medium tailored to each strain (TSB for *E. coli* and *S. aureus* and LB for *P. aeruginosa*), resulting in a final volume of 100 µL in each well, with a bacterial concentration of 5×10^5 bacteria/mL. Subsequently, the microplates were incubated under aerobic conditions at a constant temperature of 37°C for 72 h, with no agitation applied during this incubation period.

Evaluation of *L. rhamnosus* GG biofilm formation

L. rhamnosus GG biofilm formation was surveyed daily over 7 days, by measuring optical density. After the removal of the supernatant, the biofilm was fixed every day by heating at 80°C for 1 h. Thereafter, it was stained with 1 mL of 0.01% (w/v) crystal violet for 10 min at room temperature (Assaf et al. 2019, Nahle et al. 2023). The crystal violet solution was carefully discarded, and the biofilm was gently washed three times with distilled water to eliminate excess stains and non-adherent cells. An extensive pipetting was conducted to physically detach the adherent biofilm. The removed suspension was then vortexed for 1 min. The optical density was

measured at 570 nm, allowing for the quantification of the formation of *L. rhamnosus* GG biofilm (Wilson et al. 2018).

Extraction of *L. rhamnosus* GG mature biofilm

Three different types of extracts from the *L. rhamnosus* GG mature biofilm were collected between day 1 and day 7 of the formation to assess their effect on fully developed pathogenic biofilms. The extraction procedure of these extracts is summarized in Fig. 1.

Preparation of external extracts of L. rhamnosus GG mature biofilm

In this study, the external extracts of mature *L. rhamnosus* GG biofilm refer to compounds or substances derived from the outer layers or surroundings of the biofilm and secreted by the microorganisms externally through the biofilm matrix.

To obtain the external extracts from *L. rhamnosus* GG biofilm from day 1 to day 7, the medium was carefully removed from the well. Subsequently, the medium was centrifuged at 2500×*g* for 10 min at 4°C, effectively eliminating both the remaining biofilm matrix and suspended bacterial cells. The supernatant was then filtered employing filters with pore sizes 0.22 µm (Biomed Scientific, United States). The obtained supernatant now contains the biofilm external extracts that will be used for biofilm eradication testing. A sterility test of the extracts was carried out by inoculating them on TSA (Tryptic Soy Agar) (Liofilchem, Italy) to verify the absence of bacteria remaining after filtration.

Preparation of internal extracts of L. rhamnosus GG mature biofilm (with and without the mixture of lysozyme-EDTA)

In this study, internal extracts of *L. rhamnosus* GG mature biofilm refer to substances or compounds that are extracted from biofilm's interior layers or constituent parts. These extracts consist of compounds and bioactive substances derived from the microbial cells that are encased in the biofilm matrix.

The internal extracts were tested singly or mixed with lysozyme (80 mg/mL)-EDTA (Ethylenediaminetetraacetic acid) (1 mM) (lysozyme from Vivantis Technologies, Malaysia and EDTA from Sigma, St. Louis, MO, USA) (Hindieh et al. 2022). The Lysozyme (80 mg/mL)-EDTA (1 mM) mixture was defined as an antimicrobial agent that functions by breaking down the cell walls of the bacteria. This mixture was used to disrupt the membrane

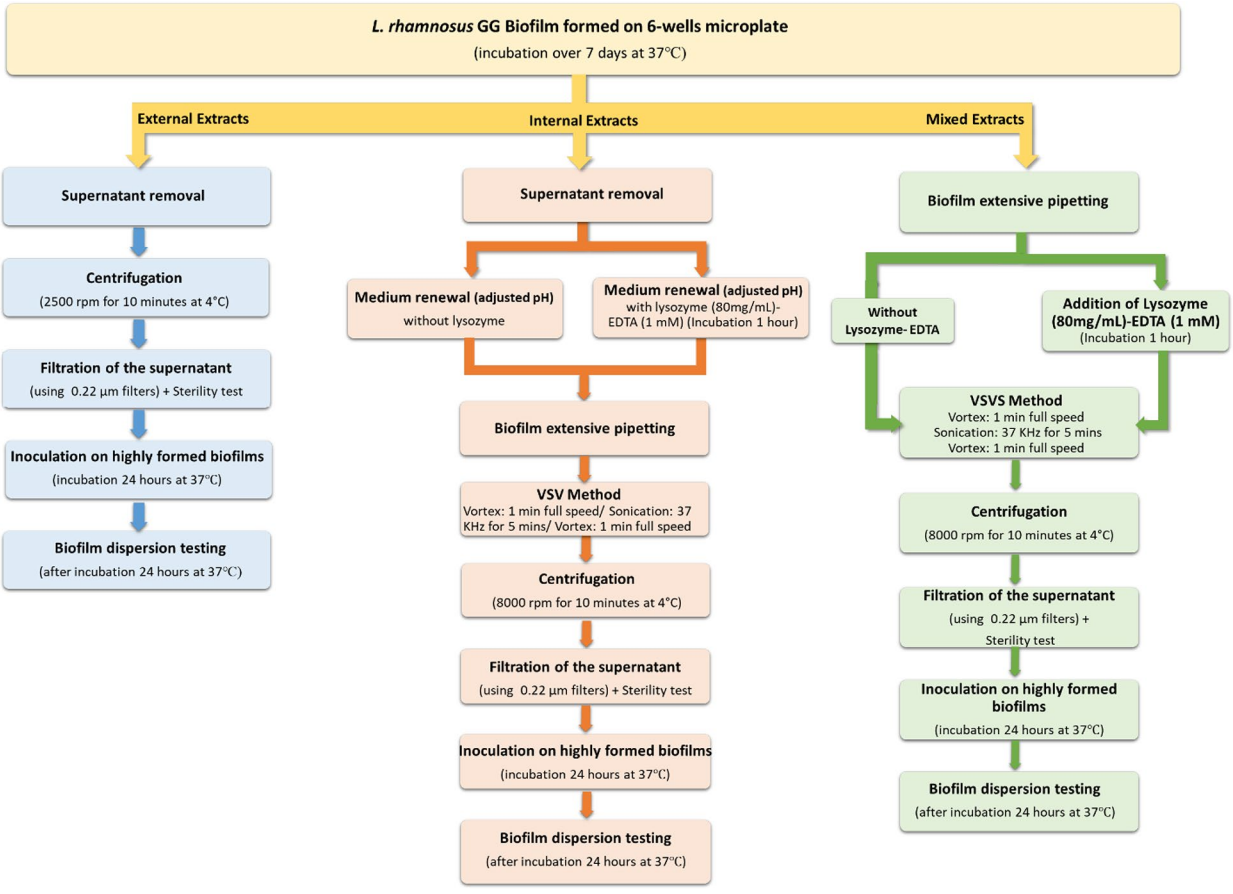

Fig. 1 Brief steps of the extraction of *L. rhamnosus* GG mature biofilm extracts

of *L. rhamnosus* GG to recuperate the cytoplasmic and, thus the internal extract (Hindieh et al. 2022).

