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Lactobacillus plantarum WCFS1 and *Lactobacillus gasseri* A237 as Therapeutic Candidates for Gut Pathogen Control: Inhibition of *Escherichia coli* and *Staphylococcus aureus* Virulence

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Abstract

The gut microbiome is a complex microbial ecosystem that requires a delicate balance to maintain host health homeostasis. Gut dysbiosis, characterized by reduced microbial diversity and shifts in the relative abundance of commensal and pathogenic species, is implicated in chronic conditions including ulcerative colitis, obesity and diabetes mellitus. Common gut pathogens include gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus*, both of which contribute to intestinal disease and systemic infection. Antibiotic therapies remain the standard of care for pathogenic infections; however, the rising threat of antibiotic resistance and the disruption of gut microbial homeostasis associated with antibiotic use warrants alternative therapeutic approaches. Probiotics represent a promising therapeutic strategy for restoring microbial balance by promoting beneficial bacteria while suppressing pathogenic activity, though additional evidence supporting strain-specific, anti-pathogenic properties is needed. This study evaluated the therapeutic potential of human-derived *Lactobacillus plantarum* WCFS1 and *Lactobacillus gasseri* A237 against the pathogenic activity of *E. coli* ATCC 25922 and *S. aureus* subsp. *aureus* ATCC 25923 via competitive adhesion and minimum inhibitory concentration assays. Overall, the probiotic strains demonstrated inhibition against pathogen activity, with greater effects observed against *S. aureus*, which prompted further evaluation of its biofilm formation and growth in the presence of probiotics. Notably, *L. gasseri* significantly reduced *S. aureus* adhesion to HT-29 intestinal epithelial cells by 55.70% ($p=0.009$), while the reduction in *E. coli* adhesion (9.84%) was not statistically significant. Probiotic cell-free supernatants suppressed *S. aureus* growth by up to 95% and reduced biofilm formation in a concentration-dependent manner, with MIC achieved at a 1:4 dilution (12.25% *v/v*) against both pathogens for all groups. Together, these findings demonstrate that *L. plantarum* WCFS1 and *L. gasseri* A237 exert anti-pathogenic effects against common human gut pathogens, supporting their potential as therapeutic candidates for the management of *E. coli* and *S. aureus* pathogens. This approach may also be used for other clinically relevant pathogenic species, warranting further investigation.

Keywords: Probiotics, Pathogens, Therapeutics, Gut Microbiome, Lactobacillus

Introduction

The human gastrointestinal (GI) tract is home to a huge and complex microbial ecosystem, harboring trillions of microorganisms, bacteria and fungi [1]. There are seven main phyla that grow within the gut microenvironment, including *Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Proteobacteria, Verrucomicrobia* and *Cyanobacteria*. Among these, *Firmicutes* and *Bacteroidetes* bacterial species constitute more than 90% of the total population. A balanced gut microbiota is imperative for host health homeostasis. A disruption to a balanced microbiome, known as microbial dysbiosis, occurs when there is a loss of beneficial bacteria, an increase of potentially pathogenic bacteria, or a reduction in overall bacterial diversity. A multitude of evidence links gut dysbiosis with various disease states, including gut-related (e.g., inflammatory bowel disease; IBD) and systemic illnesses (e.g., obesity, type 2 diabetes, neurodegenerative disorders, skin pathologies) [2, 3]. However, the exact mechanisms underlying the relationship between a dysbiotic microbiome and disease onset or progression remain largely unknown.

Proteobacteria are proposed as a microbial signature of dysbiosis, given their low abundance in the healthy gut and increased prevalence in metabolic disorders, inflammatory bowel disease, colitis and colorectal cancer [4]. *Escherichia coli* (*E. coli*), a gram-negative bacillus member of this phylum, is part of the normal intestinal flora and comprises both

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commensal and pathogenic strains [5]. Pathogenic strains carry virulence factors encoding adhesion, invasion and toxin activities [6,7], and an abundance of these genes is associated with IBD, Crohn's disease and ulcerative colitis [8]. All *E. coli* pathotypes express O-antigen lipopolysaccharides (LPS), which activate innate immune responses via Toll-like receptors [9]. *E.coli*-driven gut dysbiosis impairs the epithelial barrier and enables LPS translocation into the bloodstream, driving systemic inflammation linked to diabetes, obesity and cardiovascular disease [10]. Gram-positive pathogens similarly exploit gut dysbiosis, as seen with *Staphylococcus aureus* (*S. aureus*), an opportunistic pathogen of the Firmicutes phylum that may reside asymptomatically in the intestinal tract [11]. Contrary to the LPS-mediated effects of *E. coli*, *S. aureus* relies on protein-based virulence factors, including more than 20 heat-stable staphylococcal enterotoxins capable of causing toxin-mediated food poisoning [1213]. Gut overgrowth of *S. aureus* can result from antibiotic overexposure or immunocompromised states, causing dysbiosis, immune activation and GI distress [13,14]. In patients with impaired gut barrier integrity, *S. aureus* may translocate into the bloodstream, leading to life-threatening sepsis [13]. The rising prevalence of *S. aureus*-associated infections, including methicillin-resistant *S. aureus* (MRSA), and the limitations of current antibiotics highlight the need for novel therapeutic strategies.

