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Research Article:

Investigating the *In Vitro* Antibiofilm Potential of *Lactobacillus rhamnosus*

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Abstract

Background: Background: Biofilms formation by pathogenic bacteria such as *Escherichia coli* and *Staphylococcus aureus* imposes clinical and economical challenges in antibiotic resistance and chronic infection. Uses of probiotics and their metabolites are emerging as promising antibiofilm strategy. **Methods:** Pro-baby oral drop was used to isolate the probiotics content. One ml of the formulation was cultured on de Man, Rogosa, and Sharpe (MRS) agar plate and inspected for the grown colonies. Isolated pure cultures were sent for 16s gene sequencing. Later, probiotics supernatants were prepared at three time points: 24 h, 48 h and 72 h. Antibiofilm effect of the obtained supernatants against *S. aureus* and *E. coli* biofilm was tested via 96-well microtiter plate crystal violet staining technique. **Results:** Two *Lactobacillus rhamnosus* strains were identified genetically and designated as strain A and B. Supernatants of strain A and B significantly inhibited biofilm formation, with the highest inhibition (82.1%) reported for *S. aureus* by 48 h-old strain A supernatant. *E. coli* biofilm formation was effectively suppressed (96.45%) by 72 h-old strain A supernatant. **Conclusion:** The study suggests that *L. rhamnosus* supernatants have potent antibiofilm potential against both Gram positive and Gram negative reference pathogens. This promising finding can be further investigated on in vivo infection model.

1. Introduction

Biofilms are structured networks of extracellular polymeric substances produced by communities of microorganisms. These networks allow bacteria to adhere to both living and non-living surfaces, creating an environment that enhances protection and survival under various conditions (1). Biofilm formation influences bacterial growth, mutations, virulence, host-microbe interactions, and antibiotic resistance (2). According to the Centers for Disease Control and Prevention (CDC) in 2007, hospital-acquired infections affected approximately 1.7 million patients, leading to over 500,000 associated deaths and imposing an economic burden of approximately $11 billion due to biofilm-associated infections (3). *Staphylococcus aureus* and *Escherichia coli* are among the major biofilm-forming pathogens responsible for tissue-related infections, including osteomyelitis, lung infections in cystic fibrosis, endocarditis, dental plaque, chronic tonsillitis, chronic laryngitis, chronic wounds, and infections of the biliary and urinary tracts (4). Recent research studies have focused on exploring alternative strategies to combat biofilm-associated infections, including probiotics and their products as promising approach (5). Biofilms formation is a well programmed steps started by initial attachment on surfaces, micro-colonization and biofilms maturation (5).

Cell-free supernatants (CFS) derived from probiotic cultures have demonstrated antibacterial and antibiofilm activities due to the presence of bacteriocins, organic acids,

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biosurfactants, and hydrogen peroxide. These compounds, once considered metabolic byproducts, are now recognized for their role in shaping microbial environments and exerting specific biological functions (6). Among probiotic bacteria, certain lactobacillus species have demonstrated antibiofilm properties against different pathogenic bacteria (7). Various methods have been employed to evaluate the antibiofilm potential of probiotics, with the 96-well microtiter plate assay being one of the most widely used semi-quantitative techniques for this purpose. This method enables the assessment of biofilm formation and inhibition through crystal violet staining, providing a reliable means to compare the antibiofilm activity of different probiotic strains. For instance, a recent study conducted by Maccelli and his colleagues in 2023 (8) utilized this method to evaluate the ability of *Limosilactobacillus reuteri* DSM 17938 to inhibit biofilm formation by pathogenic bacteria. Similarly Asadzadegan et al., applied this approach to assess the antibiofilm effects of *Lactobacillus* strains against *Pseudomonas aeruginosa* (9). Given its effectiveness, we employed this method to investigate the antibiofilm potential of probiotic isolates against *Escherichia coli* and *Staphylococcus aureus* under controlled conditions.

2. Materials and Methods

2.1 Bacterial strains

Pathogenic bacteria, *Escherichia coli* and *Staphylococcus aureus*, were obtained from the stock culture of the Microbiology Laboratory/Department of Clinical laboratory Sciences, College of Pharmacy/University of Mosul. *E. coli* strain was cultured on MacConkey agar (TM Media, India), while *S. aureus* strain was cultured on Mannitol Salt Agar (Scharlau, Spain). The plates were incubated at 37°C for 24 hours under aerobic conditions using Nuve incubator (Turkey). Following incubation, the growth of the pathogenic bacteria was observed.