The extraction and manipulation of internal extracts from *L. rhamnosus* GG biofilm were carried out using the following procedure. The medium of *L. rhamnosus* GG mature biofilm was removed to eliminate the effect of the external extracts. Then, the biofilm was resuspended in a new pH-adjusted medium (MRS pH 4.5) to maintain the same conditions and eliminate the pH effect (Mgomi et al. 2023). Therefore, the pH of the biofilm medium (MRS broth) was monitored daily to subsequently adjust the newly added medium. Subsequently, the attached *L. rhamnosus* GG biofilm on the microplate was extensively pipetted until complete removal. Upon combination of the extracts with a lysozyme (80 mg/mL)-EDTA (1 mM) mixture, the resuspended medium incorporated the lysozyme-EDTA, with a 1-h incubation preceding the centrifugation. The 1-h incubation with lysozyme-EDTA allows this mixture to better reach all the bacteria within the biofilm. Consequently, after adding lysozyme-EDTA, Vortex-Sonication-Vortex Method (VSVM) was performed to further break down bacterial communities into individual cells (Okae et al. 2022). To eliminate any residual matrix and bacteria a centrifugation step was conducted at 8000×*g* for 10 min at 4°C. The resulting supernatant contained the internal extracts of the biofilm. This supernatant was then subjected to filtration using 0.22 µm filters, to ensure the complete elimination of any remaining bacteria and prevent potential growth when inoculated onto a newly formed biofilm. Finally, a sterility test of the extracts was carried out by inoculating them on TSA (Tryptic Soy Agar) (Liofilchem, Italy) to verify the absence of bacteria remaining after filtration.

Preparation of mixed extracts of L. rhamnosus GG mature biofilm (with and without the mixture of lysozyme-EDTA)

In this study, mixed extracts of *L. rhamnosus* GG mature biofilm refer to the mixture of both external and internal extracts of the biofilm.

As for the internal extracts, the mixed extracts (external and internal extracts) were tested singly or combined with the lysozyme-EDTA mixture.

The biofilm was extensively pipetted until a complete removal of all adherent biofilm was achieved. When combined with lysozyme (80 mg/mL)-EDTA (1 mM), this mixture was added to the mixed extract immediately following thorough pipetting. Then, this mixture was submitted for 1-h incubation period before the centrifugation. Subsequently, the biofilm then undergone the Vortex-Sonication-Vortex Method (VSVM) during the incubation with the mixture of lysozyme-EDTA. Moreover, to eliminate any residual matrix and bacteria and facilitate the extraction of extracellular polymeric substances (EPS), centrifugation was performed at 8000×*g* for 10 min at 4°C. The resulting supernatant containing biofilm external and internal extracts was then carefully transferred by employing filtration using 0.22 µm filters, to remove any remaining bacteria and prevent potential growth when these extracts were inoculated onto a newly formed biofilm. Finally, a sterility test of the extracts was carried out by inoculating them on TSA (Tryptic Soy Agar) (Liofilchem, Italy) to verify the absence of bacteria remaining after filtration.

Application of *L. rhamnosus* GG mature biofilm extracts on mature biofilms

100 µL of the suspensions containing external, internal, and mixed extracts were inoculated onto newly formed 3-day biofilms, consisting of *E. coli*, *S. aureus,* and *P. aeruginosa*. Following a 24-h incubation period, the biofilm eradication test was assessed by removing the supernatant, and the biofilms were fixed by heating at 80°C for 1 h. Following fixation, the microplates were stained with 100 µL of 0.1% crystal violet for 10 min at room temperature as previously described (Lim et al. 2020). Consequently, the crystal violet solution was carefully discarded, and the biofilms were gently washed three times with distilled water to eliminate excess stains and non-adherent cells. Finally, the optical density of the biofilms was measured at 570 nm, allowing for the quantification of biofilm dispersion in the experimental conditions (Hindieh et al. 2022).

Application of *L. rhamnosus* GG mature biofilm extracts on planktonic bacterial strains

Extracts from the *L. rhamnosus* GG biofilm (external, internal, and mixed extracts), obtained over a 7-day formation period, were tested on planktonic strains. Suspensions containing external, internal, and mixed extracts collected over the 7-day period of *L. rhamnosus* GG biofilm formation were inoculated with planktonic strains, including *E. coli*, *P. aeruginosa*, and *S. aureus*. The experiment was conducted using 96-well-curved bottom non-treated plates (Techno Plastic Products AG, Switzerland) as per guidelines of the National Committee for Clinical Laboratory Standards (CLSI 2012). A precise volume of the bacterial suspension was added to each well, reaching a final volume of 100 µL with a bacterial concentration of 5×10^5 bacteria/mL (CLSI 2012). Wells with only culture medium and bacterial inoculum served as a positive control, while wells with only *L. rhamnosus* GG biofilm extracts served as a negative control. Additionally, wells with only culture medium were used for sterility control. Following inoculation, the plates were incubated at 37°C for 24 h (Umerska et al. 2018). Post-incubation, the optical density was measured at 600 nm, enabling the quantification of the inhibition effect under the specified experimental conditions.

Application of increasing extract concentration on matures biofilm

After conducting experiments to assess the impact of *L. rhamnosus* GG biofilm extracts (external, internal, and mixed extracts) on the formed biofilms of the strains *E. coli*, *S. aureus*, and *P. aeruginosa* mentioned earlier over seven days, we optimized the effects by identifying the most efficient eradication result for each strain. To further concentrate the extracts with optimum eradication effect, we used a sample concentrator (MD200) (Hangzhou Allsheng Instruments Co, China), resulting in twofold and sixfold concentration levels. These concentrated extracts were subsequently subjected to the same testing protocol as previously described for each suitable biofilm strain.

Application of concentrated external extracts of *L. rhamnosus* GG mature biofilm on biofilm-associated *bacteria*

After 24 h-incubation of concentrated external extracts with biofilms, before performing the biofilm eradication test, a sample from one well of each test and a positive control well containing pathogenic biofilm were removed and placed in a 1.5 mL Eppendorf tubes (Bio Basic, Canada) to examine the killing effect of the extracts the remaining biofilm-associated bacteria. These tubes were subjected to the Vortex-Sonication-Vortex Method (VSVM) to count remaining detached microorganisms (Rosa et al. 2019). Subsequently, these samples were serially diluted, evenly spread onto Tryptic Soy Agar TSA medium (Liofilchem, Italy), and quantitatively analyzed by the colony forming units (CFU) assay by counting the colony number on TSA plates after 24 h incubation at 37℃ (Panisello Yagüe et al. 2021; Okae et al. 2022).