Antibiotic therapies remain the standard treatment for pathogenic infections; however, their repeated or long-term use disrupts the gut microbiome and contributes to antibiotic resistance, posing significant threats to host health. Irrational antibiotic use is a leading driver of this resistance. For example, in a Sudanese cohort, 87% of physicians prescribed antibiotics based on clinical judgement and fewer than 10% used culture and sensitivity testing, while 64% preferred broad-spectrum antibiotics, most commonly for cough and the common cold [15]. Moreover, 97.7% of enteropathogenic *E. coli* isolates were resistant to amoxicillin and 75.0% to ampicillin, with comparable patterns reported in the USA, where 91.0% *E. coli* isolates showed phenotypic resistance across multiple antibiotic classes. These findings emphasize the urgent need for novel therapeutic approaches to target gut pathogen activity. One promising strategy is the use of probiotics – live microorganisms that confer health benefits to the host when administered in adequate amounts [16]. Probiotics act through several mechanisms, including production of antimicrobial substances such as bacteriocins, immunomodulation, improvement of intestinal barrier function, competitive blocking of pathogen adhesion to the gut mucosa and increasing the abundance of beneficial gut microbes (i.e., *Lactobacilli* and *Bifidobacteria*) [1718,19]. Probiotics are available in the form of functional food products (e.g., yogurt, cheese, kefir), as well as dietary supplements. Specific lactic acid bacteria (LAB) may be particularly protective against pathogenic species. For example, dairy-derived *Lactobacillus plantarum* strains demonstrate antimicrobial activity against *S. aureus*, *E. coli* and *Salmonella* pathogens [20], while *L. plantarum* isolated from *Bufo gargarizans* produces bacteriocins antagonistic to *S. aureus*, *E. coli* and *Pseudomonas aeruginosa* [21]. *Lactobacillus gasseri* antimicrobial activity against oral, vaginal and urinary tract pathogens is well-documented [22,23], though its effect on gut pathogens remains poorly characterized. As probiotic properties are strain-dependent, findings cannot be extrapolated between strains, necessitating direct investigation of specific strains for clinical relevance.

The harmful effects of pathogenic *E. coli* and *S. aureus* in gut-related disease are well-established and probiotics with specific bioactivities may offer a means of controlling their growth and activity. However, the mechanisms by which probiotics counteract pathogen-induced disruption of intestinal epithelial cells (IECs) remain largely unknown. We have previously reported the IEC adhesion, anti-inflammatory and anti-oxidative properties of *Lactobacillus plantarum* WCFS1 (LpWCFS1) and *Lactobacillus gasseri* A237 (LgA237) [24], though their inhibition against pathogen virulence-associated activity and growth has not been investigated. The purpose of this study was therefore to evaluate the inhibitory potential of these human-derived strains against *E. coli* ATCC 25922 and *S. aureus* subsp. *aureus* ATCC 25923 activity. Findings support the antimicrobial activity of LpWCFS1 and LgA237 against *E. coli* and *S. aureus* and warrant further investigation of their efficacy against additional gut pathogens.

Materials and Methods

Materials and Reagents

Live probiotic strains LpWCFS1 and LgA237 were obtained from Biena Inc. (Saint-Hyacinthe, QC, Canada). Pathogenic strains – *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* subsp. *aureus* ATCC 25923 – were purchased from Cedarlane Labs (Burlington, ON, Canada; acquired November 2023). MRS powder was purchased from Millipore Sigma (CAT# 69966; Massachussetts, USA) and Luria–Bertani (LB) broth powder was purchased from Biobasic (CAT# SD7005; Ontario, Canada). Human colorectal adenocarcinoma (HT-29) cells were provided by Professor Siyaram Pandey from the Department of Chemistry and Biochemistry (University of Windsor). Other chemicals used include Dulbecco's Modified Eagle's Medium (DMEM; CAT# D6429; Sigma Aldrich, United States), sodium pyruvate (CAT# 600-110-EL, Wisent Inc., Canada), Trypsin-EDTA solution (CAT# T4049, Millipore Sigma, Canada) and Fetal Bovine Serum (FBS; CAT# FB12999102), Penicillin-Streptomycin-Glutamine 100X (CAT# 10378016) and PBS (CAT# 10010049) from Thermo Fisher Scientific (Canada). Crystal violet (CAT# CB0331) was purchased from Biobasic (Canada) and the Cell Counting Kit 8 (WST-8 / CCK8; CAT# ab228554) was acquired from Abcam (United Kingdom).

Preparation of Bacterial Strains and Culture Conditions

LpWCFS1 and LgA237 were grown anaerobically in MRS at 37°C. *E. coli* and *S. aureus* cultures were established and subcultured in LB broth at 37°C with shaking. Total bacterial colony counts were determined using spectrophotometry compared to standard curves of colony forming units (CFUs) on

the respective agar plates. For all experiments, fresh overnight bacterial cultures were centrifuged, washed twice with PBS and resuspended to the desired concentrations. To prepare the cell-free supernatant (CFS), overnight probiotic cultures were centrifuged at 4000 rpm for 30 minutes and the supernatant (native pH of 3.5-4.5) was filter-sterilized using a 0.22 µm filter and stored in -20°C.

Mammalian Cell Culture

HT-29 cells were cultured in DMEM (10% FBS, 1% penicillin-streptomycin-glutamine and 1mM sodium pyruvate). Cells from passage numbers 28-40 were grown at 37°C in a 5% CO_2 atmosphere. Media was changed every 2-3 days until cells reached confluency.

Inhibition of Pathogenic Adhesion

Probiotics were evaluated for the prevention of pathogen adhesion to IECs. HT-29 cells were seeded in a 96-well plate in DMEM (10% FBS) and grown to a confluent monolayer. Fresh subcultures of *Lactobacilli* and the pathogenic strains were resuspended to $1x10^8$ CFU/mL; 100µL of pathogen (*E. coli* or *S. aureus*) and 100µL of live probiotics (LpWCFS1 or LgA237) were added to each well ($1x10^7$ CFU/well/strain). For the combined probiotic treatment, 50µL of each probiotic were added to the well, for a total of 100µL ($1x10^7$ CFU total/well). Pathogen-only control wells received 100µL of pathogen suspension and 100µL of DMEM in place of a probiotic. Following a two-hour incubation at 37°C, the wells contents were removed and wells were washed three times with PBS. The HT-29 cells and adhered bacteria were lifted with 100µL of 0.25% trypsin following a 20-minute incubation, after which 100µL of DMEM was used to neutralize trypsin activity, giving a recovery volume of 200µL. Ten-fold serial dilutions of the lifted suspension were plated on LB agar (50µL/sample, technical duplicates), which does not support *Lactobacilli* spp. growth [25], allowing selective enumeration of the adhered pathogens. Adhered pathogen concentrations (CFU/mL) were converted to CFU/well by multiplying by the recovery volume. Percent adhesion was calculated for each technical replicate before averaging, with three biological replicates per group (n=3), using the following equation:

