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ORIGINAL RESEARCH

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Evaluation of the effects of probiotic and postbiotic toothpastes for children on cariogenic dental biofilms: an *in vitro* study

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Abstract

Background: This study aimed to evaluate the effects of commercially available probiotic- and postbiotic-containing toothpastes formulated for children on multispecies cariogenic biofilms under *in vitro* conditions. **Methods**: Multispecies biofilms composed of *Streptococcus mutans* ((American Type Culture Collection) ATCC 25175), *Lactobacillus casei* (ATCC 4646), and *Scardovia wiggsiae* ((German Collection of Microorganisms and Cell Cultures) DSM 22547) were formed on hydroxyapatite (HAP) discs. Seven experimental groups were evaluated, including a fluoride-containing positive control, a negative control, and commercially available probiotic- and postbiotic-containing children's toothpastes. The biofilms were exposed to toothpaste slurries. Biofilm biomass and live/dead cell ratios were quantitatively evaluated using confocal laser scanning microscopy (CLSM) with subsequent analysis through COMSTAT software. Statistical comparisons were performed using the Kruskal-Wallis test followed by Dunn's *post hoc* test ($p < 0.05$). **Results**: All toothpaste formulations significantly reduced live/dead bacterial biomass ratios compared with the negative control, while the fluoride-containing toothpaste (positive control) showed the strongest antibiofilm effect. **Conclusions**: Probiotic- and postbiotic-containing toothpastes modulated multispecies dental biofilm viability *in vitro* and may serve as complementary agents to fluoride-based formulations in pediatric oral care.

Keywords

Toothpaste; Antibacterial; Biofilm; Probiotic; Postbiotic

1. Introduction

Dental caries is one of the most prevalent chronic oral diseases worldwide and continues to affect a large proportion of the pediatric population [1]. The development of dental caries is a dynamic process resulting from complex interactions among acid-producing microorganisms, fermentable carbohydrates, and host-related factors such as tooth structure and saliva [2, 3].

Dental biofilm represents the primary etiological factor in the caries process. The metabolic activity of cariogenic microorganisms within the biofilm leads to acid production, which lowers the local pH and promotes demineralization of tooth mineral tissues, ultimately initiating carious lesions [2, 4, 5]. Therefore, an adequate evaluation of dental caries requires consideration of both the microbial composition of the biofilm and the interactions among cariogenic species [6].

Among these microorganisms, *Streptococcus mutans* (*S. mutans*), *Scardovia wiggsiae* (*S. wiggsiae*), and *Lactobacillus casei* (*L. casei*) play key roles at different stages of caries development. *S. mutans* is primarily involved in caries initiation, *S. wiggsiae* associated with early childhood caries due to its aciduric nature, and *L. casei* contributing to lesion progression under low pH conditions [7–9].

Although mechanical removal of dental biofilm through tooth brushing remains the most effective plaque control method, its success depends largely on individual oral hygiene practices [10]. Consequently, contemporary preventive dentistry strategies focus not on complete biofilm elimination, but on suppressing cariogenic microorganisms, reducing plaque acidity, and maintaining oral microbiota [11]. In this context, toothpastes function as essential chemical plaque control agents by limiting biofilm formation and bacterial colonization through their antimicrobial components [12].

Fluoride-containing toothpastes have long been recommended by the World Health Organization (WHO), the American Dental Association (ADA), and the World Dental Federation (FDI) because of fluoride's well-established anticariogenic properties. These toothpastes have demonstrated clinical effectiveness through both their antibacterial activity and their ability to enhance enamel remineralization [13].

In recent years, biological strategies aimed at restoring the

balance have gained increasing attention in caries prevention. Probiotics may exert anticariogenic effects by competing with pathogenic microorganisms, inhibiting biofilm formation, and modulating the host immune response. However, their clinical use presents certain limitations, including the presence of live microorganisms, challenges related to product stability and storage, and potential risks in immuno-compromised individuals [14, 15].

To overcome these limitations, postbiotics have emerged as a promising alternative. Postbiotics are biologically active microbial products that do not contain live bacteria and include cell wall components, cytoplasmic contents, and metabolites produced by probiotic microorganisms. Their favorable safety profile and structural stability make them particularly suitable for pediatric applications [16].