Statistical analysis

All tests were done in triplicate. To identify significantly different results, Two-way ANOVA was conducted using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). The results with a $P<0.05$ were considered statistically significant.

Results

Monitoring *L. rhamnosus* GG biofilm formation

Figure 2 illustrates the optical density (OD) values at 570nm wavelength over 7 days, indicating the growth of *L. rhamnosus* GG biofilm. It's being used to quantify the formation of biofilm.

The OD values were generally increasing over the 7 days. The initial significant increase ($P<0.05$) from day 1 ($OD_{570nm}=2.050$) to day 2 ($OD_{570nm}=2.167$) suggests active *L. rhamnosus* GG biofilm formation. This increase continued on day 3 ($OD_{570nm}=2.235$). However, the subsequent fluctuation in OD values was observed on days 4 ($OD_{570nm}=2.221$) and 5 ($OD_{570nm}=2.259$). From Day 5 onward, the OD values appeared relatively stable, with minor fluctuations. The OD values on Days 6 ($OD_{570nm}=2.250$) and 7 ($OD_{570nm}=2.251$) were very close, indicating a consistent level of biofilm density. This

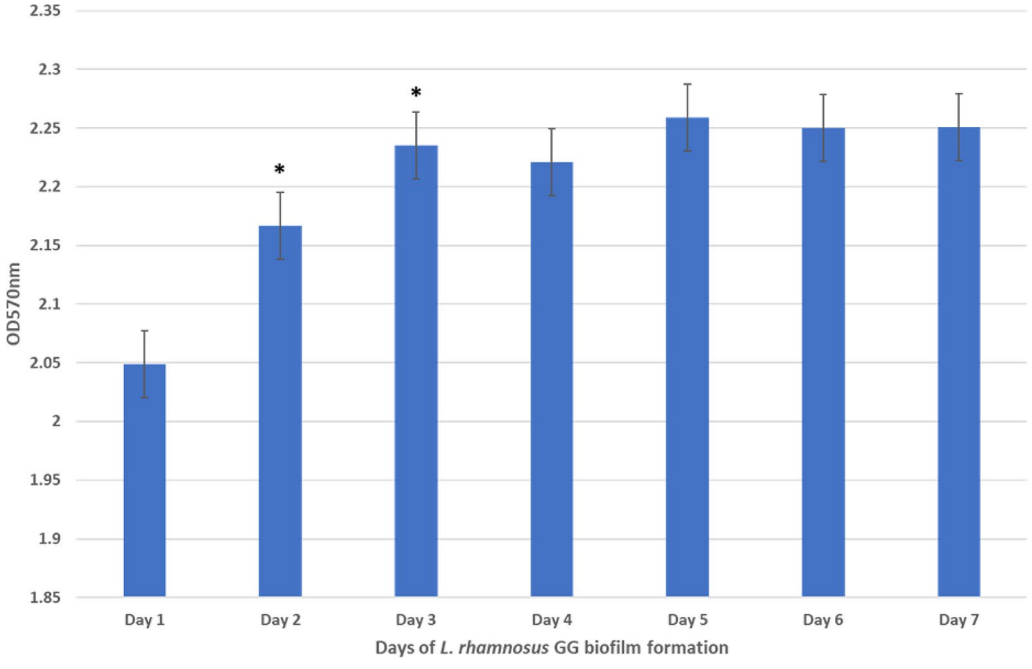

Fig. 2 Follow-up of *L. rhamnosus* GG biofilm formation over a 7 day-duration. Day 1 of biofilm formation was considered after 3 days of incubation. The optical density of 570 nm allows the quantification of the formation of *L. rhamnosus* GG biofilm. (*) indicates a significant difference ($P<0.05$) between each day and its preceding. Error bars represent the SD (standard deviation)

consistency indicates that the biofilm may have reached a relatively steady state or maximum density under the given conditions.

Effect of *L. rhamnosus* GG mature biofilm extracts on planktonic strains

By testing the extracts derived from *L. rhamnosus* GG mature biofilm on various planktonic strains (*E. coli*, *S. aureus*, and *P. aeruginosa*), their influence was found to be not significant. The bacterial growth remained the same with and without the addition of these extracts (Data not shown).

Effect of *L. rhamnosus* GG mature biofilm extracts on *E. coli* mature biofilm

Figure 3 illustrates the effect of *L. rhamnosus* GG biofilm extracts collected over 7 days of formation on highly formed *E. coli* biofilm.

In this study, the effect of *L. rhamnosus* GG biofilm extracts collected over 7 days and their modifications on *E. coli* biofilm formation was investigated. The external extracts, obtained from day 1, demonstrated a reduction in *E. coli* biofilm from 100% (positive control) to 93%. The biofilm formation significantly decreased ($P<0.05$) and reached 82% with day 2 external extracts, and days 3 and 4 external extracts did not exert a wide effect on *E. coli* biofilm formation, resulting respectively in decreasing percentages from 100% (positive control) to 92% and 91%. However, day 5 external extracts revealed a substantial drop to 70% ($P<0.05$) in formed *E. coli* biofilm. Days 6 and 7 external extracts showed considerable decreases to 75% and 85% of *E. coli* biofilm formation, respectively. Comparative analysis revealed a significant day-to-day reduction of approximately 7% ($P<0.05$) from day 1 to day 2, followed by a more considerable 21% reduction between days 4 and 5.

Internal extracts also exhibited a significant reduction ($P<0.05$) by around 80% in *E. coli* formed biofilm with days from 1 to 4. Accordingly, a considerable diminution of the formation ($P<0.05$) of about 9% was observed with internal extracts from day 4 to day 5, with stability, thereafter, ending at 82% of *E. coli* biofilm formation with those from day 7.