Probiotic strains were isolated from an oral probiotic drop formula (PROB-baby-BIOPHARMA,Serbia) (**Figure 1a**). One milliliter of the formula was mixed with one milliliter of sterile distilled water in a sterile plain tube to achieve proper dilution. The diluted mixture was spread onto MRS agar (Neogen, USA) using a sterile cotton swab. The inoculated plates containing the probiotic bacteria were incubated at 37 °C for 24 h or 48 h. Following incubation, the growth of the probiotic bacteria was observed. Probiotic colonies were isolated from the MRS agar and identified by 16S gene sequencing.

2.2 Probiotic Supernatant Preparation

Few colonies of fresh probiotic culture strains (strain A and strain B) were suspended in sterile normal saline to achieve a turbidity equivalent to 0.5 McFarland standard, which corresponds to approximately 1.5×10^6 colony-forming unit (CFU) per milliliter. A total of six sterile plain tubes were prepared, with three tubes designated for each strain. Each tube was filled with three milliliters of Brain Heart Infusion (BHI) broth (Neogen, USA) and inoculated with the respective probiotic suspensions. The tubes were incubated aerobically at 37 °C for three incubation periods: 24 hours, 48 hours, and 72 hours. After each incubation period, the contents of the tubes were centrifuged at 1000 rpm for 10 minutes at 10 °C. The supernatant was collected, filter-sterilized using Pall-Gelman filters (USA) with a pore size of 0.22 micrometers, aliquoted and stored at -20 °C for subsequent use.

2.3 Antibiofilm assay

To evaluate the antibiofilm potential of probiotic's supernatant, antibiofilm assay was performed by co-incubating the pathogenic strains with the probiotic's supernatant using 96-well plate (10) Briefly, bacterial suspensions of *E. coli* and *S. aureus* were prepared, adjusted to a 0.5 McFarland turbidity standard (equivalent to approximately 1.5×10^8 CFU/mL). A one hundred µL aliquot of each bacterial suspension was pipetted into the respective wells of a sterile polystyrene flat-bottomed 96-well plate (Citotest, China) in triplicate. Subsequently, 100 µL of probiotic supernatant at 24, 48, or 72 h concentrations was added to the wells. Wells containing only the bacterial suspension served as the positive controls, while wells containing only brain heart infusion (BHI) broth served as the negative controls and incubated under aerobic conditions at 37 °C for 24 h. At the end of the incubations period, wells content were gently pipetted out followed by washing three times with distilled water to remove unattached cells. The wells were air dried and fixed with 96% methanol (Ajax Finechem, Australia) for 10 minutes. After emptying the wells, the adherent cells were stained by pipetting a volume of 200 µL of 0.1% crystal violet solutions for 15 minutes. Stained wells were washed to decant the excess stain and left to air-dry overnight. Finally, the stained biomass was re-solubilized by pipetting 200 µL of 30% acetic acid into each stained well and optical density was recorded using micro-plate reader (BIOBASE, China) at 600 nm. The percentage of biofilm formation inhibition was calculated according to the following formula:

% inhibition= [(absorbance growth control − absorbance sample) / absorbance growth control] x 100; where absorbance is the mean reading of the three repeats (11).

2.4 Statistical analysis

Data were represented as mean ± standard deviation of the three experimental repeats. Percentage was used to demonstrate the antibiofilm potential of the probiotic's supernatant and statistically assessed using Dunnett's one way ANOVA (GraphPad version 4) in comparison to the untreated growth control at $p > 0.05$ was considered significant.

3. Results

3.1 Probiotic isolates

Two morphologically different colonies were observed on MRS agar plate after 48 h incubation. They were designated as strain A (orange arrow) and strain B (blue arrow) as shown in **Figure 1b**. Pure culture was prepared from strain A colonies and strain B colonies on MRS agar medium and stocked in the fridge for subsequent use. The two isolated probiotic strains were identified by 16S sequencing. Two strains of *L. rhamnosus* were identified and designated as A and B.

