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Research Article

Effects of Postbiotics Derived from *Lactobacillus plantarum* and *Bifidobacterium bifidum* on Biofilm Formation and Virulence Gene Expression of *Enterococcus faecalis*

Samira Saedi [1], Javad Nezhadi [2], Roya Abedi Soleimani [3], Mahdi Asghari Ozma [1], Hossein Samadi Kafil [2,4,*]

[1] Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran
[2] Department of Clinical Microbiology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[3] Department of Food Science and Technology, Faculty of Nutrition and Food Sciences, Tabriz University of Medical Sciences, Tabriz, Iran
[4] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

[*] ***Corresponding Author:*** Department of Clinical Microbiology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran. Email: kafilhs@tbzmed.ac.ir

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Abstract

Background: *Enterococcus faecalis* is an opportunistic pathogen capable of forming biofilms and developing antibiotic resistance, which complicates infection treatment.

Objectives: This study investigates the effect of postbiotics derived from *Lactobacillus plantarum* and *Bifidobacterium bifidum* on biofilm formation and virulence gene expression in *E. faecalis*.

Methods: In the present study, standard strains of *L. plantarum* ATCC 8014, *B. bifidum* ATCC 15696, and *E. faecalis* ATCC 29212 were used. Postbiotics/cell-free supernatants (CFSs) were prepared from probiotics and added to *E. faecalis*. The contents of the obtained postbiotics were evaluated by GC-MS. Biofilm formation was examined using the microtiter plate method, and the expression of endocarditis- and biofilm-associated pilus A (*ebpA*), enterococcal fibronectin-binding antigen A (*efaA*), aggregation substance (*asa*), and adhesin to collagen of *Enterococcus* (*ace*) genes was assessed by real-time (RT)-PCR.

Results: The CFSs significantly reduced biofilm formation in a dose-dependent manner. *Bifidobacterium bifidum* CFSs at 20, 10, and 5 mg/mL significantly decreased biofilm formation. Similarly, *L. plantarum* CFSs at 20 and 10 mg/mL showed a significant inhibitory effect. The qRT-PCR analysis revealed that *L. plantarum* CFSs downregulated *efaA*, *asa*, and *ace* genes but had no effect on the *ebpA* gene. Conversely, *B. bifidum* CFSs reduced *ebpA* and *ace* gene expression but did not significantly alter *efaA* and *asa* genes.

Conclusions: These findings suggest that postbiotics may help reduce the pathogenicity of *E. faecalis*, particularly in preventing infections caused by *E. faecalis*.

Keywords: *Enterococcus faecalis*, *Lactobacillus plantarum*, *Bifidobacterium bifidum*, Postbiotics, Virulence Genes

1. Background

Enterococcus faecalis is a Gram-positive bacterium commonly found in the large intestine, where it aids digestion and vitamin production. However, it can act as an opportunistic pathogen, especially in hospitalized or immunocompromised patients (1, 2). Its ability, similar to that of *Escherichia coli*, to acquire antibiotic resistance genes increases the risk of severe hospital-acquired infections, particularly during prolonged antibiotic therapy (3, 4). *Enterococcus faecalis* can cause urinary tract infections (UTIs), bacteremia, meningitis, endocarditis, wound infections, and gastrointestinal infections, especially after complex surgeries or organ transplants (5). Another key virulence factor of *E. faecalis* is its strong biofilm-forming ability. Biofilms are organized communities of microorganisms embedded in a self-produced extracellular polymeric substance

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(EPS) and attached to living or non-living surfaces. This structure protects microorganisms from adverse environmental conditions, disinfectants, and host immune responses.

Once *E. faecalis* forms a biofilm, treatment becomes extremely difficult, as antimicrobials cannot effectively penetrate its depths. Consequently, the infection may become chronic and easily spread through the bloodstream or other tissues (6, 7). In addition to biofilm formation, *E. faecalis* expresses various virulence genes that significantly contribute to its pathogenicity. By combining biofilm formation, virulence factor expression, and the acquisition of antibiotic-resistance genes, this bacterium poses serious clinical challenges (8). Given its role in life-threatening infections — particularly in immunocompromised patients — there is an increasing focus on developing new therapeutic approaches to combat *E. faecalis* (9).