Introducing lysozyme (80 mg/mL)—EDTA (1mM) to internal extracts significantly improved the reduction of *E. coli* biofilm ($P<0.05$), initiating at 42% of formation instead of 82% without the additives and exhibited fluctuations within the range of 40–45% throughout the entire 7-day duration. Mixed extracts from days 2 and 3 of *L. rhamnosus* GG biofilm, induced important reductions of *E. coli* formed biofilm ($P<0.05$), reaching 78% and 88% respectively. Subsequent days demonstrated a notable and significant decrease of *E. coli* biofilm ($P<0.05$) to around 77% with mixed extracts from days 4, 5, and 6, ultimately reaching 85% with those obtained from day 7. The addition of lysozyme (80 mg/mL)—EDTA (1mM) to mixed extracts significantly improved the reduction of *E. coli* biofilm ($P<0.05$), starting at 60% as opposed to 90%

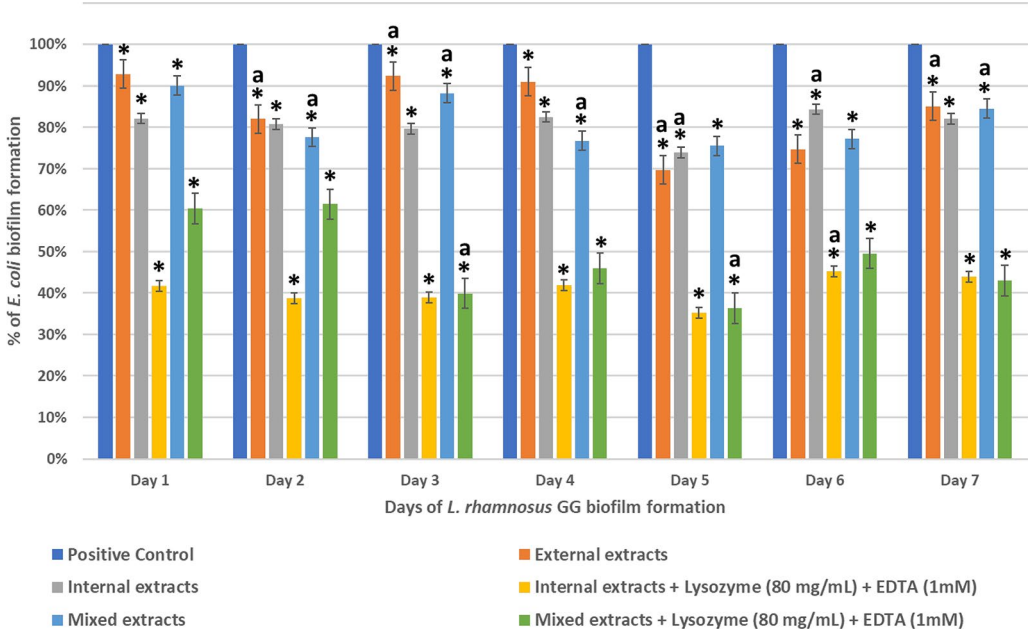

Fig. 3 Effect of different *L. rhamnosus* GG biofilm extracts collected over a 7 day-duration on highly formed *E. coli* biofilm. Internal extracts and mixed extracts were tested alone and combined with a lysozyme (80 mg/mL)-EDTA (1 mM) mixture. The results are mean values of three replicates. (*) indicates a significant difference ($P<0.05$) between each test and the positive control (without extracts and lysozyme). The lowercase letter (a) indicates a significant difference ($P<0.05$) between each day and its preceding. Error bars represent the SD (standard deviation)

(without lysozyme-EDTA mixture), and exhibiting fluctuations in the range of 40–43% throughout the 7-day duration.

It is quite noticeable that *L. rhamnosus* GG biofilm external extracts obtained from day 5 presented the most effective eradication effect on *E. coli* biofilm. They dispersed about 30% of *E.coli* formed biofilm, as shown in Fig. 3

Effect of *L. rhamnosus* GG mature biofilm extracts on *S. aureus* mature biofilm

The effect of *L. rhamnosus* GG biofilm different extracts on highly formed *S. aureus* biofilm is highlighted in Fig. 4.

In the evaluation of various extracts derived from the *L. rhamnosus* GG biofilm concerning *S. aureus* biofilm formation, distinct effects were discerned. External extracts collected over the 7 days of *L. rhamnosus* GG biofilm formation presented a significant increase in biofilm eradication ($P<0.05$). Compared to the positive control (100%), the percentage of *S. aureus* biofilm formation decreased to 71% with external extracts from day 1, gradually declining to a range of 68%-70% on days 2, 3, and 4. However, a much greater reduction ($P<0.05$) to 48% was observed on day 5, compared to both the control and the preceding day. The biofilm formation percentages with external extracts on days 6 and 7 were 78% and 80%, respectively. Internal extracts from the *L. rhamnosus* GG biofilm demonstrated a remarkable decrease ($P<0.05$) in *S. aureus* formed biofilm, reaching around 80% from day 2 to 4. A pronounced additional decrease of 27% ($P<0.05$) from day 4 to day 5 led to 56% of *S. aureus* biofilm with internal extracts from day 5 *L. rhamnosus* GG biofilm. Days 6 and 7 internal extracts also exhibited reductions in biofilm formation to 89% and 87%, respectively. A significant decrease ($P<0.05$) of *S. aureus* formed biofilm was noticed after the incorporation of lysozyme (80 mg/mL)-EDTA (1mM) into internal extracts, with the percentage fluctuating between 30 and 44% throughout the 7 days, highlighting the sustained effectiveness of the lysozyme and EDTA combination in reducing *S. aureus* biofilm formation. Mixed extracts from the *L. rhamnosus* GG biofilm had a comparatively less impactful effect, reducing *S. aureus* biofilm to 94%, 89%, 98%, and 90% on days 1, 2, 3, and 6, and 87% on days 4 and 7. Notably, mixed extracts from day 5 significantly decreased ($P<0.05$) *S. aureus* biofilm to 73%. Introduction of the lysozyme-EDTA mixture to mixed extracts exerted an important reducing effect on *S. aureus* biofilm formation, initiating at 67% with day 1 extracts, reaching a minimum of 38% with day 3 extracts, and fluctuating between 44 and 69% on subsequent days. It is observed that external extracts of *L. rhamnosus* GG extracts obtained from day 5 formation, presented the highest eradication effect about 48% of *S. aureus* formed biofilm, as shown in Fig. 4.

