

## Control of Opportunistic Oral Cavity Infections Using Postbiotics Secreted by Aerobic Oral Flora, with Minimal Impact on Host Cells

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### ABSTRACT

**Introduction:** Postbiotics derived from the natural oral microbiome offer a potential solution for chemotherapy-induced oral dysbiosis by restoring microbial balance. These compounds hold promise for managing oral infections, particularly in vulnerable populations, by restoring microbial balance, directly inhibiting pathogen growth, and promoting a healthy mucosal immune response. This study evaluates the antimicrobial activity of postbiotics derived from aerobic oral bacteria against *Staphylococcus aureus* and assesses their cytotoxicity in human cells, contributing to the development of new therapeutic strategies for oral infections. **Methods:** Specific strains of aerobic oral bacteria were isolated and cultured in nutrient broth at 37°C in a 5% CO<sub>2</sub> atmosphere. After 24 h of incubation, the supernatant containing postbiotics was collected and centrifuged, followed by filtration to isolate the postbiotics. The antibacterial activity of these postbiotics was assessed against *S. aureus* isolated from oral wounds using the agar well diffusion method on Mueller-Hinton agar plates. In parallel, the cytotoxic effects on Normal Human Dermal Fibroblasts (NHDF) were evaluated through MTT viability assays to measure metabolic activity, and SYBR Green staining to quantify DNA content, and assess cell membrane integrity. Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test, with significance set at  $P < 0.05$ . **Results:** Postbiotics exhibited significant antimicrobial activity against *S. aureus*, as evidenced by a mean zone of inhibition of 17.0 mm ( $\pm 1.4$  mm), while no inhibition was observed in saline controls. Furthermore, postbiotics treatment resulted in NHDF cell viability of 94% ( $\pm 1.6\%$ ) compared to the untreated controls 75.2% ( $\pm 0.7\%$ );  $P < 0.0001$ , demonstrating their biocompatibility. **Conclusion:** This study demonstrates the promising therapeutic potential of postbiotics for combating opportunistic oral infections, exhibiting negligible cytotoxicity towards host cells. Future research will explore isolating and identifying the active components of these postbiotics and evaluating their efficacy *in vivo* models and human oral cell lines.

### INTRODUCTION

Cancer is currently the second leading cause of death globally and is projected to potentially become the leading cause by the year 2060 [1]. The increase in cancer prevalence underscores the need for effective management of cancer-related complications, particularly opportunistic oral infections, which significantly impact quality of life and treatment outcomes. These infections, often caused by pathogens like *S. aureus*, pose a substantial threat to cancer patients, especially those undergoing chemotherapy. *S. aureus* is a concerning

pathogen known to cause severe oral infections, including abscesses, cellulitis, and osteomyelitis. While *S. aureus* can be part of the normal flora in some healthy individuals, it is rarely dominant in the oral cavity of the general healthy population. However, its colonization increases significantly in older adults and immunocompromised patients, such as cancer patients undergoing chemotherapy, due to their weakened immune systems [2, 3]. These infections can progress to mucosal ulcerations, resulting in pain, malnutrition, and

heightened risk of secondary infections, which can ultimately interrupt cancer treatment and worsen patient outcomes [1, 4, 5].

Typically, treatments for oral infections rely on antimicrobials, anti-inflammatories, and cytoprotectives, but these treatments may adversely affect the viability and function of normal oral cells, underscoring the need for safer, more targeted therapies that selectively eliminate pathogens while preserving host tissues [6]. In recent years, postbiotics, which are metabolic byproducts or components released by probiotics that exert beneficial effects on the host, have emerged as a focus of research as they are recognized for their safety profile and stability compared to live probiotics. Postbiotics offer several advantages, including an extended shelf life, enhanced stability during storage and transport, and a diminished risk of transferring antibiotic resistance genes. Moreover, postbiotics have demonstrated promising antibacterial, antioxidant, and immune-modulating properties [7, 8]. In the context of oral infections, postbiotics demonstrate potential in selectively targeting *S. aureus* through their bioactive compounds, comprising organic acids, bacteriocins, and cell wall components, which can disrupt the bacterial cell membrane, inhibit growth, or interfere with virulence factors, while sparing healthy host cells [9]. Central to the oral epithelial defense are various components, including antimicrobial peptides, the innate immune response, and MUC16, a transmembrane mucin, plays a pivotal role by forming a glycan-rich barrier on the epithelial surface, which effectively prevents pathogen contact and subsequent infection [10]. Studies have demonstrated the crucial role of MUC16 in protecting against oral infections caused by *S. aureus*. For example, the suppression or reduction of MUC16 expression results in increased *S. aureus* adherence, underscoring its importance in defense against staphylococcal infection [11].