One of the most promising new therapeutic methods that has attracted the attention of researchers in recent years is the use of probiotics and postbiotics to treat diseases (10, 11). Probiotics are live microorganisms that provide health benefits to the host, while postbiotics — their metabolic products — support immune function, inhibit pathogens, and reduce inflammation (12). Among the common microorganisms used in the production of postbiotics are *Lactobacillus plantarum* and *Bifidobacterium bifidum*, which have gained much attention due to their unique properties in producing antibacterial compounds and regulating the immune system (13, 14). As previously mentioned, postbiotics refer to compounds or products obtained from the metabolic activity of probiotics, which can have beneficial effects on host health (15). These compounds include proteins, peptides, fatty acids, enzymes, and various metabolites that can directly or indirectly enhance antibacterial, anti-inflammatory, and immunogenic activities in the body (16).

2. Objectives

This study investigated the effects of *L. plantarum* and *B. bifidum* postbiotics on *E. faecalis* virulence genes — endocarditis- and biofilm-associated pilus A (*ebpA*), enterococcal fibronectin-binding antigen A (*efaA*), aggregation substance (*asa*), and adhesin to collagen of *Enterococcus* (*ace*) — as well as on biofilm formation. The findings provide insights into the potential use of postbiotics as therapeutic or preventive agents against *E. faecalis* and related pathogens. By reducing biofilm formation and downregulating virulence genes, these postbiotics demonstrate preliminary in vitro promise as adjunct strategies to decrease bacterial pathogenicity, potentially leading to alternative treatments with fewer side effects than conventional antibiotics.

3. Methods

3.1. Bacterial Strains

All experiments were performed on standard laboratory strains. To investigate the effects of postbiotics derived from probiotics on the expression of virulence genes and biofilm-forming ability in the *E. faecalis* ATCC 29212 strain, the *L. plantarum* ATCC 8014 and *B. bifidum* ATCC 15696 strains were used.

3.2. Preparation of Cell-Free Supernatants

To prepare CFSs, *L. plantarum* ATCC 8014 and *B. bifidum* ATCC 15696 were grown on De Man-Rogosa-Sharpe (MRS) agar at 37°C for 18 - 24 h. Colonies were transferred into MRS broth (Merck, Germany) supplemented with 0.6% yeast extract (Sigma-Aldrich) and incubated overnight at 37°C and 150 rpm. Cultures were centrifuged (6000 × g, 10 min, 4°C), and the supernatants were filtered (0.22 µm, Millipore), aliquoted, and stored at -20°C.

3.3. Determination of Cell-Free Supernatant Concentration

To determine the CFS concentration, supernatants were obtained by centrifugation and sterile filtration, then freeze-dried or low-temperature dried to yield a powder. Dry weight was measured, and the powder was reconstituted in sterile medium to the desired concentration, expressed as mg dry matter/mL. This procedure ensured accurate application of CFS bioactive components and improved experimental reproducibility.

3.4. GC-MS Postbiotic Metabolite Identification

GC-MS analysis of postbiotics was performed using a Shimadzu QP-5050 apparatus, a Gas Chromatograph GC-17A with an HP-5 capillary column (phenylmethyl siloxane, 30 m, 0.25 mm internal diameter), and a mass spectrometer with a mass range of 50 - 600 m/z. Helium served as the carrier gas at 1 mL/min with a 1:30 split ratio. The injector and detector were calibrated at 250°C

and 280°C, respectively. The column temperature was programmed to rise linearly at 5°C per minute from 60°C to 250°C and held at 250°C for 10 minutes. Retention indices were determined based on the retention times of injected n-alkanes under identical chromatographic conditions prior to sample injection. By comparing mass spectra with Willey (n17) and Adams libraries, GC-MS identified and characterized the compounds (17).