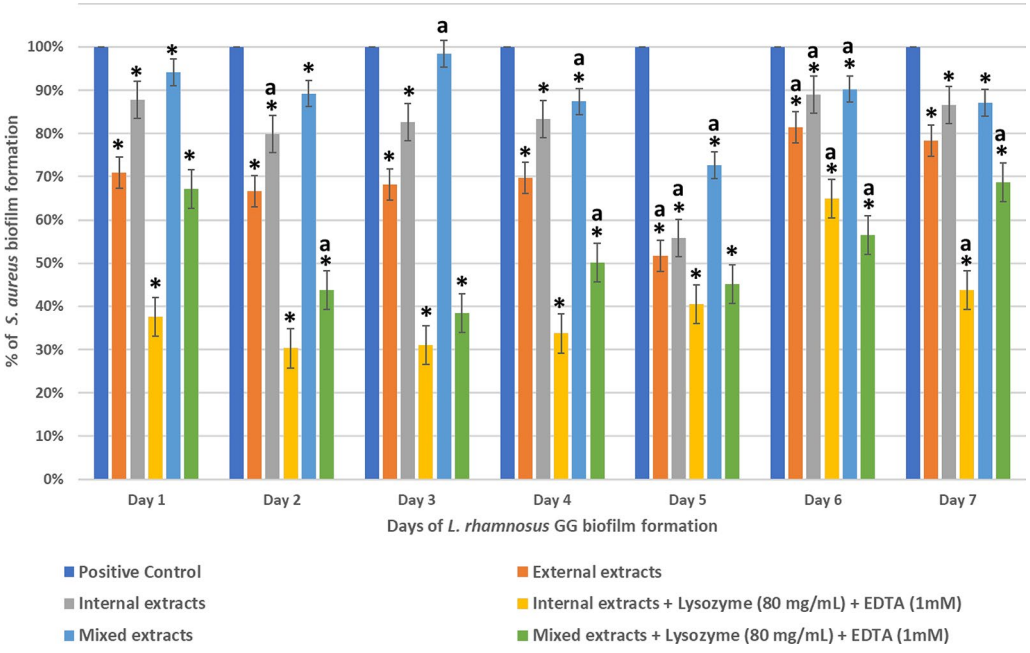

Fig. 4 Effect of different *L. rhamnosus* GG biofilm extracts collected over a 7 day-duration on highly formed *S. aureus* biofilm. Internal extracts and mixed extracts were tested alone and combined with a lysozyme (80 mg/mL)-EDTA (1 mM) mixture. The results are mean values of three replicates. (*) indicates a significant difference ($P<0.05$) between each test and the positive control (without extracts and lysozyme). The lowercase letter (a) indicates a significant difference ($P<0.05$) between each day and its preceding. Error bars represent the SD (standard deviation)

Effect of *L. rhamnosus* GG biofilm extracts on *P. aeruginosa* mature biofilm

The effect of *L. rhamnosus* GG biofilm different extracts on *P. aeruginosa* highly formed biofilm is shown in Fig. 5.

L. rhamnosus GG external extracts, collected over 7 days, demonstrated a significant effect ($P<0.05$) against *P. aeruginosa* biofilm formation, with notable variations observed between extraction days, particularly between days 2 and 3 ($P<0.05$). The biofilm formation exhibited a fluctuating pattern, starting at 56% with days 1 and 4 external extracts, peaking at 66% with day 2 extracts, and maintaining around 70% with external extracts from days 5, 6, and 7. The lowest biofilm formation percentage, 54%, was observed when incubated with external extracts from day 3. Similarly, internal extracts displayed a significant reduction effect ($P<0.05$) on *P. aeruginosa*-formed biofilm compared to the positive control, demonstrating a moderate impact. Biofilm formation varied from 62 to 80%, reaching its lowest (62%) with day 3 extracts. The addition of the lysozyme (80 mg/mL) and EDTA (1mM) mixture to internal extracts showed a significant reduction ($P<0.05$) in the percentage of biofilm formation. It initiated at 30% with day 1 extracts and gradually increased to 42% with day 7 internal extracts. Mixed extracts consistently led to higher biofilm formation percentages compared to other extracts, with significant variations ($P<0.05$) ranging between 70 and 85% for extracts from days 1, 2, 4, and 5, ultimately reaching 89% with days 6 and 7 mixed extracts. The lowest biofilm formation percentage, 65%, occurred when incubated with mixed extracts from *L. rhamnosus* GG biofilm day 3. However, when combined with lysozyme and EDTA, the mixed extracts exhibited a notable decreasing effect ($P<0.05$) on *P. aeruginosa* biofilm formation. This started at 46% with extracts from day 1, reached a minimum of 29% on day 4, and fluctuated between 46 and 63% thereafter. External extracts collected from day 3 *L. rhamnosus* GG biofilm showed the most significant eradication about 46% of *P. aeruginosa* biofilm.

Effect of increasing external extract concentration on biofilm formation

The *L. rhamnosus* GG biofilm extract exhibiting the greatest effect in reducing biofilm formation was selected for optimization across the tested strains. Notably, the external extracts obtained on the fifth day of *L. rhamnosus* GG biofilm formation proved to be the most effective in dispersing mature biofilms of *E. coli* and *S. aureus*, while those from the third day of formation showed optimal effectiveness in eradicating *P. aeruginosa* biofilm.

Additional experiments were conducted to investigate the effect of increasing the concentration of these external extracts on eradicating pathogenic biofilms. Additionally, the inhibitory and killing effects of these concentrated extracts on the viable cells within the biofilms were also assessed. The extracts were concentrated two and six times more than their initial concentration, and each concentration was assessed on the respective biofilm. The results of these optimization efforts are illustrated in Fig. 6.

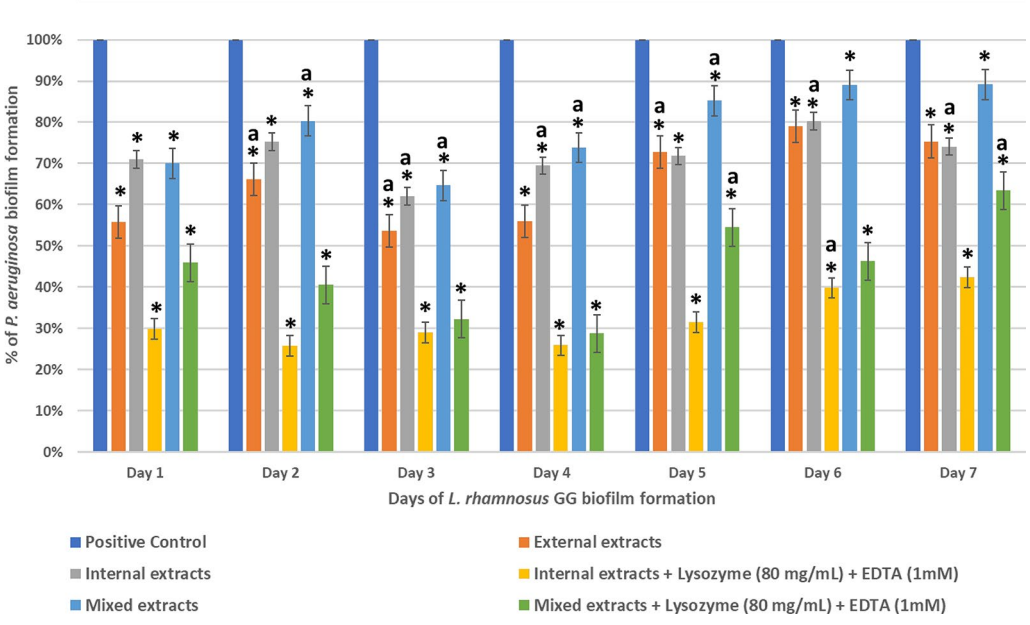

Fig. 5 Effect of different *L. rhamnosus* GG biofilm extracts collected over a 7 day-duration on highly formed *P. aeruginosa* biofilm. Internal extracts and mixed extracts were tested alone and combined with a lysozyme (80 mg/mL)-EDTA (1 mM) mixture. The results are mean values of three replicates. (*) indicates a significant difference ($P<0.05$) between each test and the positive control (without extracts and lysozyme). The lowercase letter (a) indicates a significant difference ($P<0.05$) between each day and its preceding. Error bars represent the SD (standard deviation)