Furthermore, macrophage colony-stimulating factor (M-CSF) regulates immune cell proliferation and differentiation. Researchers have identified M-CSF as a potential marker for the presence or severity of opportunistic infections, including those caused by *S. aureus* [3]. This suggests that M-CSF, which is abundantly expressed in various tissues and is also secreted into saliva, plays a significant role in the host response to these infections. Its presence in saliva serves as a diagnostic indicator of oral dysbiosis and *S. aureus* infection, particularly in immunocompromised individuals, such as older adults with periodontal disease or individuals with HIV-1 infection [12, 13].

Therefore, postbiotics derived from the natural oral microbiome of healthy individuals hold promise as a novel therapeutic approach to counteract chemotherapy-induced dysbiosis by selectively inhibiting the growth of opportunistic pathogens and promoting the restoration of a healthy oral microbiota. These postbiotics have the potential to restore microbial balance, eliminate

pathogenic overgrowth, and enhance mucosal immunity. Thus, this study investigates the antimicrobial activity of postbiotics derived from aerobic oral flora against *S. aureus* and assesses their safety in human cells, with the aim of developing new therapeutic strategies to manage oral infections.

## MATERIAL AND METHODS

**Isolation of oral bacteria.** Oral bacteria were collected from three healthy female volunteers (n=3, mean age 23 ± 7.8 years) with no history of oral diseases or current medication use, including antibiotics. Samples were collected from four distinct oral sites: the gums, palate, tongue, and buccal mucosa, using sterile cotton swabs. The swabs were then aseptically transferred into 1 mL of nutrient broth: containing gelatin peptone (5g/L), beef extract (1g/L), yeast extract (2g/L), and sodium chloride (5g/L); QuLab, USA, and incubated at 37°C with shaking at 180 rpm for 24 h to promote bacterial growth.

**Extraction of postbiotics.** After 24 h of incubation, 1 mL of the bacterial culture was transferred to 50 mL of fresh nutrient broth and incubated at 37°C with shaking at 180 rpm for 18-24 h. This extended incubation promotes the secretion of metabolites. After removal of bacterial cells, these metabolites constitute the postbiotics. Following incubation, bacterial cells were pelleted by centrifugation at 10,000 × g for 15 min at 4°C. The supernatant was then aseptically filtered through a 0.22 µm PES syringe filter and chilled on ice to minimize degradation of heat-sensitive postbiotics, to remove any remaining bacterial cells. The filtered postbiotic solution was stored at -20°C, which minimizes enzymatic activity and degradation, preserving postbiotic stability for further analysis.

**Isolation of opportunistic pathogens.** *S. aureus* was isolated from an oral wound located on the periodontal tissues of a 13-year-old boy diagnosed with leukemia undergoing chemotherapy. A sterile Rayon swab was used to sample the wound site. The swab was immediately inoculated onto Mueller-Hinton agar plates (Merck, Germany) and incubated aerobically at 37°C for 24 h to allow bacterial growth. The resulting colonies were subcultured onto fresh Mueller-Hinton agar plates using a sterile loop. The resulting pure cultures underwent Gram-staining. Identification of *S. aureus* was confirmed using standard biochemical tests. Only isolates confirmed as *S. aureus* (Gram-positive, catalase-positive, coagulase-positive) proceeded to further analysis. To further confirm identification and assess strain variations, isolates underwent additional tests for urease, indole, methyl red, Voges-Proskauer (VP), Simmons' citrate, and motility. Antibiotic susceptibility testing was performed using the Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.