3.5. Preparation and pH Adjustment of Cell-Free Supernatants for Biofilm Formation Assay

The CFSs from *L. plantarum* and *B. bifidum* were collected under standard sterile laboratory conditions, including controlled temperature, pH, and incubation time. Their naturally acidic pH was neutralized to approximately 7 with NaOH to avoid acidity-related effects on biofilm formation. The neutralized CFSs were then applied in assays evaluating their impact on *E. faecalis* biofilm formation (18).

3.6. Biofilm Formation Assay

The effect of CFSs on *E. faecalis* ATCC 29212 biofilm formation was evaluated using a 96-well microtiter plate assay. A bacterial suspension (0.5 McFarland, ~10^7 CFU/mL, OD600 = 0.08 - 0.1) in trypticase soy broth (TSB) containing 0.5% glucose was prepared, and 10 µL were added to 90 µL of medium per well. Then, 100 µL of CFSs were added, and the plates were incubated at 37°C for 24 h, with untreated wells serving as controls. After incubation, wells were washed with saline, fixed with methanol, stained with 0.1% crystal violet, washed, air-dried, and destained with 33% acetic acid. Biofilm biomass was quantified at OD570.

3.7. Quantitative Real-time PCR Assay

Enterococcus faecalis ATCC 29212 was grown on blood agar (BA) at 37°C under aerobic conditions until reaching the mid-logarithmic phase (OD600 ≈ 0.5). The samples were then treated with CFSs derived from *L. plantarum* and *B. bifidum* at a concentration of 20 mg/mL. *Enterococcus faecalis* ATCC 29212 was exposed to the CFSs for 4 hours, allowing sufficient time for the transcriptional response while avoiding late-stage stress effects.

A q-real-time (RT)-PCR assay was performed using the SYBR Premix EX Taq II, Tli RNase H+ (Takara Bio Inc.), and a RT-PCR machine (Applied Biosystems StepOnePlus™). In the present study, the universal gene (16S rRNA) was used as the reference gene (19). DNA-free RNA was used for cDNA synthesis according to the manufacturer's instructions (Yekta Tajhiz Azma, Tehran, Iran; Cat. No. YT4500). RNA extraction was performed using the Favorgen kit (Favorgen, Pingtung, Taiwan).

The quality and purity of the extracted RNA were assessed using a NanoDrop spectrophotometer (Thermo Scientific, USA) by measuring the absorbance ratio at 260/280 nm. RNA samples with an A260/A280 ratio between 1.8 and 2.1 were considered acceptable. To eliminate potential genomic DNA contamination, all RNA samples were treated with DNase I (Acell Teb Rad Med) prior to reverse transcription. The cDNA synthesis was performed using the Parstous cDNA Synthesis Kit (Parstous Co., Iran) following the manufacturer's instructions. As a negative control, a no-RT control (without reverse transcriptase enzyme) was included for each sample to confirm the absence of DNA contamination in downstream qPCR reactions. The reaction protocol consisted of an initial temperature of 95°C for 15 minutes, followed by 40 cycles of 30 s at 95°C, 45 s at 60°C, and 45 s at 72°C.

3.8. Statistical Analysis

GraphPad Prism (v.9.4.1) and the Statistical Package for the Social Sciences (SPSS) v.20.0 (SPSS, Inc., Chicago, IL, USA) were employed for statistical analysis. The two groups were compared using Student's *t*-test. Data normality was assessed using the Shapiro-Wilk test, and since the data were normally distributed, one-way ANOVA was applied to compare the means between independent groups and stages. A P-value of less than 0.05 was considered statistically significant. Furthermore, all experiments were conducted using three independent biological replicates.

4. Results

4.1. Metabolite Profiling of Postbiotics by GC-MS

Table 1 shows the bioactive compounds identified in the postbiotic samples, as revealed by GC-MS analysis. By comparing the metabolite profiles of *B. bifidum* and *L. plantarum*, two postbiotic sources, it is evident that they possess distinct metabolic capabilities.