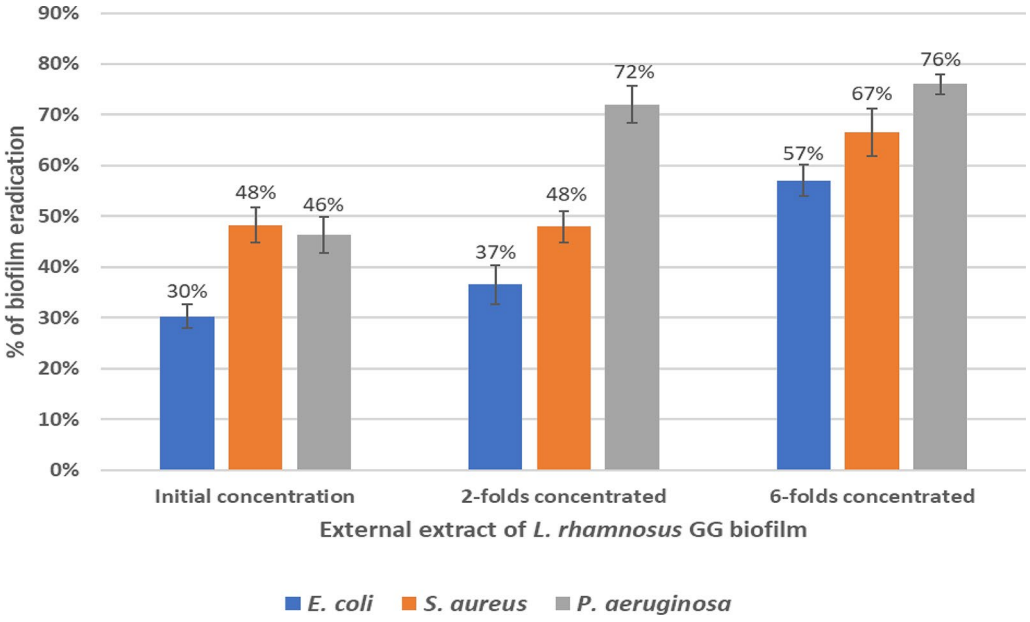

Fig. 6 Effect of increasing the external extract of *L. rhamnosus* GG biofilm concentration into twofold and sixfold concentrations. The external extract effectively dispersed mature *E. coli* and *S. aureus* biofilms on day 5, while on day 3, it effectively dispersed mature *E. coli* and *S. aureus* biofilms on day 5, while on day 3, it effectively dispersed the *P. aeruginosa* biofilm. The results are mean values of three replicates. Error bars represent the SD (standard deviation).

An eradication of 30% of *E. coli* biofilm was observed by approximately 30% using the initial concentration of *L. rhamnosus* GG biofilm external extract (from day 5 of its formation). The eradication percentage increased to 37% and 57% with twofold and sixfold concentrated same external extracts respectively (from day 5 of *L. rhamnosus* GG formation). Moreover, the initial concentration of *L. rhamnosus* GG biofilm external extract (from day 5) eradicated by 48% of the *S. aureus* biofilm. The percentage of eradication remained constant (48%) with a twofold concentration of the same extract but increased to 67% with a sixfold concentration. Likewise, the percentage of eradication of *P. aeruginosa* biofilm was 46% with *L. rhamnosus* GG biofilm day 3 external extracts. This percentage significantly increased to 72% with a twofold concentration and further to 76% with a sixfold concentration of the external extract. Hence, increasing the concentration of the external extracts led to an increased eradication.

Table 1 Comparative table between the killing effects of *L. rhamnosus* GG concentrated external extracts on the bacteria remaining viable in the biofilm after eradication of the three tested biofilm strains. The results are mean values of three replicates

Bacterial Biofilm		***E. coli***	***S. aureus***	***P. aeruginosa***
Positive control	Colony Forming Unit	2×10^{7} (±0.0145)	5×10^{7} (±0.0132)	8×10^{8} (±0.0172)
External Extract				
twofold concentrated	Colony Forming Unit	6×10^{6} (±0.1195)	4×10^{6} (±0.0903)	6×10^{1} (±0.0959)
	Killing ratio	$\mathbf{0.3\times10^{1}}$	$\mathbf{1.25\times10^{1}}$	$\mathbf{1.3\times10^{7}}$
	Killing percentage	**70%**	**92%**	**99.99%**
sixfold concentrated	Colony Forming Unit	8×10^{3} (±0.1167)	7×10^{3} (±0.1055)	2×10^{1} (±0.0920)
	Killing ratio	$\mathbf{2.5\times10^{3}}$	$\mathbf{7.14\times10^{3}}$	$\mathbf{4\times10^{7}}$
	Killing percentage	**99.96%**	**99.98%**	**99.99%**

The bold font is used to highlight the importance of the results

Effect of concentrated external extracts of *L. rhamnosus* GG biofilm on biofilm-associated *bacteria*

The killing effect was also performed with the external extract's concentrations. Notably, the external extracts presented an important killing effect for the three strains (Fig. S5). It was clearly observed that concentrated external extracts of *L. rhamnosus* GG biofilm killed some of the remaining bacterial cells in the eradicated biofilms. Compared to the positive control, the colonies of *E. coli* and *S. aureus* decreased with external extracts twofold concentrated and showed a significant bactericidal effect with sixfold concentrated extracts. Particularly, *P. aeruginosa* showed the most sensitive strain against these extracts. Detailed results are provided in Table 1.

These findings indicate the potent killing effect of the external extracts on the survival of bacterial cells, with *P. aeruginosa* showing particular sensitivity.

Upon application of twofold concentrated external extracts, a significant reduction in CFUs was observed for all strains. For *E. coli* and *S. aureus*, the killing rates were 0.3×10^{1} and 1.25×10^{1}, resulting in 70% and 92% of

killing percentages, respectively. In the case of *P. aeruginosa*, a remarkable 1.3×10^7 killing rate was achieved (corresponding to a 99.99% killing percentage). Further concentration to sixfold resulted in more substantial reductions in CFUs. For *E. coli, S. aureus*, and *P. aeruginosa* there were 2.5×10^3 (corresponding to a 99.96% killing percentage), 7.14×10^3 (corresponding to a 99.98% killing percentage), and 4×10^7 (corresponding to a 99.99% killing percentage) as killing rates respectively.