The following antibiotics were tested at their standard concentrations: ampicillin, cefoxitin, clindamycin, erythromycin, trimethoprim-sulfamethoxazole, and penicillin. The isolated *S. aureus* strain was susceptible to ampicillin, cefoxitin, clindamycin, erythromycin, and trimethoprim-sulfamethoxazole, but resistant to penicillin, emphasizing the importance of developing alternative therapies for penicillin-resistant *S. aureus* infections.

**Antimicrobial activity assay.** The antimicrobial activity of the extracted postbiotics was assessed using the agar well diffusion method. Sterile wells with a diameter of 6 mm were aseptically created on Mueller-Hinton agar plates using a sterile pipette tip. A bacterial suspension of *S. aureus*, cultured in nutrient broth for 18-24 h, was prepared in saline. The bacterial concentration was determined by measuring the optical density (OD) at 600 nm and adjusted to CFU/mL based on a pre-established standard curve. The suspension was then standardized to a final concentration of approximately  $1.5 \times 10^8$  CFU/mL by dilution with sterile saline. A 10  $\mu$ L sample of the extract was diluted in 100  $\mu$ L of saline (1:10 dilution) to test the postbiotics. This 1:10 dilution minimizes the potential influence of the nutrient broth used during extraction, which could affect postbiotic diffusion or exhibit antimicrobial activity. Then, 100  $\mu$ L of the diluted postbiotics solution was aseptically added to each well. As a negative control, two wells were filled with 100  $\mu$ L of nutrient broth diluted 1:10 in saline. The plates were incubated at room temperature for 5 min to allow diffusion of the solutions into the agar, then incubated at 37°C for 24 h. After 24 h of incubation, the diameters of the inhibition zones around the wells were measured, indicating antibacterial activity.

#### Cytotoxicity assays

**MTT assay.** The cytotoxic effects of the extracted postbiotics on NHDF were assessed using the MTT assay. NHDF cells were seeded at a density of 5,000 cells per well in a 96-well plate. Cells were cultured in high-glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and a 1% penicillin-streptomycin solution (100 units/mL penicillin, 100  $\mu$ g/mL streptomycin). The cells were incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> for 24 h to allow cell attachment and proliferation. Next, the culture medium was replaced with fresh DMEM containing a 10% dilution of the postbiotic solution (n=5 wells per treatment). The 10% dilution was prepared by mixing 10  $\mu$ L of postbiotic solution with 90  $\mu$ L of fresh DMEM. A vehicle control, consisting of a 10% dilution of nutrient broth in DMEM, was tested to account for potential effects from the extraction process. Ten wells received only fresh DMEM as negative controls. After 48 h of treatment, the medium was carefully aspirated, and 100  $\mu$ L of 0.5 mg/mL MTT solution (Sigma, Germany) was added to each well. The plates were incubated for 4 h at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> to promote

formazan crystal formation. After aspirating the MTT solution, 100  $\mu$ L of DMSO was added to each well. The plate was then incubated at 37°C for 10 min with gentle shaking to dissolve the formazan crystals. Finally, the absorbance at 570 nm was measured using an ELISA reader (BioTek, USA). Cell viability was calculated as the percentage of the absorbance value of each treatment group relative to the negative control (untreated cells), which was set as 100% viability.

**SYBR Green staining.** To assess the effects of postbiotics on cell membrane integrity, SYBR Green staining was performed to differentiate between live and dead cells using fluorescent microscopy. SYBR Green (Jena Bioscience, Germany) was diluted in phosphate-buffered saline (PBS) according to the manufacturer's instructions. NHDF cells were seeded at a density of 10,000 cells per well in a 48-well plate and cultured at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> until they reached approximately 90% confluence. After treatment, the cells were trypsinized and centrifuged at 400  $\times$ g for 5 min to remove the supernatant containing trypsin. The cell pellet was resuspended in PBS to a concentration of  $1 \times 10^6$  cells/mL. 100  $\mu$ L of the cell suspension was stained with 100  $\mu$ L of SYBR Green solution. The cells were then incubated with SYBR Green in the dark for 10 min at room temperature. After incubation, the cells were washed once with PBS to remove any excess dye or debris and then centrifuged again. The stained cells were observed under a fluorescent inverted microscope (Motic AE31E series) using the GFP filter, which is optimal for detecting SYBR Green fluorescence.