Unlike conventional antimicrobial agents that exert non-selective bactericidal effects, probiotic and postbiotic approaches aim to modulate oral biofilms by supporting microbial homeostasis. Accordingly, evaluation of their efficacy should involve not only assessment of antibacterial inhibition but also analysis of biofilm viability and the distribution of live and dead bacteria. Multispecies biofilm models provide a more clinically relevant framework by reflecting interbacterial interactions and the ecological complexity of oral biofilms [5, 11, 17].

Based on these considerations, a multispecies cariogenic dental biofilm model incorporating *S. mutans*, *L. casei*, and *S. wiggsiae*, selected due to their complementary roles in cariogenic biofilms, was used. The aim of this study was to comparatively evaluate the antibacterial and biofilm-modulating effects of probiotic- and postbiotic-containing toothpastes formulated for children under *in vitro* conditions.

The null hypothesis of this study was that there would be no significant differences in antibacterial efficacy and biofilm viability among probiotic-, postbiotic-, and fluoride-containing toothpastes.

2. Materials and methods

2.1 Study design and experimental groups

This *in vitro* study evaluated the effects of probiotic- and postbiotic-containing toothpastes on multispecies dental biofilm viability and biomass using a standardized cariogenic biofilm model relevant to pediatric oral health.

Seven experimental groups were included in the study. Six commercially available toothpaste formulated for children were tested, and distilled water was used as the negative control. A fluoride-containing toothpastes was included as the positive control. Details regarding the tested toothpastes, including active ingredients, and manufacturers are presented in Table 1.

All experimental materials were prepared and sterilized at the Department of Pediatric Dentistry Clinic, Faculty of Dentistry, Yeditepe University. Biofilm assays were performed at the Department of Oral Microbiology, Faculty of Dentistry, Istanbul University.

The required sample size was determined using G*Power 3.1 software (Heinrich Heine University Düsseldorf, Düsseldorf, NRW, Germany). The effect size (f) was set at 0.50, based on assumptions consistent with previous *in vitro* studies evaluating antimicrobial efficacy against *S. mutans* [18]. The alpha error probability was set at 0.05 and the statistical power at 0.80. Based on these parameters, the required sample size was calculated as 10 specimens per group. Accordingly, 10 specimens were prepared for each group. Of these, 8 specimens per group were included in the final quantitative biofilm analysis, while the remaining specimens were not included in the quantitative dataset.

2.2 Preparation of bacterial suspensions

S. mutans (ATCC-25175), *L. casei* (ATCC 4646) and *S. wiggsiae* (DSM 22547) were the strains used in this study. These strains were obtained from the bacterial collection of the Faculty of Dentistry, Microbiology Research Laboratory, Istanbul University. *S. wiggsiae* was cultured on Brucella agar (Merck KGaA, DE, Darmstadt, Germany) supplemented with 5% sheep blood, 5 mg/mL hemin, and 5 mg/mL vitamin K. *S. mutans* and *L. casei* were cultured on brain-heart infusion (BHI) agar (Merck) in an anaerobic chamber with a gas mixture of 80% nitrogen (N_2), 10% carbon dioxide (CO_2) and 10% hydrogen (H_2) at 37 °C for 48 h.

Bacterial suspensions were prepared by transferring colonies from incubated plates into BHI broth supplemented with yeast extract, hemin, and vitamin K. Each bacterial strain was individually adjusted to the appropriate turbidity using the McFarland standard (*S. mutans* and *L. casei*: 0.5 McFarland; *S. wiggsiae*: 1 McFarland) prior to dilution. The suspensions were then diluted 100-fold to obtain a final concentration of 1×10^6 CFU/mL. To verify the accuracy of the inoculum size, serial dilutions followed by plate counting were performed. The standardized suspensions were subsequently mixed in equal proportions (1:1:1 ratio) and inoculated simultaneously to establish the multispecies biofilm model. Biofilms were formed in BHI broth supplemented with 5% sucrose under anaerobic conditions at 37 °C. The biofilm model was maintained under static conditions, and the culture medium was not renewed during the 24-hour incubation period.