Discussion

The fluctuations in *L. rhamnosus* GG biofilm formation shown in Fig. 2, may indicate variability or changes in the biofilm growth dynamics. The provided results suggest an active biofilm formation with fluctuations and a potential stabilization in later days. It is evident that the dispersed state was not achieved, and the biofilm remained mature, as indicated by the consistently high optical density observed over these 7 days.

Furthermore, following 3 days of incubation, *L. rhamnosus* GG biofilm may have accumulated quorum-sensing signaling molecules in the surrounding environment due to an elevated bacterial density. Upon reaching a minimal threshold, these molecules bind to receptor proteins, consequently activating the expression of genes linked to biofilm formation (Rutherford and Bassler 2012). Additionally, lactic acid bacteria biofilms are recognized for their secretion of antimicrobial agents such as bacteriocins, bacteriocins-like substances, biosurfactants, H_2O_2, and metabolites including organic acids (Mgomi et al. 2023). Enzymes such as proteases, peptidases, polysaccharide-degrading enzymes, ureases, lipases, amylases, esterases, and phenoloxidases are also generated by lactic acid bacteria biofilms (Padmavathi et al. 2018).

Despite the notable effects of the extracts, particularly external and internal, derived from *L. rhamnosus* GG biofilm within the three different bacterial biofilms, their influence on the same strains in planktonic forms was not significant. This observation may be attributed to the extracts' specificity in targeting biofilm forms rather than planktonic forms of bacteria.

However, over the past years, LAB bioactive compounds such as bacteriocins, lactic acid, and other molecules, have garnered significant interest for their considerable potential, particularly as innovative therapeutic antibiotics (Assaf et al.2018; Darbandi et al. 2022). Notably, numerous studies have demonstrated their antibacterial effects against *E. coli*, *S. aureus,* and other pathogens (Gao et al. 2019; Dejene et al. 2021).

After incubating *L. rhamnosus* GG extracts (external, internal, and mixed) with different pathogenic biofilms including *E. coli*, *S. aureus*, and *P. aeruginosa* (presented in Figs. 3, 4, and 5), the intriguing aspect lies in the dynamic and fluctuation patterns observed both within the same extracts from day to day and among diverse extracts. This phenomenon may be attributed to the complex nature of the biofilm, characterized by many different gene expressions and the release of diverse molecules. An explanation for this variability could be linked to the initial stages of *L. rhamnosus* GG biofilm formation, where molecules were likely confined to the intracellular space (W. Jon windsor 2020; Mao et al. 2023). Subsequently, these molecules were secreted to the extracellular environment, evident in the most significant effect observed with external extracts (Figs. 3, 4, 5).

However, as the formation of *L. rhamnosus* GG biofilm progressed, the extracellular concentration of these secreted molecules could reach a "critical mass" (Salas-Jara et al. 2016). At this point, the intracellular molecules might cease their exit from the cell, whether through diffusion or transport, leading to an increase in their intracellular concentration. This scenario may provide insight into the sporadic, yet significant effects observed with internal extracts (Figs. 3, 4, and 5). Also, changes in the expression of the regulatory genes, including MaAb, LuxS, and the extracellular polysaccharides (EPS) gene cluster, may be involved in biofilm formation and secretion of the implicated molecules (Nahle et al. 2023). Also, these extracts may have decreased the expression of genes related to virulence factors, adhesion, biofilm formation, metabolism, and antimicrobial resistance in biofilm-forming cells and suspended cells (Qian et al. 2021).

Nonetheless, the restricted effect of the mixed extracts on the three different biofilms illustrated in Figs. 3, 4, and 5, may be due to a competition between molecules, leading to an antagonist effect, or it may be due to the binding of the molecules to the biofilm matrix or the cell wall. It can also be a response to genetic suppression or overexpression. All these suggestions require genetic and molecular studies to be able to understand the mechanisms of action, the related genetic expressions, and the released molecules that occur throughout the formation of the *L. rhamnosus* GG biofilm.

As it is evident, *L. rhamnosus* GG biofilm extracts have shown an important eradication effect on *S. aureus* biofilm (Fig. 4) as that on *E. coli* and *P. aeruginosa* biofilms (Figs. 3, 5). Hence, it is important to mention that these extracts are effective against gram-positive bacteria as well as gram-negative bacteria. Overall, the results highlight the varied and often significant effects of *L. rhamnosus* GG biofilm different extracts on dispersing and eradicating different pathogenic biofilms. Day-to-day variability in biofilm formation may indicate the dynamic nature of the biofilm system.

After detecting the significant eradication effect of *L. rhamnosus* GG biofilm extracts against different biofilm strains, several hypotheses can be postulated. The initial deduction is that the extracts exhibited an impact against

biofilms rather than planktonic bacteria. Subsequently, it can be asserted that these extracts specifically target systems associated with biofilm formation (Gondil and Subhadra 2023). Hence, these extracts may affect the quorum sensing of biofilms (Rémy et al. 2018).

However, communication through quorum sensing in planktonic free bacteria is considerably limited and even negligible (Solano et al. 2014; Preda and Săndulescu 2019). Quorum-sensing allows individual bacteria within colonies to coordinate and carry out colony-wide functions such as biofilm formation (W. Jon Windsor 2020). It seems that *L. rhamnosus* GG biofilm, recognized for its diverse array of biologically active compounds, may also serve as a potential origin of secreted metabolites with anti-quorum sensing (QS) properties. A recent study by Marques et al. 2023, demonstrated that the *L. rhamnosus* GG genome showed 33 proteins predicted as potential QS signaling peptides. However, *L. rhamnosus* GG biofilm may have released antimicrobial substances, metabolites and enzymes displaying anti-QS effect (Mao et al. 2023; Marques et al. 2023). The specific mechanisms of the antibiofilm functions of *L. rhamnosus* GG biofilm is not well understood; however, further approaches may be proposed to be involved in antibiofilm and antimicrobial effects. First, it may be due to the role of matrix-degrading enzymes, secreted by *L. rhamnosus* GG extracts, specifically external extracts, which dispersed the biofilm. Therefore, quorum sensing disruption may be caused by QS inhibitor molecules released by *L. rhamnosus* GG extracts, thus making bacteria inside more sensitive to specific antimicrobial compounds (bacteriocins, peptides, enzymes, etc.), achieving biofilm disruption and bacterial inhibition (Song et al. 2019). It is important to mention that maybe one molecule or a pool of molecules are engaged in QS disruption and biofilm dispersion. Thus, further studies and investigations are necessary. Moreover, the accelerated eradication may be also through QS regulation caused by *L. rhamnosus* GG biofilm extracts alone or added to the effect of the extracts already released by the other biofilm. Hence, when introducing mature *L. rhamnosus* GG biofilm extracts into another mature biofilm already secreting external autoinducers, there might be a potential increase in bioactive molecules, particularly after increasing the concentration of the extracts (Mukherjee and Bassler 2019). Instinctively, when the concentration of autoinducers reaches a critical threshold, it triggers specific responses in gene expression (Solano et al. 2014; Zhou et al. 2020), leading to coordinated actions such as biofilm formation, maturation, and disassembly (Shoji and Chen 2020; Samrot et al. 2021). Consequently, the quorum sensing system may interpret an inaccurate signal regarding QS autoinducers, leading to a directive for self-disassembly after maturation. Therefore, the bacteria communicating via quorum sensing (QS), switched to a floating state, directly encountering specific antimicrobial compounds (such as enzymes, bacteriocins, and others) released by *L. rhamnosus* GG biofilm. This exposure led to their inhibition or killing.