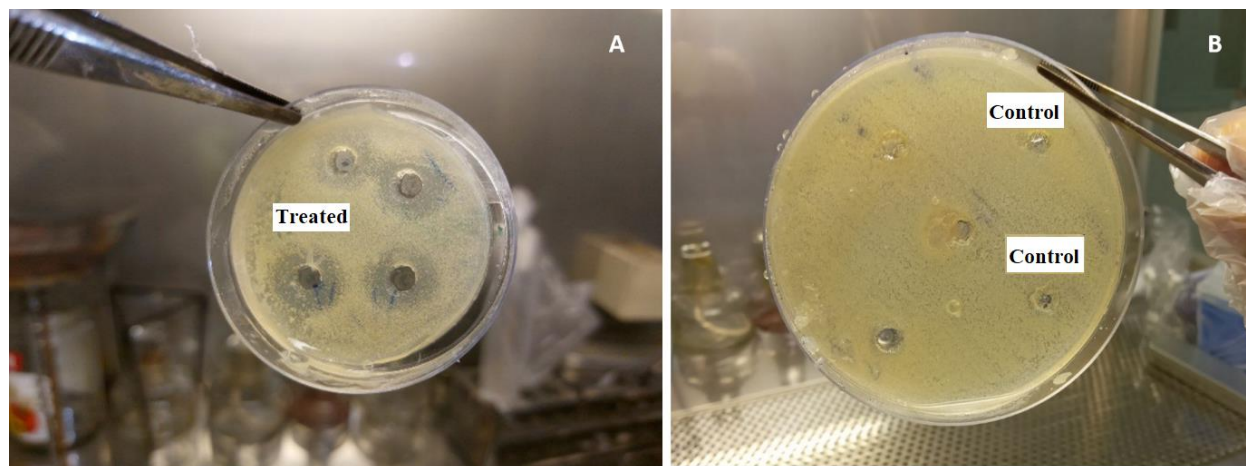
**Statistical analysis.** The results were expressed as mean  $\pm$  standard deviation (SD). Statistical analysis and data visualization were performed using GraphPad Prism software (version 10.3.1.509). Differences between treatment groups in the MTT assay, including postbiotics-treated, vehicle control (nutrient broth dilution), and untreated (negative control) groups, were assessed using one-way ANOVA. Tukey's post-hoc test was used to determine the significance of pairwise comparisons between treatment groups. A *P*-value < 0.05 was considered statistically significant.

**Ethical considerations.** This study was approved by the Ethics Committee of Tehran University of Medical Sciences (Ethics ID: IR.TUMS.SPH.REC.1403.039) and conducted in accordance with its ethical guidelines. Informed written consent was obtained from all participants, including healthy volunteers and the patient with the oral wound, prior to sample collection. All participants were provided with a detailed information sheet explaining the study's purpose, procedures, and potential risks and benefits, and their consent was documented in writing.

## RESULTS

**Antimicrobial activity of postbiotics against opportunistic oral infections.** The extracted postbiotics demonstrated potent antibacterial effects against *Staphylococcus aureus*, evidenced by distinct zones of

inhibition surrounding the sample wells (Figure 1). The mean diameter of these inhibition zones was  $17.0 \text{ mm} \pm 1.41 \text{ mm}$  (SD), significantly surpassing the negative control, which exhibited no measurable inhibition zone ( $P < 0.001$ ). This indicates a robust antimicrobial response with minimal variability in the postbiotic effectiveness.



**Fig. 1.** Demonstration of antimicrobial efficacy of postbiotics via agar well diffusion assay. (A) Clear zones of inhibition around wells filled with postbiotic solutions, indicating effective antibacterial action against *S. aureus*; (B) Control wells filled with nutrient broth show no inhibition zones, underscoring that the nutrient broth, used as a vehicle control, does not possess inherent antibacterial properties, thereby confirming the specific activity of the postbiotics.