$$\%\ Adhered = \frac{adhered\ CFU/well}{1X10^7\ CFU\ added}\ X\ 100\%$$

Minimum Inhibitory Concentration of Bacterial Strains

To determine the minimum inhibitory concentration (MIC) of the probiotic CFS, a broth microdilution assay was performed, according to CLSI guidelines [26]. Overnight cultures of pathogenic *S. aureus* and *E. coli* were resuspended to $1X10^8$ CFU/mL and diluted 1:100 in MH broth to a working density of $1X10^6$ CFU/mL. Probiotic CFS or tetracycline (100µL) was added to the first well of a 96-well plate and serial two-fold dilutions were performed across the row in 50µL of MH broth. Each well then received 50µL of the pathogen suspension, yielding a final well volume of 100µL and an inoculum density of $5X10^5$ CFU/mL. Final CFS concentrations ranged from 50% to 0.049% (*v/v*) and tetracycline from 32 to 0.031 µg/mL. Inoculated broth without treatment served as the growth (positive) control and uninoculated broth as the negative control. Plates were incubated at 37°C for 24 hours, after which the MICs were defined as the lowest concentrations with no visible bacterial growth and confirmed by the absence of metabolically active cells using the CCK-8 assay (absorbance at 450 nm). The mean absorbance values among biological replicates (n=4) are reported as raw readings for each group.

Crystal-Violet Biofilm Assay and Growth Inhibition of *S. aureus*

To determine the impact of probiotics on pathogenic biofilm formation, a biofilm assay was performed using crystal-violet stain according to previous studies [27], with minor modifications. A 1% inoculum of *S. aureus* (10^7–10^8 CFU/mL) was made in cation-adjusted MH II broth. A 96-well plate was inoculated with the *S. aureus* solution and treated with probiotic CFS at 10% or 20% (*v/v*) or MRS broth. Incoulated media with PBS served as the growth (positive) control, while uninoculated media with PBS acted as the negative control. The plates were incubated for 24 hours at 37°C. After measuring bacterial growth via absorbance (OD_{600} nm), the plates were washed with PBS (200µL) and heat-fixed at 65°C. The wells were then stained with 150µL of 2% crystal violet solution for 10 minutes, washed with PBS (200µL) and dried at 65°C. The biofilms were dissolved using 150µL of 33% acetic acid for 30 minutes in room temperature. After transferring 125µL of each well into a new plate, absorbance was read at 540 nm using a multiplate reader. Absorbance values were corrected by subtraction of the negative control and expressed as biofilm formation relative to the growth control as percent means among the biological replicates (n=6); the MRS broth-treated group had n=4 replicates due to technical error. Corrected values are presented graphically, while uncorrected absorbance values for biofilm biomass and the corresponding growth measured in the same wells are reported as a table.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 10.2.2 for macOS (GraphPad Software, Boston, MA, USA). Each assay was conducted as a single experiment. Data are expressed as mean ± standard error of the mean (SEM). Welch's one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was used to assess statistical significance for bacterial adhesion assays. The CFU counts and calculated percent adhered were analyzed as numerical values. A two-way ANOVA with Tukey's post-hoc comparison was performed to assess between group differences for biofilm and growth inhibition assays. Differences were considered significant at $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

Results

Lactobacillus gasseri A237 Inhibits Pathogen Adhesion to Intestinal Cells

The impact of the probiotics on pathogenic adhesion to IECs was evaluated. *S. aureus* adhered 1.26X10^6 CFU/mL to HT-29 cells after two hours (Figure 1). When co-incubated with LgA237, a significant reduction in adhesion occurred (5.57X10^5 CFU/mL; *p=0.009*), reducing the total adhered amount by 55.79% compared to control. Treatment with LpWCFS1 reduced *S. aureus* adhesion to 1.19X10^6 CFU/mL, or 1.10X10^6 CFU/mL when combined with LgA237, though neither reached statistical significance compared to control. Similarly, *E. coli* adhered to HT-29 cells at a concentration of 8.03X10^5 CFU/mL (Figure 2). LgA237 reduced *E. coli* adherence by 9.84% (7.24X10^5 CFU/mL), though the effect was not statistically significant. Treatment with LpWCFS1 or the combined probiotics did not influence *E. coli* adherence, enabling 1X10^6 and 1.39X10^6 CFU/mL to adhere, respectively. Interestingly, the percent of *E. coli* adhered was the highest following the combined probiotic treatment (2.78%), followed by LpWCFS1 (2.0%), though the difference was not significant compared to control (1.61%). Overall, LgA237 had the greatest inhibitory activity against pathogenic adhesion to IECs, while LpWCFS1 alone and combined with LgA237 did not affect adherence of *S. aureus* or *E. coli*.

Lactobacilli Strains Exhibit Antimicrobial Action Against *E. coli* and *S. aureus*

To assess the antimicrobial activity of LpWCFS1 and LgA237, the MICs of their CFS were determined against *S. aureus* and *E. coli*, using a broad-spectrum antibiotic, tetracycline, as a reference. The individual and combined CFS demonstrated antimicrobial activity against both pathogens, with an MIC of ¼ for both *S. aureus* and *E. coli* (Figure 3; Table 1), corresponding to 12.5% (*v/v*). Partial growth inhibition was observed at the