Table 1. Postbiotic GC-MS-Identified Major Chemicals

No.	*Bifidobacterium bifidum*	*Lactobacillus plantarum*
1	Lactic acid	Lactic acid
2	Glycolic acid	Acetic acid
3	3-Hydroxybutyric acid	Succinic acid
4	Succinic acid	Butyric acid
5	3-Hydroxypropionic acid	2,3-Butanediol
6	Stearic acid	Diacetyl
7	Heptadecanoic acid	Phenyllactic acid

4.2. Biofilm Formation

The biofilm formation assay confirmed the inherent ability of *E. faecalis* to form biofilms, which play a crucial role in its pathogenicity and resistance to antimicrobial treatments. Treatment with CFSs derived from *L. plantarum* and *B. bifidum* resulted in a concentration-dependent reduction in biofilm formation, as indicated by decreasing optical density (OD) values. Notably, CFSs from *B. bifidum* exhibited stronger inhibitory effects than those from *L. plantarum* (Figure 1S in Supplementary File).

Furthermore, it was determined that postbiotics derived from *B. bifidum* exhibited a greater capacity to inhibit the formation of *E. faecalis* biofilms than those derived from *L. plantarum*. Statistical analyses revealed that concentrations of 20 ($P < 0.0001$), 10 ($P < 0.0001$), and 5 mg ($P = 0.04$) of *B. bifidum* CFSs significantly decreased biofilm formation compared to the control group. Likewise, concentrations of 20 ($P = 0.003$) and 10 mg ($P = 0.01$) of *L. plantarum* CFSs significantly reduced biofilm formation relative to the control group (Figure 1, Table 2).

Table 2. Effect of Different Cell-Free Supernatant Concentrations on Biofilm Formation Reduction in *Enterococcus faecalis* [a]

CFSs Concentration (mg/mL)	Biofilm Reduction of *Enterococcus faecalis* (%)
Lactobacillus plantarum	
20	44
10	27
5	14
2.5	7
Bifidobacterium bifidum	
20	56
10	50
5	18
2.5	7

Abbreviation: CFS, cell-free supernatant.

[a] Evaluated with all experiments performed in triplicate to ensure reproducibility.

4.3. q-Real-time PCR

4.3.1. The Ability of Lactobacillus plantarum Cell-Free Supernatants to Reduce the Expression of Virulence Genes

The analysis of *E. faecalis* isolates revealed a significant reduction in the expression of key virulence genes — *efaA*, *asa*, and *ace* — following exposure to CFSs produced by *L. plantarum*, as compared to the control group. The downregulation of *efaA* ($P < 0.0001$), *asa* ($P = 0.01$), and ace ($P = 0.001$) suggests that *L. plantarum* CFSs may interfere with mechanisms associated with bacterial adhesion, invasion, and biofilm formation, all of which are critical for *E. faecalis* pathogenicity. These findings align with the hypothesis that probiotic-derived postbiotics can mitigate bacterial virulence factors, thus offering potential therapeutic strategies for controlling *E. faecalis*-associated infections.

However, no significant difference was observed in the relative expression of the *ebpA* gene ($P > 0.05$) in the treated group compared to the control. This may indicate that *L. plantarum* CFSs selectively target certain virulence factors more effectively than others, or that the *ebpA* gene — associated with biofilm formation — may require higher concentrations or longer exposure to fully modulate its expression. It is also possible that different bacterial strains respond variably to postbiotic treatments. Overall, these results suggest that while *L. plantarum* CFSs can significantly reduce the expression of several critical virulence genes, factors such as exposure time, dosage, and strain variability may influence the full extent of their effects (Figure 2A, C, E, and G).