**Cytotoxicity assessment of postbiotics on NHDF cells.** An MTT assay evaluated the impact of a 10% dilution of extracted postbiotics on NHDF cell viability, comparing it with a 10% dilution of nutrient broth (vehicle control) and cells left untreated (negative control). Postbiotic-treated cells exhibited significantly higher viability than untreated cells (mean viability increase = 18.76%; 95% CI: 17.40% to 20.13%;  $P < 0.0001$ ). Similarly, the vehicle control group demonstrated enhanced viability over the untreated group (mean viability increase = 17.26%; 95% CI: 15.90% to 18.63%;  $P < 0.0001$ ). Although cells treated with postbiotics showed a marginal increase in viability compared to those treated with the vehicle control, this improvement was not statistically significant (mean difference = 1.50%; 95% CI: -0.08% to 3.08%;  $P = 0.0635$ ) (see Table 1 and Figure 2 for detailed data).

Overall, the MTT assay results indicated that both the postbiotic-treated group and the vehicle control group

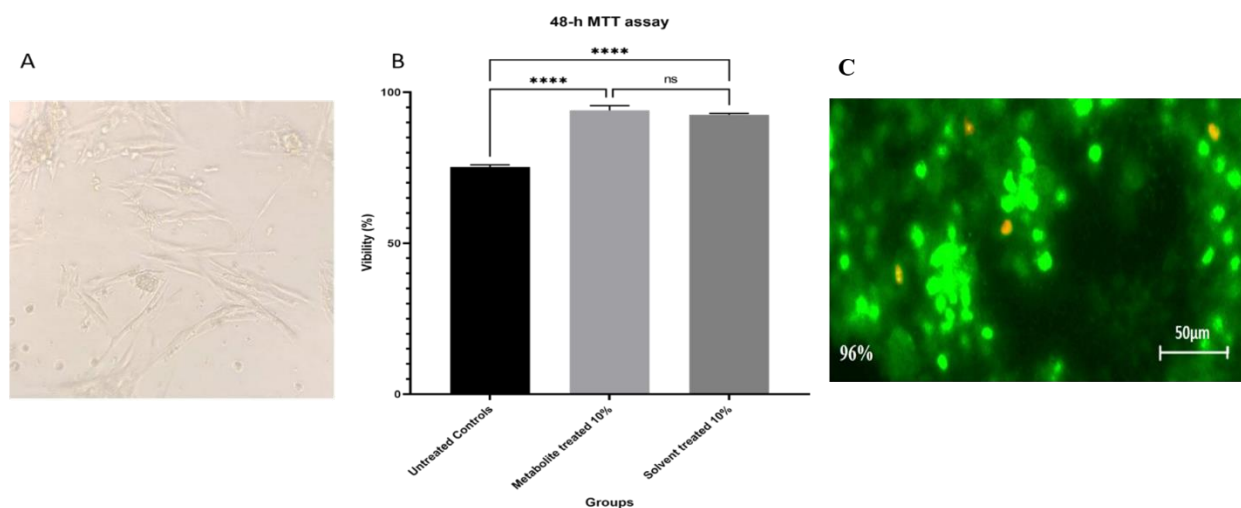
(1:10 dilution of Tryptic Soy Broth (TSB)) displayed significantly enhanced cell viability relative to the untreated control group after 48 hours ( $P < 0.0001$ ). The elevated viability in the vehicle control might stem from the nutrient broth's composition, including growth factors like peptides and amino acids, or other nutritive elements such as vitamins and minerals, which are known to bolster cell survival. Additionally, this enhancement might reflect a hormetic response, where mild exposure to stressors like postbiotics could trigger cellular defense mechanisms, potentially altering proliferation rates or metabolic activities. Further research is warranted to elucidate the precise mechanisms, including any hormetic effects and the involvement of specific cellular pathways. As depicted in Figure 2, the 10% dilution of antimicrobial postbiotics did not significantly compromise NHDF cell viability, suggesting a favorable safety profile at the tested concentration.