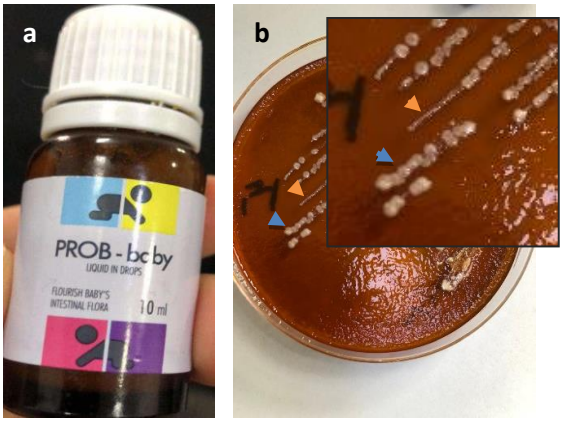


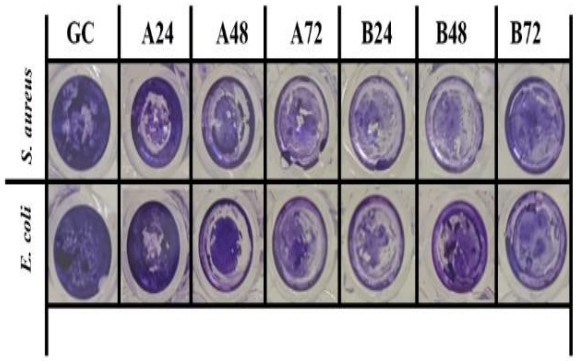
Figure 1. a: Pro-baby formula for probiotics isolation. **b**: Growth of two morphologically different colonies on MRS agar designated as strain A (orange arrow) and strain B (blue arrow).

3.2 Inhibition of biofilm formation

The ability of the probiotic strain A and strain B supernatant to *in vitro* inhibit pathogenic biofilm establishment was appraised using the microtiter plate with crystal violet staining for semiquantitative assessment. Crystal violet-stained wells are represented in **Figure 2** and a statistical comparison of the semi-quantified biofilm biomass is illustrated in **Figure 3.**

Table 1 depicts the percentage of inhibition of the treated biofilm compared to the untreated control. *S. aureus* and *E. coli* demonstrated the potential to grow as biofilm on the polystyrene wells of the used 96-well plate, but that of *S. aureus* was found denser (**Figure 3**). However, the biomasses of the dense biofilms of the former pathogens significantly declined when co-cultured with the probiotics' supernatants at the three time points but at varying degree.

The six supernatant products were found significantly able to suppress *S. aureus* biofilm formation with minor differences (**Table 1**). However, *E.coli* biofilm formation was



suppressed significantly by A72 and B72 (96.45% and 75.02%, respectively) while A24 and B48 supernatant products suppress *E. coli* biofilm formation by only 11%.

Figure 2. Antibiofilm potential of probiotic strain A and B supernatants at three time points (24 h, 48 h and 72 h) against *S. aureus* and *E. coli* biofilm formation. Prevention of biofilm formation was assessed by crystal violet assay. **GC:** growth control; **A24, A48, A72:** probiotic strain A supernatant at three time points (24 h, 48 h and 72 h, respectively); **B24, B48, B72:** probiotic strain B supernatant at three time points (24 h, 48 h and 72 h, respectively).

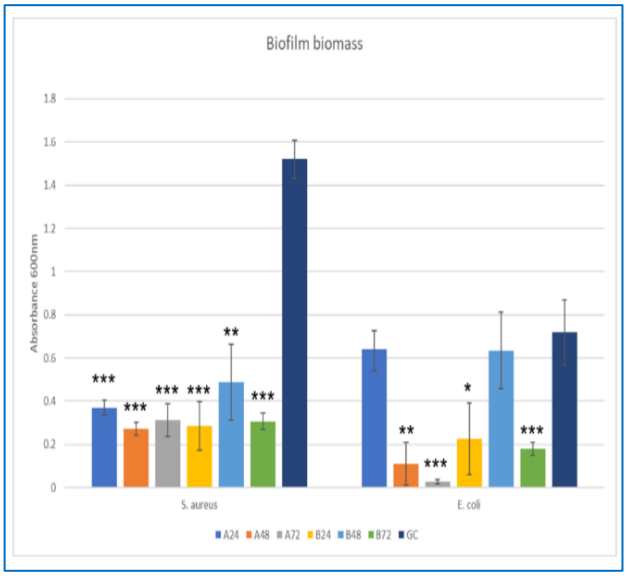


Figure 3. The repressive effects of the probiotics' supernatants against *S. aureus* and *E. coli* biofilm formation. Semi-quantitative assessment by crystal violet staining of the biofilm biomass. **GC**: growth control; **A24, A48, A72**: probiotic strain A supernatant at three time points (24 h, 48 h and 72 h, respectively); **B24, B48, B72**: probiotic strain B supernatant at three time points (24 h, 48 h and 72 h, respectively). *; $p > 0.05$, **; $p > 0.005$, ***; $p > 0.001$ using Dunnett's one way ANOVA.