4.3.2. The Ability of Bifidobacterium bifidum Cell-Free Supernatants to Reduce the Expression of Virulence Genes

The gene expression analysis of *E. faecalis* isolates showed that exposure to CFSs derived from *B. bifidum* led to a significant reduction in the expression of the *ebpA* ($P = 0.0003$) and *ace* ($P = 0.0001$) genes compared to the control group. These findings suggest that *B. bifidum* CFSs may interfere with key virulence mechanisms of *E. faecalis*, including biofilm formation and adhesion, which are crucial for bacterial persistence and pathogenicity.

On the other hand, no significant difference was observed in the relative expression of the *efaA* and *asa* genes ($P > 0.05$) in the treated group compared to the

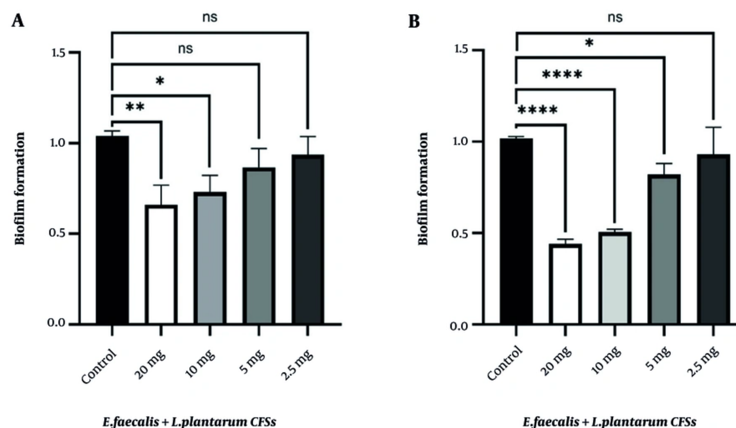

Figure 1. Statistical analysis revealed that *Lactobacillus plantarum* and *Bifidobacterium bifidum* cell-free supernatants (CFSs) significantly reduced *Enterococcus faecalis* biofilm formation in a dose-dependent manner, with *B. bifidum* showing a stronger inhibitory effect at equivalent concentrations. All experiments were performed in triplicate to ensure result accuracy and reliability (* P-value = 0.01, ** P-value = 0.001, **** P-value = 0.0001).