**Table 1.** Effects of postbiotics on NHDF cell viability: results from Tukey's multiple comparisons test

Comparison	95% CI of difference (%)	Summary	Adjusted P-value
Untreated control vs postbiotics	-20.13 to -17.40	****	<0.0001
Untreated control vs vehicle control	-18.63 to -15.90	****	<0.0001
Postbiotics vs vehicle control	-0.07602 to 3.076	ns	0.0635

• ns = no significant.

**Note:** This table summarizes the outcomes of Tukey's multiple comparisons test post-ANOVA, evaluating the viability differences among NHDF cells in three conditions: untreated, treated with a 10% dilution of postbiotics, and treated with a 10% dilution of the vehicle control.



**Fig. 2.** Impact of postbiotics on NHDF cell viability. (A) Light microscopy captures NHDF cells after 48 h of exposure to postbiotics, vehicle control, and in an untreated state. (B) MTT assay results (Mean Viability %  $\pm$  SD) illustrate that both postbiotic-treated cells ( $94\% \pm 1.581$ ) and vehicle control groups significantly enhance cell viability relative to untreated controls ( $75.24\% \pm 0.7484$ ) ( $****P < 0.0001$ ). Although postbiotic-treated cells displayed a trend towards improved viability over the vehicle control, this was not statistically significant (ns). (C) Fluorescence microscopy with SYBR Green staining highlights DNA content, indicating cell numbers post-treatment, reinforcing that the antimicrobial postbiotics at the tested concentration do not significantly impair NHDF cell viability.

**Fluorescence microscopy analysis.** To further evaluate the cytotoxic potential of postbiotics on NHDF cells, fluorescence microscopy with SYBR Green was employed. SYBR Green, a nucleic acid stain, fluoresces green upon binding to nucleic acids, with healthy cells displaying bright, uniform fluorescence, whereas cells with compromised membranes or undergoing cell death show altered staining patterns. As depicted in Figure 2C, postbiotic-treated NHDF cells exhibited bright, consistent SYBR Green fluorescence, akin to untreated controls, indicating intact cell membranes and nucleic integrity. This observation, in alignment with the MTT assay outcomes, supports that the postbiotic concentration tested does not significantly induce cytotoxicity in NHDF cells.

## DISCUSSION

Chemotherapy-related side effects, particularly opportunistic oral infections, significantly impair the effectiveness of cancer treatment and adversely affect patients' quality of life [14]. To tackle this issue, our study explored the potential of postbiotics secreted by aerobic oral flora as a novel strategy for managing these infections. We assessed the effectiveness of these postbiotics against *S. aureus*, a prevalent agent of opportunistic oral infections, alongside examining their cytotoxic impact on NHDF cells.

The findings from our research underscore the potential of postbiotics, extracted from the oral microbiome of healthy individuals, as an effective antimicrobial approach against oral dysbiosis, particularly the type exacerbated by chemotherapy. Our results align with those reported by Khani *et al.* (2023) [15], where we

observed an antimicrobial effect against *S. aureus* with an inhibition zone of  $17.0 \pm 1.411$  mm. This activity mirrors the outcomes seen with postbiotic cell-free supernatants from *Lactobacillus* species, which Khani *et al.* found to produce inhibition zones between 15.32 and 23.21 mm against *S. aureus* and other pathogens [15].

Our study has shown that postbiotics secreted by oral aerobic microbiota effectively curb the growth of *S. aureus*, with efficacy akin to that of postbiotics from *Lactobacillus* species as described by Khani *et al.* These findings reinforce the therapeutic potential of oral microbiota-derived postbiotics in preventing and treating *S. aureus* infections, thereby enhancing oral health and aiding infection management in vulnerable groups like chemotherapy patients. Although this study did not delve into identifying the specific bioactive compounds due to resource constraints, this gap paves the way for future research. Investigating these components could lead to the development of more precise and effective postbiotic therapies for oral infections. Previous research, including that by Khani *et al.* (2023) [15], has identified compounds like lactic acid, laurostearic acid, and isopropylidene-3,3-dimethylglutaric acid as key players in the antimicrobial activity of *Lactobacillus*-derived postbiotics, suggesting similar or novel compounds might be at work in our extracts.