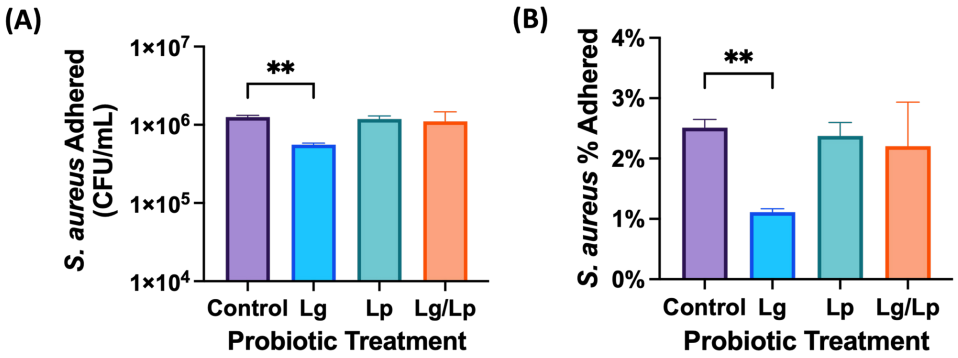


Figure 1. Adhesion of *Staphylococcus aureus* subsp. *aureus* ATCC 25923 to HT-29 colon cells, in the presence and absence of probiotic cells, represented as (A) CFU/mL and (B) percent adhered. Cells were treated with *S. aureus* (1X10^7 CFU/well) and LgA237 or LpWCFS1 (1X10^7 CFU/well), or both strains combined (5X10^6 CFU/well/strain; 1X10^7 CFU/well total), and incubated for two hours. *S. aureus* alone with DMEM acted as the control. Bars indicate significant differences between groups and errors bars represent standard error among independent samples (n=3; **p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*). CFU: colony forming units. Lg: *Lactobacillus gasseri* A237. Lp: *Lactobacillus plantarum* WCFS1.

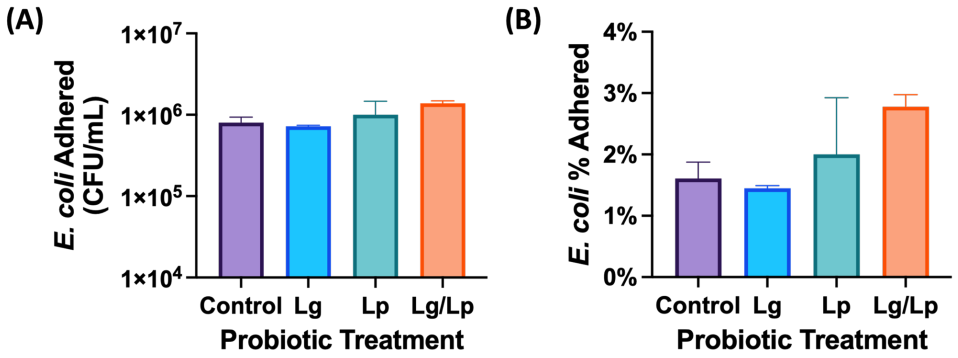


Figure 2. Adhesion of *Escherichia coli* ATCC 25922 to HT-29 colon cells, in the presence and absence of probiotic cells, represented as (A) CFU/mL and (B) percent adhered. Cells were treated with *E. coli* (1X10^7 CFU/well) and LgA237 or LpWCFS1 (1X10^7 CFU/well), or both strains combined (5X10^6 CFU/well/strain; 1X10^7 CFU/well total), and incubated for two hours. *E. coli* alone with DMEM acted as the control. Bars indicate significant differences between groups and errors bars represent standard error among independent samples (n=3; **p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*). CFU: colony forming units. Lg: *Lactobacillus gasseri* A237. Lp: *Lactobacillus plantarum* WCFS1.

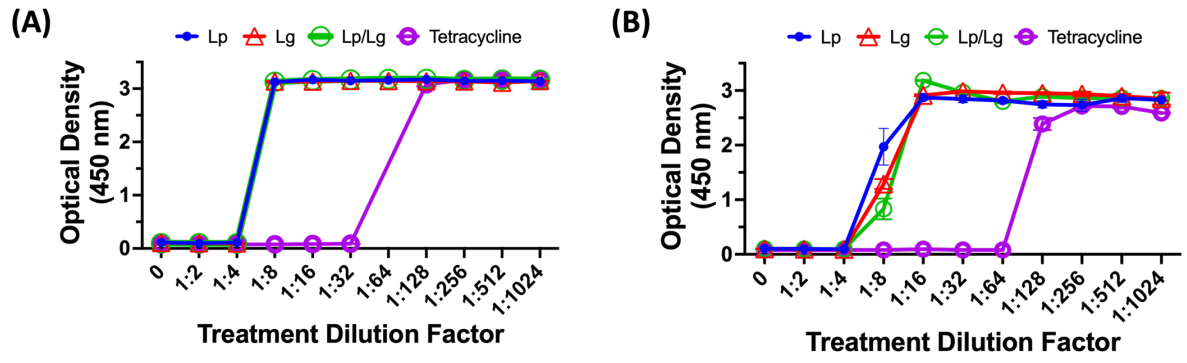


Figure 3. Minimum inhibitory concentrations of cell-free supernatants from *Lactobacillus plantarum* WCFS1 and *Lactobacillus gasseri* A237 against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* subsp. *aureus* ATCC 25923. Serial two-fold dilutions of individual or combined (1:1) probiotic CFS, or tetracycline, were inoculated with (A) *E. coli* or (B) *S. aureus* for 24 hours. Errors bars represent standard error among the independent samples (n=4). CFS: cell-free supernatant. Lp: *Lactobacillus plantarum* WCFS1. Lg: *Lactobacillus gasseri* A237.

Table 1. Minimum inhibitory concentration of probiotic cell-free supernatants against gram-negative *Escherichia coli* ATCC 25922 and gram-positive *Staphylococcus aureus* subsp. *aureus* ATCC 25923 pathogenic species. Serial two-fold dilutions of individual probiotic cell-free supernatant or Tetracycline were inoculated with *S. aureus* or *E. coli* for 24 hours. The minimum inhibitory concentrations were detected by visual turbidity and confirmed with absorbance measurements (450 nm). SEM: standard error of the mean. MIC: minimum inhibitory concentration.