Table 1. Percentage (%) of inhibition of *S. aureus* and *E. coli* biofilm biomass.

Test Microorganism	Probiotic Supernatant					
	A24	A48	A72	B24	B48	B72
S. aureus	75.84 %	82.10 %	79.52 %	81.22 %	67.94 %	79.90 %
E. coli	11.02 %	84.67 %	96.45 %	68.55 %	11.78 %	75.02 %

4. Discussion

In recent decades, antibiotic resistance is considered as a growing health crisis with biofilm associated infection playing a critical role in bacterial resistance and antibiotic failure (12). *S. aureus*, *E. coli* can form biofilms *in vivo* causing a variety of chronic infections such as UTI and mastitis. They can also form pathogenic biofilms on medical devices such as catheters (13,14,15). This makes infections and medical device related infection difficult to treat (16). Given limitations of conventional therapies, alterative options such as probiotics derived metabolites are gaining attention for their potential anti-biofilms effects (16).

During our study, PROB-baby probiotic product was selected based on the assumption that it contains two probiotic strains, *L. rhamnosus* and *L. reutri* as labelled on the product container. However, gene sequencing demonstrated that the product contained two different strains of *L. rhamnosus*. This discrepancy raises concerns about the accuracy of labeling and potential issues related to product formulation, storage conditions, or manufacturer credibility. Such inconsistencies can significantly impact research outcomes and highlight the need for rigorous verification of probiotic content before experimental use (11).

The current study findings demonstrate that both *S. aureus* and *E. coli* biofilms were effectively inhibited by *L. rhamnosus* supernatants with varying degree of effectiveness over different incubation periods where *S. aureus* showed the highest percentage of inhibition at 48 h while *E. coli* biofilm significantly inhibited at the 72 h-old supernatants showing a time dependent efficacy.

The antibiofilm activity observed in this study may be attributed to the bioactive compounds present in the *L. rhamnosus* supernatant. *L. rhamnosus* is known to produce a variety of antimicrobial metabolites, including proteins, bacteriocins, organic acids, and biosurfactants, which play a crucial role in disrupting biofilm formation (17). These compounds have been found to interfere with bacterial adhesion, degrade the extracellular polymeric matrix, and disrupt quorum sensing, thereby preventing biofilm maturation and persistence (18,19).

Bacterial cell wall nature may also contribute to the susceptibility to the probiotic byproducts. Gram negative bacteria have additional outer membrane that exclude macromolecules like bacteriocin. However, the presence of lactic acid acts as permeabilizer that facilitates outer membrane penetrations, leading to bacterial weakening and suppressing biofilm production (20). While in Gram positive bacteria, it has been shown that the presence of peptidoglycan and its susceptibility to acidic nature of lactic acid potentiates its biofilm inhibition (21).

Components and nature of the probiotics spent culture can also be affected by incubation periods such as temperature, pH, types of culture medium used, treatment with other substances which all affects type and concentrations of secreted products of the lactobacilli (22,23) which in turns can affect their antibiofilm potentials.

Some organic acids and exopolysaccharide (EPS) have been shown to exert their antibiofilm activity via down regulating biofilms and quorum sensing (QS) genes. For instance, EPS produced from probiotic bacteria reduced biofilm formation of enterohemorrhagic *E. coli* O157:H7 via repression of the curli production genes. Inhibition of cheY gene, which contributes to the microbe attachment ability, has also been reported to associate with biofilm inhibition in *Pseudomonas aeruginosa* via hindering biofilm mushroom cap formation (24,25). Some probiotics-secreted proteins also exhibited antibiofilm activity (26).

S. aureus and *E. coli* biofilm have been noticed to respond differently to treatment with the supernatants of the two study strains (A and B) at the three time points. This could be due to differences in biofilm nature of the two pathogenic strains in terms of biomass thickness or components of the extracellular matrix and adhesion potential to surfaces in which *E. coli* showed higher adhesion to surfaces and thicker biofilm biomass than that of *S. aureus* (27).