In addition to their antimicrobial capabilities, postbiotics play a role in modulating the oral microbiome, thereby enhancing oral health. Research, including that by Zhao *et al.* (2023), has illustrated that postbiotic preparations, such as the supernatant from *Lactobacillus paracasei* ET-22, can significantly curb the growth of *Streptococcus mutans*, a leading cause of dental caries.

This supernatant not only inhibited bacterial growth but also modulated biofilm formation, indicating that postbiotics might offer a viable approach for oral health maintenance and caries risk reduction. The capacity of postbiotics to influence biofilm dynamics could extend to preventing the establishment of other opportunistic pathogens, like *S. aureus*, thereby underscoring their broader potential in oral infection control [16].

To identify the bioactive constituents within the postbiotics from *L. paracasei* ET-22, we employed non-targeted LC-MS/MS analysis, which identified phenyllactic acid as a significant organic acid in the supernatant. Phenyllactic acid is recognized for its wide-ranging inhibitory effects on biofilm formation across various pathogenic bacteria. Its effectiveness against oral pathogens, including *S. aureus* and *Actinobacillus actinomycetemcomitans*, has been documented, reinforcing its role in oral health maintenance [16, 17].

The enhanced viability of NHDF cells treated with postbiotics, as opposed to untreated controls (Figure 2), underscores their potential safety and biocompatibility at the tested concentration. This finding supports the non-cytotoxic nature of the postbiotics on NHDF cells. The antimicrobial efficacy of these postbiotics might also relate to their impact on epithelial barrier function, particularly via the modulation of MUC16, a crucial glycoprotein in epithelial defense. MUC16 creates a glycan-rich shield on epithelial surfaces, deterring pathogen contact [10, 18]. Postbiotics could bolster this defense by enhancing MUC16 expression or its glycosylation state, or by preventing its degradation, thus fortifying resistance against pathogens like *S. aureus* [19]. Given MUC16's role in oral epithelial integrity, future studies should explore how postbiotics from healthy oral microbiota affect MUC16 dynamics. Such research could include *in vitro* assessments with oral epithelial cells and *in vivo* experiments in models of oral infection or dysbiosis. Additionally, examining the therapeutic potential of postbiotics in populations with compromised oral health, like chemotherapy or radiotherapy patients, could reveal new avenues for infection prevention and treatment. If postbiotic applications indeed elevate MUC16 levels, they might significantly enhance mucosal barrier functions, potentially lowering infection risks in vulnerable individuals.

Future research must focus on elucidating the active components within the postbiotic extract through advanced analytical methods like high-performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS). Following identification, research should delve into the mechanisms by which these compounds exert their effects on bacterial proliferation, biofilm dynamics, and host immune modulation. For clinical translation, it's imperative to further validate the antimicrobial efficacy and biocompatibility of these postbiotics. This validation should employ advanced models, such as 3D oral epithelial cultures, organoids, or

*in vivo* animal models of oral infection, which better mimic the intricate interactions within the oral cavity. These systems offer a more realistic assessment of how postbiotic therapies might perform in actual clinical scenarios, considering the dynamic between the oral microbiome, host tissues, and pathogenic agents.

This study has showcased the capability of postbiotic preparations sourced from the oral microbiota of three healthy subjects to significantly curb the proliferation of *S. aureus*, an opportunistic pathogen. The antimicrobial prowess of these postbiotics was confirmed, showing no compromise in efficacy. Crucially, these preparations displayed negligible cytotoxicity against NHDF cells, preserving cell viability at levels substantially higher than those observed in untreated controls.

Our findings underscore the promise of postbiotics as both safe and potent antimicrobial agents, evidenced by their excellent biocompatibility with NHDF cells. Derived from a healthy oral microbiome, these postbiotics present a novel approach for mitigating chemotherapy-induced oral dysbiosis. They have the potential to foster a balanced oral microbiome, thereby decreasing the frequency and intensity of opportunistic infections, which could significantly enhance patient recovery and quality of life.

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#### CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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