Probiotic(s) or Antibiotic	*Escherichia coli* ATCC 25922			*Staphylococcus aureus* subsp. *aureus* ATCC 25923		
	MIC	**Corresponding In-Well Concentration**	**Mean Optical Density (450 nm)** ± SEM	**MIC**	**Corresponding In-Well Concentration**	**Mean Optical Density (450 nm)** ± SEM
Lactobacillus plantarum WCFS1	1:4	12.5% (*v/v*)	0.111 ± 0.003	1:4	12.5% (*v/v*)	0.092 ± 0.001
Lactobacillus gasseri A237	1:4	12.5% (*v/v*)	0.095 ± 0.003	1:4	12.5% (*v/v*)	0.091 ± 0.001
Lactobacillus plantarum WCFS1 & *Lactobacillus gasseri* A237	1:4	12.5% (*v/v*)	0.095 ± 0.001	1:4	12.5% (*v/v*)	0.097 ± 0.002
Tetracycline	1:32	1 µg/mL	0.089 ± 0.004	1:64	0.5 µg/mL	0.080 ± 0.000

1:8 dilution for *S. aureus* by the individual and combined CFS, as evidenced by reduced but detectable turbidity, while complete inhibition was confirmed at a 1:4 dilution (Figure 3B). The MIC of tetracycline was observed at the 1:32 dilution (1 µg/mL) for *E. coli* and 1:64 dilution (0.5 µg/mL) for *S. aureus*.

LpWCFS1 and LgA237 Reduce Biofilm Formation of *Staphylococcus aureus*

Upon adhesion to the intestinal wall, pathogens form biofilms to evade the host's immune response and antimicrobial compounds, while enabling virulence factor activity. Given the significant reduction in *S. aureus* adhesion observed by LgA237, the effect of the probiotic CFS on *S. aureus* biofilm formation was investigated. While probiotic treatment at the lower concentration (10% *v/v*) did not impact biofilm formation, significant effects were found following treatment with 20% (*v/v*) of the individual and combined probiotics (Figure 4). Specifically, treatment with LpWCFS1 led to the greatest reduction in biofilm formation ($p<0.0001$), followed by LgA237 alone and combined with LpWCFS1 ($p=0.0015$ and $p=0.0016$, respectively). The effects of each probiotic treatment at 20% (*v/v*) were significant compared to their 10% (*v/v*) counterparts. Biofilm

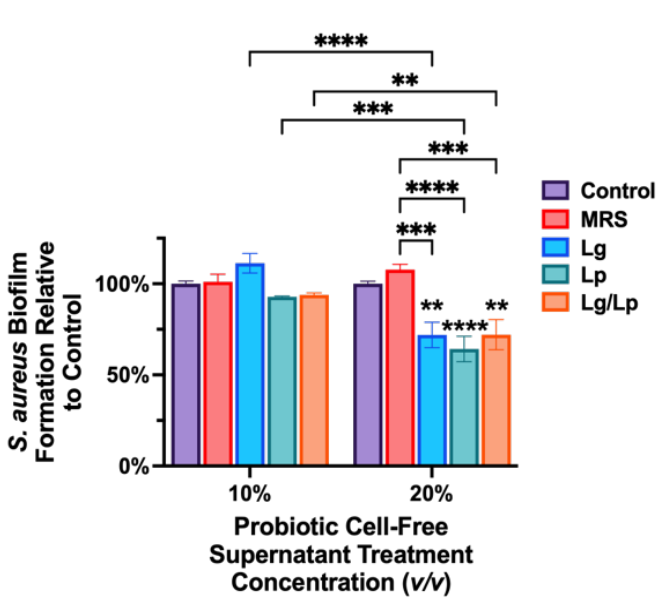


Figure 4. The effect of probiotic cell-free supernatant treatment on *Staphylococcus aureus* biofilm formation. Live pathogenic *S. aureus* cells were inoculated with CFS from LpWCFS1 and/or LgA237 (10% or 20% *v/v*) or MRS broth for 24 hours, stained with crystal violet and measured for absorbance (540 nm). Stars indicate significance relative to control (PBS; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$), bars indicate variations between groups and errors bars represent standard error among independent samples (n=6). CFS: cell-free supernatant. Lg: *Lactobacillus gasseri* A237. Lp: *Lactobacillus plantarum* WCFS1. MRS: de Man, Rogosa and Sharpe.

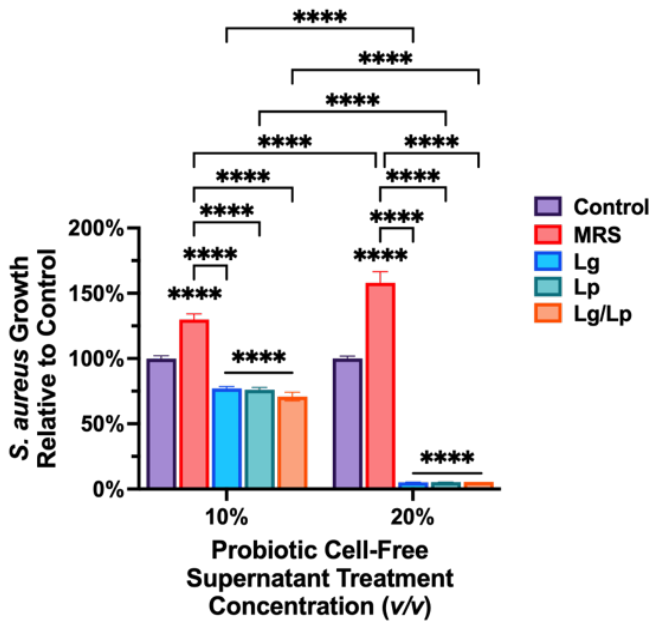


Figure 5. Growth inhibition of *Staphylococcus aureus* subsp. *aureus* ATCC 25923 following treatment with probiotic cell-free supernatants. Live pathogenic *S. aureus* cells were inoculated with CFS from LpWCFS1 and/or LgA237 (10% or 20% *v/v*) or MRS broth for 24 hours and bacterial growth was quantified (OD600 nm). Stars indicate significance relative to control (PBS; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$), bars indicate variations between groups and errors bars represent standard error among independent samples (n=6). CFS: cell-free supernatant. Lg: *Lactobacillus gasseri* A237. Lp: *Lactobacillus plantarum* WCFS1.