2.3 Artificial saliva preparation

Artificial saliva was prepared in the laboratory according to the formulation described by of Pratten *et al.* [19], consisting of Lab-lemco powder (1 g/L), yeast extract (2 g/L), proteose peptone (5 g/L), porcine gastric mucus (2.5 g/L), sodium chloride (0.35 g/L), calcium chloride (0.2 g/L) and potassium chloride (0.2 g/L). After autoclaving, 1.25 mL of a 40% urea solution was added, and the pH was adjusted to 7. The solution was subsequently sterilized by filtration through a 0.22-member filter.

2.4 Preparation of toothpastes

Toothpaste slurries were prepared by mixing 2 g of each toothpaste with 5 mL of distilled water to obtain a homogeneous suspension. This ratio was selected to simulate the dilution conditions occurring during toothbrushing in the oral environment. The mixture homogenized using vortex mixer

TABLE 1. Active ingredients, and manufacturer information of the toothpastes used in the study.

Toothpaste Commercial Name	Group Code	Active Ingredients	Manufacturer
Glimo Pi First Teeth Probiotic Natural Kids Toothpaste	P1	Bacillus sp. (probiotic), Xylitol, Propolis extract, Elderberry extract	Haltron Pharmaceuticals Industry and Trade Inc., Turkey
Nordics Kids Toothpaste Strawberry Splash Probiotic	P2	Lactobacillus Ferment (probiotic), Xylitol, Calcium Lactate, Stevia extract	Norte Ltd., Sofia, Bulgaria
Henry Blooms Kids Probiotic Toothpaste	P3	Lactobacillus paracasei GMNL-33 (probiotic), Xylitol, Coconut oil, Aloe vera	Henry Blooms, Australia
Dentiste Kids Orange Flavored Toothpaste +1 Year	PO1	Lactobacillus Ferment Filtrate (postbiotic), Xylitol, Herbal extracts	Siam Cosmeceutical Co., Ltd. Lamlukka, Prathumthan, Thailand
Dentiste Junior Toothpaste +6 Years Mixed Fruit Flavor	PO2	Sodium Fluoride (1000 ppm fluoride), Lactobacillus Ferment Filtrate (postbiotic), Xylitol, Herbal extracts	Siam Cosmeceutical Co., Ltd. Lamlukka, Prathumthan, Thailand
Colgate Big Kids' Smiles Toothpaste +6 Years Mint Toothpaste	PC	Sodium Monofluorophosphate (1450 ppm fluoride), Arginine	Colgate Palmolive, USA

P1, Glimo Pi First Teeth Probiotic Natural Kids Toothpaste; P2, Nordics Kids Toothpaste Strawberry Splash Probiotic; P3, Henry Blooms Kids Probiotic Toothpaste; PO1, Dentiste Kids Orange Flavored Toothpaste +1 Year; PO2, Dentiste Junior Toothpaste +6 Years Mixed Fruit Flavor; PC, Colgate Big Kids' Smiles Toothpaste +6 Years Mint Toothpaste.

to obtain uniform suspensions. All toothpaste slurries were freshly prepared immediately prior to application to ensure consistency across experimental groups.

2.5 Biofilm formation

Sterile 24-well plates (Greiner, Sigma-Aldrich) were used for the *in vitro* biofilm experiment. One sterile hydroxyapatite disc (HAD40, Himed, USA) was placed into each well. For salivary pellicle formation, 500 μL of artificial saliva was applied onto the surface of each disc, followed by incubation at 37 ℃ for 1 hour on a shaker.

After pellicle formation, the discs were gently rinsed with phosphate buffered saline (PBS) and transferred to new wells. Subsequently, 1.6 mL of sucrose-containing BHI Broth and 200 μL of bacterial suspension were added to the discs placed in the new well. All plates were incubated at 37 ℃ under anaerobic conditions for 24 hours. After incubation, the discs were rinsed with 2 mL of PBS and put in new wells.

2.6 Application of toothpastes on biofilm-formed discs

A volume of 500 μL of toothpaste slurry was applied to the biofilm-coated discs in the wells, and the specimens were incubated at 37 ℃ for 2 minutes in an incubator shaker set to 90 rpm. After treatment, the discs were rinsed three times with sterile saline for 10 seconds per rinse.