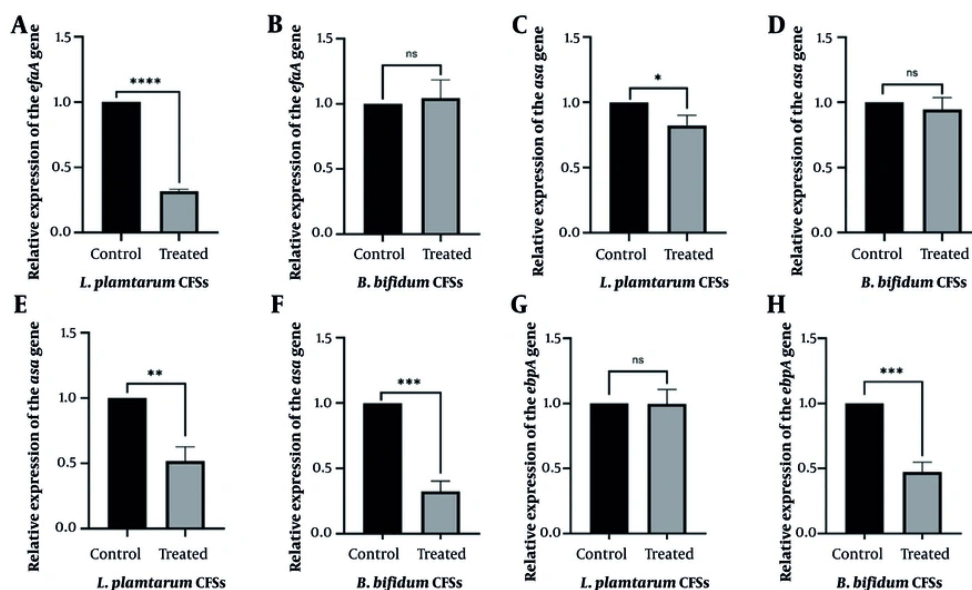

Figure 2. A, C, E, G, the effect of cell-free supernatants (CFSs)-derived from *Lactobacillus plantarum* on the expression of virulence genes including enterococcal fibronectin-binding antigen A (*efaA*), aggregation substance (*asa*), adhesin to collagen of *Enterococcus* (*ace*), and endocarditis- and biofilm-associated pilus A (*ebpA*); B, D, F, H, the effect of CFSs-derived from *Bifidobacterium bifidum* on the expression of virulence genes including *efaA*, *asa*, *ace*, and *ebpA* (the student's *t*-test was used to compare the groups; * P-value = 0.01, ** P-value = 0.001, *** P-value = 0.0001 - 0.0003, and **** P-value < 0.0001; Abbreviation: ns, not significant).

control. This could imply that the postbiotic compounds derived from *B. bifidum* selectively target specific virulence factors, while others — such as those involved in stress response or other regulatory pathways — may be less sensitive to this treatment. It is also possible that higher concentrations or prolonged exposure may be required to significantly modulate the expression of these genes.

Overall, these results indicate that *B. bifidum* CFSs exhibit selective anti-virulence activity, particularly affecting genes related to biofilm formation and adhesion. These findings suggest a potential strategy for managing *E. faecalis*-associated infections (Figure 2B, D, F, and H).

5. Discussion

The observed inhibitory effects on biofilm formation and virulence gene expression suggest that postbiotics interfere with bacterial communication and metabolic pathways. Postbiotics contain various bioactive compounds, such as short-chain fatty acids (SCFAs), peptides, and bacteriocins, which can influence bacterial physiology (20). Studies have shown that postbiotic compounds can disrupt quorum sensing (QS), a key regulator of biofilm formation, thereby reducing bacterial adhesion and aggregation. Bacteriocins, organic acids (e.g., lactic and acetic acids), and SCFAs can interfere with QS in *E. faecalis* by blocking QS receptors, disrupting cell membranes, altering intracellular pH, or modulating global regulatory systems. Some *Lactobacillus*-derived metabolites inhibit the FSR QS system, which controls virulence factors such as gelatinase (gelE) and serine protease (sprE). Differences in gene expression between *L. plantarum* and *B. bifidum* CFSs may reflect variations in their bioactive compound profiles and interactions with pathogen regulatory pathways (21). Additionally, specific metabolites from *B. bifidum* and *L. plantarum* may modulate bacterial gene expression, leading to a decrease in virulence factor production (13).

This study examined the effects of *L. plantarum* and *B. bifidum* CFSs on the expression of key *E. faecalis* virulence genes (*efaA*, *asa*, *ace*, and *ebpA*), which are essential for adhesion, biofilm formation, and survival in hostile environments, while also analyzing the CFS composition using GC-MS. The *efaA* gene encodes a surface protein that mediates adhesion to host cells and supports stable biofilm formation, an essential step in infection. Since adhesion is central to many virulence processes, targeting *efaA* can reveal how postbiotics may inhibit this mechanism (3, 22). The *asa* gene encodes an adhesin that enables *E. faecalis* to attach to host cells and promote biofilm formation. Adhesins play critical roles in chronic infections, as biofilms enhance bacterial resistance to treatment and immune evasion. Studying this gene can clarify how postbiotics may modulate or inhibit adhesion and biofilm formation, offering potential for new therapeutic strategies (19, 23).

Biofilms not only confer antibiotic resistance but also protect bacteria from host immune responses. Therefore, studying the impact of postbiotics on this gene can help identify new ways to interfere with biofilm formation and improve the treatment of resistant infections (22). Finally, the *ebpA* gene is involved in the production of proteins that enable *E. faecalis* to bind to host cells. These proteins directly contribute to adhesion and biofilm formation processes. Examining the effects of postbiotics on this gene can help us better understand the molecular pathways involved in virulence and could lead to the development of targeted therapies (24). The selected genes play crucial roles in virulence processes such as host cell adhesion, biofilm formation, and treatment resistance. Our results showed that postbiotics reduce the expression of some of these genes and inhibit biofilm formation, highlighting their potential as biological agents to control *E. faecalis* infections. Notably, *B. bifidum* postbiotics had a stronger effect on biofilm reduction than those from *L. plantarum*.