Table 2. Biofilm biomass and corresponding growth of *Staphylococcus aureus* subsp. *aureus* ATCC 25923 treated with probiotic cell-free supernatants. Cell-free supernatants from *Lactobacillus plantarum* WCFS1 and/or *Lactobacillus gasseri* A237, or MRS broth, were co-incubated with live *S. aureus* at 10% or 20% (*v/v*) for 24 hours. Biofilm biomass is reported as the raw absorbance values following crystal violet staining of the wells (OD_{540} nm), alongside corresponding growth measured by absorbance (OD600 nm) from the same wells. Data is reported as means of independent samples (n=6) ± SEM.

Probiotic Treatment	Concentration (*v/v*)	Biofilm Biomass (OD_{540} nm)	Corresponding Growth (OD_{600} nm)
Growth control	10%	0.951 ± 0.005	0.472 ± 0.009
	20%	0.935 ± 0.007	0.419 ± 0.007
MRS	10%	0.954 ± 0.013	0.569 ± 0.017
	20%	0.970 ± 0.013	0.629 ± 0.031
Lactobacillus plantarum WCFS1	10%	0.928 ± 0.001	0.374 ± 0.007
	20%	0.770 ± 0.032	0.075 ± 0.000
Lactobacillus gasseri A237	10%	0.987 ± 0.017	0.377 ± 0.006
	20%	0.806 ± 0.032	0.075 ± 0.000
Lactobacillus plantarum WCFS1 & *Lactobacillus gasseri* A237	10%	0.931 ± 0.003	0.352 ± 0.014
	20%	0.806 ± 0.038	0.076 ± 0.000
Uninoculated control	10%	0.632 ± 0.077	0.059 ± 0.001
	20%	0.475 ± 0.092	0.056 ± 0.000

formation following MRS treatment did not differ from control at either concentration, while at 20% (*v/v*) the MRS-mediated biofilm production was significantly higher than that of CFS from LpWCFS1 ($p<0.0001$), as well as LgA237 ($p=0.0002$) and the combined treatment ($p=0.0003$). Numerical values of the biofilm biomass, represented by the crystal violet absorbance, are presented in Table 2.

Probiotic Cell-Free Supernatants Suppress *S. aureus* ATCC 25923 Growth

The ability of LpWCFS1 and LgA237 to inhibit pathogenic *S. aureus* growth was investigated. Treatment with CFS from the individual and combined probiotics significantly reduced *S. aureus* growth (i.e., 10 and 20% *v/v*; $p<0.0001$; Figure 5). At 10% (v/v), *S. aureus* growth was reduced by approximately 23% for the individual CFS and 30% for the combined treatment, though the differences between individual and combined CFS did not reach statistical significance. Growth inhibition at 20% (v/v) was significantly greater compared to 10% (*v/v*) for all three CFS treatment groups ($p<0.0001$), each inhibiting *S. aureus* growth by approximately 95%. Contrarily, co-incubation with MRS broth promoted *S. aureus* growth relative to the control and all three CFS groups at either concentration ($p<0.0001$), with 20% (*v/v*) producing a greater increase than 10% (*v/v*) ($p<0.0001$). The numerical values representing *S. aureus* bacterial growth are reported in Table 2.

Discussion

The global rise in antibiotic resistance and the disruptive effects of broad-spectrum antibiotics on the gut microbiome highlight the need for alternative therapeutic strategies for pathogenic gut infections. GI infections arising from food-borne pathogens, intestinal disease or other physiological stressors can disrupt microbial balance and lead to gut dysbiosis, further complicating host health recovery. Naturally-derived agents and formulations with antimicrobial efficacy against food-borne and plant pathogens are increasingly reported in the literature, such as *Corchorus olitorius* leaf and seed extracts and plant extracts of *Tamarix ramosissima*, *Rosmarinus officinalis*, *Chelidonium majus*, *Silybum marianum*, *Satureja hortensis* essential oil and propolis[28, 29]. Probiotics represent another promising approach to limit pathogenic growth and activity, though strain-specific evidence targeting gut pathogens remains limited.

Probiotic Effects on Pathogen Adhesion to IECs

Adhesion of pathogenic species to the gut intestinal lining is the first step towards colonization and probiotics may competitively exclude pathogens by occupying intestinal binding sites [35]. We tested the adherence of *S. aureus* and *E. coli* to IECs in the presence of live probiotic cells. The adhesion rates observed in this study are consistent with previously reported counts of pathogen bacterial cell adhesion to HT-29 cell monolayers [36, 37]. Our results revealed LgA237 significantly reduces *S. aureus* adhesion by 55.79%, while it modestly reduces *E. coli* adhesion by 9.84%, lacking significance. However, LpWCFS1 did not significantly influence adhesion of either pathogen. Moreover, the combined probiotic treatment did not alter pathogen adhesion, with a slight increase observed for *E. coli* adhesion. This may reflect the reduced concentration of each strain relative to the single-strain groups (i.e., $5X10^6$ versus $1X10^7$ CFU/strain), which was necessary to maintain a consistent total probiotic load of 1×10^7 CFU/well. As between-group differences were not statistically significant, this observation is attributed to assay variability, rather than enhanced *E. coli* adhesion. The differential activity between LpWCFS1 and LgA237 may reflect strain-specific differences in surface properties. For example, other *L. gasseri* strains rely on sortase-dependent surface proteins or production of exopolysaccharides (EPS) to enhance probiotic adhesion to the intestinal epithelium and compete with pathogens [38, 39]. Azacarate-Peril et al. reported that *L. gasseri* strains harbor a substantial number of mucus-binding proteins and genes that encode EPS production, though their adhesion to Caco-2 cells was strain-specific [40]. *L. gasseri* also modulates the expression of E-cadherin, a critical adhesive protein in the intestine, to mediate its adhesion to the gut mucosa [41]. While genomic evidence suggests strong mucin-binding potential in *L. gasseri*, functional adhesion is strain and cell-line dependent. For example, the strain *L. gasseri* K7 successfully interfered with *E. coli* O8:K88Ent– adhesion to Caco-2 cells [42], yet we found LgA237 did not strongly impact *E. coli* adhesion. Identifying the genes encoded by LgA237 that act as adhesion factors would be an essential next step. The lack of influence of LpWCFS1 on pathogen adhesion was surprising, due to its excellent adhesion capacity to HT-29 cells, which exceeds that of LgA237 [24]. However, LpWCFS1 may adhere to IECs through distinct receptor-mediated mechanisms that do not directly compete with *S. aureus* or *E. coli* binding sites, leading to anti-pathogenic activity through immune modulation and antimicrobial compound production (e.g., LamD558) once adhered, rather than through prevention of pathogen binding [43]. Another study reported a significant inhibition of *S. aureus* and *E. coli* adhesion to HT-29 cells, following a four-hour pretreatment with *L. plantarum* L125 via moonlighting proteins with adhesive functions [44]. Thus, exclusion and displacement adhesion assays should be performed to further understand the adhesion mechanisms of these strains. Investigating probiotic-pathogen adhesion activity in other cell lines, such as a mucus-producing model (e.g., HT-29-MTX) or a gut co-culture system that offers a heterogenous cell surface (e.g., Caco-2/HT-29), would also be beneficial [45].