Probiotics byproducts vary according to the kinetic followed by the producing strain and thereby their biological activity varies accordingly such as the antibiofilm activity. Probiotics biosurfactants have been documented to be produced at different growth phases and the difference in the antibiofilm activity of the two tested strains supernatants reported in this study can be attributed to this reason. These biosurfactants can be cell-bound or extracellular and exert antimicrobial and antiadhesive/antibiofilm effect by reducing surface tension and soap-like property (28). In a study conducted by Sambanthamoorthy and his colleagues showed that biosurfactants produced in 48 h-incubation supernatant of *L. rhamnosus* were able to significantly reduce biofilm of *Acinetobacter baumannii*, *E. coli* and *S. aureus* (29). Moreover, *L. rhamnosus* biosurfactant (BSLR) showed significant biofilm inhibition of *S. aureus* and *E. coli* at 50 mg/ml, with partial inhibition for *S. aureus* at 25 mg/mL in the same study.

Different probiotics strains also exhibit difference in antibiofilm activity due to genetic properties inherent to the

species itself and extends within different strains resulting in probiotic strain-specific property (10). This might explain the variable antibiofilm effect obtained from the two *L. rhamnosus* strains studied. Similarly, Asadzadegan and his colleagues, documented that different probiotic strains of the same species showed a wide range of biofilm inhibition (9).

5. Conclusion

This study demonstrates the significant antibiofilm properties of two strains of *L. rhamnosus* supernatants against *S. aureus* and *E. coli* biofilms. The findings support the potential use of bacterial supernatants as standalone treatment or as adjunct to antibiotics to combat biofilm-associated antimicrobial resistance. Further investigations are needed to identify the active components responsible for this effect and explore their broader therapeutic applications.

Future studies should focus on optimizing supernatant composition, assessing its efficacy in vivo, and exploring its integration with advanced technologies such as nanotechnology and surface-coating strategies. These approaches could enhance the application of probiotic-derived supernatants in biomedical fields, including the prevention of biofilm formation on medical devices such as catheters and implants. Additionally, their potential use in the food industry to improve product stability and sterility warrants further exploration.

Acknowledgment

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إمكانات *Lactobacillus rhamnosus* كعامل مضاد للأغشية الحيوية

خلاصة

المقدمة: يُشكّل تكوّن الأغشية الحيوية بواسطة البكتيريا المُمرضة، مثل الإشريكية القولونية والمكورات العنقودية الذهبية، تحديات سريرية واقتصادية في مقاومة المضادات الحيوية والعدوى المزمنة. وتُعدّ استخدامات البروبيوتيك ومستقلباتها استراتيجيةً واعدةً لتكوين الأغشية الحيوية المُضادة. **الطرق**: استُخدمت قطرة برو-بيبي الفموية لعزل محتوى البروبيوتيك. زُرعت 1 مل من التركيبة على طبق أجار دي مان، روجوسا، وشارب(MRS) ، وفُحصت المستعمرات النامية. أُرسلت المزارع النقية المعزولة لإجراء تسلسل الجينات 16 ثانية. لاحقًا، حُضّرت رواسب البروبيوتيك العلوية عند ثلاث فترات زمنية: 24 ساعة، 48 ساعة، و72 ساعة. اختُبر تأثير الرواسب العلوية المُتحصل عليها كمضادات حيوية ضد الأغشية الحيوية للمكورات العنقودية الذهبية والإشريكية القولونية باستخدام تقنية التلوين البلوري البنفسجي لطبق ميكروتيتر 96 بئرًا. **النتائج**: تم تحديد سلالتين من بكتيريا Lactobacillus rhamnosus وراثيًا، وتم تصنيفهما على أنهما السلالتان A و .Bتَبّطت الرواسب الطافية من السلالتين A و B بشكل ملحوظ تكوين الأغشية الحيوية، مع تسجيل أعلى نسبة تثبيط (82.1%) لبكتيريا S. aureus بواسطة الرواسب الطافية من السلالة A التي يبلغ عمرها 48 ساعة. كما ثبّطت الرواسب الطافية من السلالة A التي يبلغ عمرها 72 ساعة تكوين الأغشية الحيوية لبكتيريا E. coli بفعالية (96.45%). **الخلاصة**: تشير الدراسة إلى أن الرواسب الطافية لبكتيريا L. rhamnosus تتمتع بقدرة فعالة كمضاد حيوي ضد كل من مسببات الأمراض المرجعية موجبة وسالبة الجرام. يمكن دراسة هذه النتيجة الواعدة بشكل أعمق باستخدام نموذج العدوى داخل الجسم الحي.

الكلمات المفتاحية: الأغشية الحيوية، الإشريكية القولونية، Lactobacillus rhamnosus، المكورات العنقودية الذهبية، الرواسب الطافية.