Moreover, the lysozyme-EDTA combination, previously investigated for its antimicrobial and antibiofilm effects (Hindieh et al. 2022), demonstrated an augmented effect with the internal and mixed extracts of *L. rhamnosus* GG biofilm on the three tested biofilms (Figs. 3, 4, 5). This synergistic effect could be attributed to EDTA's proficiency in chelating Mg^{2+} and Ca^{2+} within the biofilm's extracellular polymeric substances (EPS). Consequently, EDTA rendered the matrix more permeable to lysozyme, enabling the disruption of cell walls and facilitating the extraction of a greater quantity of biofilm internal extracts, and cytoplasmic contents. Thus, mixing it with these novel biofilm extracts may serve as an alternative or potentially reduce antimicrobial resistance issues (Hindieh et al. 2022).

Furthermore, by increasing the concentration of the external extracts, as observed in Fig. 6, higher eradication percentages of approximately about 20% can be achieved for *E. coli* and *S. aureus* biofilms. On the other hand, this is not the case for *P. aeruginosa* which may have reached a maximum of eradication explained by the negligible difference (8%) in the percentages of eradication between the twofold and sixfold concentrations. Consequently, Table 1 indicates that external extracts of *L. rhamnosus* GG biofilm also present bactericidal properties, since the killing rates and percentages (around 99.9%) have been achieved by increasing the concentration of the extracts.

The concentration-dependent nature of the reductions emphasizes the potential of these extracts in combating bacterial cells in biofilm formation (Fig. S5), providing valuable insights for further research and potential applications in biofilm control strategies. Therefore, to reach even larger eradication and killing percentages for *E. coli* and *S. aureus*, an increase in the concentration may be useful. Nevertheless, the bactericidal effect (Table 1) of the extracts seems to be correlated with the eradication effect (Fig. 6). Hence, a slower decrease in eradication and viable cells was observed with twofold-concentration when tested with *E. coli* and *S. aureus*. Conversely to *P. aeruginosa*, a twofold concentration highly eradicated the biofilm and killed its cells. It may be explained by the variability between strains.

However, when exposed to the same extracts, *E. coli*, *S. aureus*, and *P. aeruginosa* exhibit distinct behaviors, highlighting the diversity in their responses to these interventions. This differential response may be attributed to variations in their biofilm structures, genetic makeup, and signaling mechanisms. Each bacterium possesses distinct features that influence its susceptibility to

specific antibiofilm and antimicrobial agents. Scientific studies, such as those by (Vestby et al. 2020; Karygianni et al. 2020; Hindieh et al. 2022), have demonstrated the complexity of biofilm formation and the diversity in microbial responses. Furthermore, the wide-ranging environmental adaptations and surface interactions of biofilms contributed to the observed differences in antibiofilm efficacy across bacterial species. Understanding these variations is crucial for developing targeted and effective antimicrobial interventions tailored to the specific characteristics of each pathogen.

In this study, we proposed new dimensions in beneficial biofilm applications. It also opens the way for innovative, targeted solutions to combat biofilm-associated infections. The potential of *L. rhamnosus* GG biofilm extracts to eliminate pathogenic biofilms (*E. coli*, *S. aureus*, and *P. aeruginosa*) and destroy the cell growth of these strains. The addition of various extracts (external, internal, and mixed) from *L. rhamnosus* GG resulted in a noticeable reduction in biofilm formation through exclusion mechanisms. Notably, external extracts exhibited the most significant eradication effect against all three strains. The optimal eradication was achieved with a sixfold concentrated external extract, resulting in eradication percentages of 57%, 67%, and 76% for *E. coli*, *S. aureus*, and *P. aeruginosa* biofilms, respectively. Additionally, substantial killing rates and bactericidal effects of 99.9% were observed against the three strains in this case. Importantly, extracts from *L. rhamnosus* GG or other lactic acid bacteria biofilms are predicted as anti-Quorum Sensing compounds. Further research should be undertaken to investigate the predicted association of *L. rhamnosus* GG biofilm extracts and the real effect in reducing pathogenic biofilm formation by disrupting and altering the QS system. Recently, the use of LAB biofilms for biocontrol against pathogenic biofilm bacteria is an emerging research field. The progressive discovery of the benefits of such biofilms suggests their potential for novel biotechnological applications in various fields. Consequently, *L. rhamnosus* GG biofilms are proving to be new anti-biofilm strategies. Interestingly, it appears that a combination of molecules from *L. rhamnosus* GG biofilm extracts with antimicrobials might be a promising fresh perspective for the eradication of bacterial biofilms and inhibition of bacterial growth. Thus, further assays by adding these novel extracts from *L. rhamnosus* GG biofilm to lysozyme and EDTA or minimal amounts of antibiotic may also reduce antimicrobial resistance worldwide problems. Similarly, this innovative strategy offers a more straightforward application compared to the extraction of single molecules, with higher efficiency. Notably, its significance lies in the lack of specificity for pathogenic strains, as evidenced by successful testing on both gram-positive and gram-negative strains, yielding crucial results. Moreover, the specificity of this approach is directed towards bacterial biofilm, distinguishing it from planktonic free cells. Further genetic and molecular experiments are needed to identify the molecules in charge of the antibiofilm capabilities. More structural investigations into the bioactive substances (metabolites, proteins, and other compounds) of *L. rhamnosus* GG biofilm extracts are required. It would also be extremely important to intensify the knowledge about *L. rhamnosus* GG biofilm, which could enhance their antibiofilm and antimicrobial activities, especially for the treatment of mixed bacteria biofilm infections on antibiotic-resistant bacteria strains.

Supplementary Information

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Supplementary Material 1

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Author contributions
JCA, JY, and AEK conceived and designed the experiments. PH performed the experiments. JCA, PH, AC, AA, AEK, JY, and NL analyzed the data. PH wrote the paper. JY, JCA, AC, AA, NL, and AEK reviewed the manuscript.

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Availability of data and materials
Authors can confirm that all relevant data are included in the article and its additional files.

Declarations

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