Minimum Inhibitory Concentrations of Probiotic CFS Against *S. aureus* and *E. coli*

The production of antimicrobial compounds by probiotic CFS may directly inhibit pathogenic growth. MIC was therefore determined for both *S. aureus* and *E. coli* using the CCK-8 assay, an established method for quantifying viable, metabolically active bacterial cells [46]. Complete growth inhibition of both pathogens was achieved at a 1:4 dilution of all CFS treatments,

corresponding to a concentration of 12.5% (*v/v*). These findings are consistent with previously reported MIC values for LAB CFS, including *L. plantarum* strains against *S. aureus, E. coli* and other opportunistic pathogens, where MIC values typically range from 1:2 to 1:8, with 1:4 being most reported [47, 48]. In the present study, the probiotic CFS groups produced partial inhibition of *S. aureus* at a 1:8 dilution (6.25% *v/v*), with the most pronounced effect seen by the combined CFS treatment, where OD_{450} values remained below 1.0. Saidi et al. reported complete eradication of *S. aureus* growth by *L. casei* and *L. rhamnosus* CFS at a MIC of 1:8, although the inoculum density was approximately 0.7 $\log_{10}$ lower than the concentration used here [49]. This suggests that a lower MIC of LpWCFS1 and LgA237 CFS may be obtained against a reduced pathogen load; testing the CFS against a range of pathogen inoculum densities would help clarify the antimicrobial potencies of the probiotics investigated herein. Given the growing concern over antibiotic resistance, investigating the live bacterial cells and CFS as adjunctive therapies to conventional antibiotic treatments would also be a practical next step towards clinical translation. Previously, the combination of gentamicin with a multi-strain probiotic showed superior antimicrobial efficacy against *E. coli* O157:H7 infections, compared to gentamicin alone [50]. Administering probiotics with antibiotics may also help mitigate antibiotic-associated side effects, including gut dysbiosis and GI distress, by supporting the restoration of microbial balance during treatment, representing a promising therapeutic approach [51, 52]. However, co-administration with antibiotics can compromise the viability of live probiotics. Encapsulation of probiotics within biopolymers, such as the chitosan-alginate system demonstrated by Thirumalai et al.[53], can protect probiotics from antibiotic exposure while enhancing their therapeutic efficacy against pathogens.

Probiotic CFS Effects on *S. aureus* Biofilm Formation and Growth

Bacterial adhesion to host surfaces is a critical precursor to biofilm development, which encases the bacteria within a protective matrix and limits the efficacy of traditional antimicrobial therapies [54]. Given the significant reduction in *S. aureus* adhesion to IECs observed by LgA237, *S. aureus* was prioritized for subsequent assessment of biofilm formation in the presence of probiotic CFS. Probiotic-derived compounds may interfere with biofilm development through mechanisms such as quorum sensing interference rather than direct cell wall disruption. *S. aureus* biofilm formation is largely regulated by its quorum sensing system, or intracellular signaling, whereby accumulation of autoinducing peptides at sufficient population densities coordinates the expression of virulence genes involved in biofilm development [55]. Our study showed an inhibitory effect of *S. aureus* biofilm formation by LpWCFS1, LgA237 or their combination. Specifically, treatment with 20% (*v/v*) probiotic CFS significantly reduced *S. aureus* biofilm formation, while 10% (*v/v*) had no significant effect. Compared to control, MRS had no effect on *S. aureus* biofilm formation, confirming the treatment effects to be from the CFS itself. These findings are aligned with several earlier reports of biofilm inhibition by LAB strains [56-58]. Moreover, biofilm biomass in the 20% (*v/v*) treatment groups was reduced considerably less than pathogen growth (i.e., roughly 28-36% compared to 95%; Table 2), suggesting *S. aureus* cells surviving at MIC-level exposure may upregulate biofilm production per cell as a compensatory stress response, a mechanism previously reported following antimicrobial exposure at inhibitory concentrations [59, 60]. Thus, the reduction in total biofilm biomass at 20% (*v/v*) may have been influenced by growth inhibition rather than direct biofilm-disrupting activity by the probiotic CFS. Antibiofilm activity cannot be fully separated from reduced bacterial load without further investigation, such as exposure of pre-formed biofilms to CFS, or treatment at sub-inhibitory concentrations (i.e., ½, ¼ and 1/8 MIC), with biofilm biomass normalized to viable cell counts. Such approaches would also enable application of the specific biofilm formation index [61]. Other mechanisms underlying biofilm reduction by these probiotics should be studied, such as the production of quorum-quenching molecules which have been found in *Bacillus subtilis* 6D1 to drive *S. aureus* biofilm inhibition [62]. An earlier study revealed LpWCFS1 encodes genes sensitive to a quorum sensing molecule from *P. aeruginosa* [63], however, its capability to sense similar molecules produced by *E. coli* or *S. aureus* has yet to be determined. Probiotic strains can also be genetically modified to enhance antimicrobial efficacy. For example, LpWCFS1 was recently engineered to express *S. aureus*-sensing biosensor circuits coupled with antimicrobial compound production, demonstrating its potential as a targeted therapeutic platform against *S. aureus* infection [64]. *L. plantarum* also produces lipoteichoic acid, antimicrobial peptides and EPS, which may contribute to pathogen biofilm inhibition, though this activity is strain-specific and should be investigated for LpWCFS1, specifically [65, 66]. Similarly, biosurfactants isolated from *L. gasseri* BC9 were shown to disperse preformed biofilms and inhibit biofilm development of different *S. aureus* strains, including several of MRSA origin [67]. LgA237 may also contain membrane vesicles in its CFS capable of disrupting *S. aureus* ATCC 25923 and *Gardnerella vaginalis* biofilms, as shown by *L. gasseri* ATCC 19992 [68], though direct investigation is needed. Moreover, future studies can extend investigation of the probiotics' CFS against *E. coli* biofilm formation, as the CFS may harbor anti-biofilm activity despite the probiotic cells' lack of effect on IEC adhesion.