Specifically, *L. plantarum* postbiotics decreased *efaA*, *asa*, and ace expression but did not affect *ebpA*, whereas *B. bifidum* postbiotics reduced *ebpA* and ace expression without significantly impacting *efaA* and *asa*. These differences likely stem from variations in their composition and metabolic activities. Our findings on *L. plantarum* postbiotics align with Kim et al., who reported that lactic acid bacteria postbiotics suppress biofilm formation in mastitis-associated bacteria, including *E. faecalis* (25). Similarly, Nezhadi and Ahmadi found that postbiotics derived from *L. plantarum* can prevent biofilm formation in nosocomial bacteria, including *E. faecalis* and *Pseudomonas aeruginosa* (1). Furthermore, Knysh et al. demonstrated that postbiotics derived from *B. bifidum* effectively prevented biofilm formation in pathogenic bacteria, including *E. coli* and *P. aeruginosa*, and significantly reduced the biofilm formation rate compared to the control group (26). Additionally, a study by Asghari Ozma et al. showed that postbiotics derived from lactic acid bacteria such as *B. bifidum* can play a significant role in inhibiting biofilm formation and serve as novel therapeutic agents for treating infections caused by *Clostridium difficile* (27).

This study represents the first investigation into the effects of postbiotics derived from *B. bifidum* and *L.*

plantarum on the expression of virulence genes, including *efaA*, *ebpA*, *asa*, and *ace*. Nevertheless, several previous studies have demonstrated that *L. plantarum* can influence the expression of other virulence genes. For example, in a study conducted by Oumaima et al., the effect of *L. plantarum* was investigated on *P. aeruginosa*, revealing that *L. plantarum* can reduce the activity of the MexXY-OprM efflux pump in this bacterium, thereby aiding in overcoming antibiotic resistance (28). Furthermore, a study by Zabolyova et al. showed that postbiotics (enterocins) can positively influence the treatment of methicillin-resistant *S. aureus* strains and contribute to overcoming antibiotic resistance (29). Additionally, Ishikawa et al. reported that postbiotics produced by lactobacilli modify the transcription of virulence genes in *Aggregatibacter actinomycetemcomitans*, thereby reducing its pathogenicity and antibiotic resistance (30).

Our results indicate that postbiotics from *B. bifidum* were more effective than those from *L. plantarum* in reducing *E. faecalis* biofilm formation. The strains exerted distinct effects on virulence gene expression, likely reflecting differences in their metabolic profiles. Postbiotics from *L. plantarum* reduced *efaA*, *asa*, and *ace*, but not *ebpA*, whereas *B. bifidum* reduced *ebpA* and *ace* without altering *efaA* and *asa*. A limitation of this study is that it did not assess the synergistic effects of postbiotics with antibiotics or other drugs; this will be addressed in future research.

5.1. Conclusions

This study provides new insights into the potential of postbiotics from *L. plantarum* and *B. bifidum* to reduce the pathogenicity of *E. faecalis* by inhibiting biofilm formation and suppressing the expression of key virulence genes. These findings support the growing interest in postbiotics as a novel and sustainable alternative to combat bacterial infections. Further research, particularly clinical studies, is required to confirm these results and explore the practical applications of postbiotics in future healthcare.

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Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

Footnotes

Authors' Contribution: S. S. and J. N.: Data curation, investigation, methodology, resources, software, validation, visualization, and writing-original draft; R. A. S. and M. A. O.: Writing-original draft, methodology, and resources; H. S. K.: Project administration, resources, software, and supervision.

Conflict of Interests Statement: The authors declare no conflict of interest.

Data Availability: The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

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