Reducing pathogen cell density through growth inhibition and competitive adhesion to intestinal binding sites can limit biofilm formation and enhance susceptibility to antimicrobial treatment. Our findings of reduced biofilm formation after CFS treatment suggest a possible contribution of growth inhibition. All three CFS treatments significantly suppressed *S. aureus* growth at both 10% and 20% (*v/v*), with the higher concentration achieving an astounding 95% reduction in pathogenic growth. These findings are comparable with previously reported growth inhibition activity of the *L. plantarum* and *L. gasseri* species against *S. aureus* [69, 70]. Moreover, the growth-promoting

effects of MRS broth alone indicate that the CFS-mediated inhibition is from the crude CFS rather than the media itself. In general, the antimicrobial activity of LAB CFS can be attributed to the production of organic acids, hydrogen peroxide, bacteriocins and EPS; these components act together by lowering environmental pH, disrupting bacterial membranes and interfering with pathogen colonization [71, 72]. This activity depends on the bacterial strain and its concentration, which is consistent with the dose-dependent growth inhibition observed in this study. Thus, the importance of strain selection and its dosage is also critical for developing probiotic-based antimicrobial strategies.

Future Perspectives and Limitations

Despite the anti-pathogenic potential demonstrated by the probiotics, several limitations must be acknowledged. Our study evaluated crude CFS which has an acidic pH. Further studies comparing native and pH-neutralized (pH of 7.0) CFS are critical to determine whether the observed antimicrobial effects are attributable to organic acids or to other probiotic metabolites, such as bacteriocins. For example, Mirnejad et al. reported a loss of antimicrobial efficacy of *L. casei* CFS against clinical pathogens after neutralizing the CFS, concluding the lack of involvement of bacteriocins and attributing the activity instead to organic acids [73]. Moreover, the combined treatments contained each probiotic, as live cells or CFS, at half of the concentration of the individual treatments; future work should test the combination prepared at full individual concentrations to determine additive or synergistic effects. As well as testing a range of probiotic and pathogen treatment inoculums, various CFS concentrations, beyond 10% and 20% (*v/v*), can be investigated to determine the minimum and maximum therapeutic dosages. The assays presented herein should also be performed using both live probiotic cells and CFS, to distinguish the contribution of the cells themselves from their secreted compounds. Additionally, agar-based assays – such as agar dilution to determine MIC and disk diffusion to screen antimicrobial activity – may be performed to confirm our conclusions. Additional characterization via imaging, such as scanning electron microscopy of pathogenic biofilms, would be beneficial to observe morphological changes of the pathogenic cells in response to probiotic treatment.

Our findings demonstrate antimicrobial potential of LpWCFS1 and LgA237 against gut pathogens, though further investigation is required before clinical translation can be considered. *S. aureus*- or *E. coli*-colonized murine models can be used to assess the safety, colonization and therapeutic efficacy of LpWCFS1 and LgA237 under physiological conditions, as demonstrated for other probiotic-pathogen interactions [74, 75], while enabling microbial sequencing to characterize their gut-modulatory properties. Simpler animal models, such as *Drosophila melanogaster* or zebrafish, could also be used to examine changes in microbial composition, intestinal morphology and immune responses following probiotic treatment [76, 77]. However, inter-species differences in the intestinal microbiota warrant investigation in humans; clinical trials in healthy or pathogen-colonized individuals may evaluate efficacy against gastrointestinal symptoms [78], either as an alternative or adjunct to antibiotic therapy. Several challenges should be considered when translating these findings to *in vivo* or clinical settings. The antimicrobial effects observed herein were achieved in nutrient-rich media under static conditions and may not be reproduced under the harsh conditions of the GI tract. Pathogen adhesion may also differ *in vivo*, given the mucus layer absent in HT-29 cells, while the host immune response may further modulate the probiotics' effects. Finally, the resident microbial community in animals and humans introduces competition that may limit probiotic efficacy against the pathogens. Despite these limitations, animal and clinical studies are needed to translate these findings for clinical use.

Conclusion

Managing pathogenic infections and overgrowth in the GI tract is critical for host health homeostasis. The global rise in antibiotic resistance limits current treatment options and warrants the development of novel, alternative therapies to manage pathogen virulence. This study demonstrates, for the first time, anti-pathogenic activity of LpWCFS1 and LgA237 against human gut pathogens, *E. coli* ATCC 25922 and *S. aureus* ATCC 25923, through inhibition of *S. aureus* adhesion to IECs and concentration-dependent growth inhibition of both pathogens, with defined MICs. Greater activity against *S. aureus* prompted assessment of biofilm formation, which was reduced by probiotic CFS, though the accompanying decrease in bacterial load requires further investigation to confirm specific antibiofilm effects. Overall, our findings support the potential of these human-derived strains as targeted therapeutic candidates for gut pathogen management against *E. coli*, *S. aureus* and potentially other pathogens. Further investigation in animal and human models is required before the clinical potential of this approach can be fully realized.

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Conflicts of Interest

The authors declare no conflicts of interest.

Ethical Approval

Not applicable.

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