2.7 Evaluation with confocal laser scanning microscope (CLSM)

Eight hydroxyapatite discs were evaluated for each of the seven experimental groups (n = 8), totaling 56 discs. Each disc was processed individually and analyzed as a separate experimental unit using a confocal laser scanning microscope (CLSM) (Leica CLSM, Leica Microsystems, Heidelberg, BW, Germany). The discs were transferred into sterile 24-well tissue culture plates (Greiner, Sigma-Aldrich, Kremsmünster, OÖ, Austria) and 20 μL of the LIVE/DEAD BacLight Kit (lot number: 1910798, Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA) staining solution was added. Plates containing samples were covered with aluminum foil and kept in a dark environment for 15 minutes, then stored at 4 ℃ until imaging. In a light-free laboratory, the samples were imaged using the CLSM with optical lenses at 10× magnification and at wavelengths of 488 nm and 532 nm. Images were acquired from three distinct regions of each sample, and a series of optical sections was captured to visualize bacterial viability, with live and dead cells appearing in green and red, respectively. The biomass of the multispecies biofilm on the sample surface was measured using the COMSTAT software (version 2.0, Technical University of Denmark, Kongens Lyngby, Denmark) ($\mu m^3/\mu m^2$). A schematic overview of the experimental workflow is shown in Fig. 1.

2.8 Statistical analysis

In this study, statistical analyses were performed using NCSS 2007 statistical software (Number Cruncher Statistical System, Kaysville, UT, USA). Descriptive statistical methods (mean, standard deviation, median and interquartile range) were used to evaluate the data. The normality of the data distribution was assessed using the Shapiro-Wilk test. For variables that did not show a normal distribution, the Kruskal-Wallis test was used for intergroup comparisons, followed by Dunn's multiple comparison test for pairwise comparisons. Statistical significance was set at $p < 0.05$.

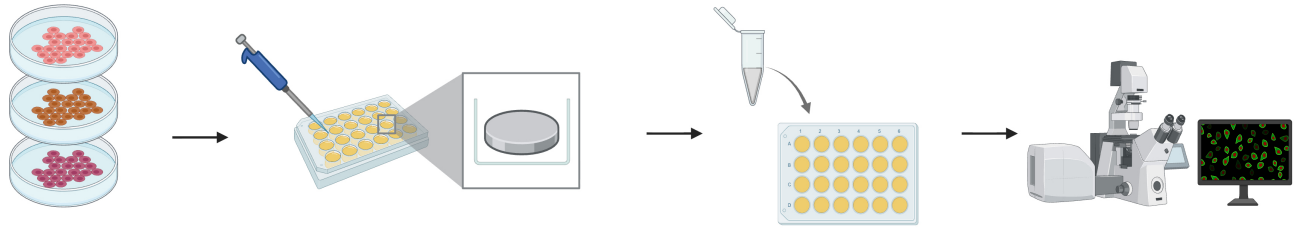

FIGURE 1. Experimental workflow of multispecies biofilm formation and analysis. Multispecies bacterial suspensions consisting of *Streptococcus mutans*, *Lactobacillus casei*, and *Scardovia wiggsiae* were inoculated onto hydroxyapatite (HAP) discs placed in 24-well plates for biofilm formation. Following incubation, established biofilms were treated with toothpaste slurries according to experimental groups. Biofilm viability and biomass were analyzed using confocal laser scanning microscopy (CLSM) and quantified with COMSTAT software. HAP, hydroxyapatite; CLSM, confocal laser scanning microscopy.

3. Results

3.1 Effects of toothpastes on biofilm viability

A CLSM analysis of images of live and dead cells in bacterial biofilms formed on HAP discs was performed. In the live/dead cell-staining procedure, the green dots indicate live cells, the red dots indicate dead cells, and the yellow dots indicate both live and dead cells.

After evaluation of all experimental groups, the highest density of viable bacterial cells was observed in the negative control group. Biofilms exposed to probiotic- and postbiotic-containing toothpastes exhibited a reduced density of viable cells and an increased presence of non-viable bacteria. The most pronounced reduction in biofilm viability was observed in the fluoride-containing toothpaste group (positive control group), which showed the highest proportion of dead bacterial cells among all groups (Fig. 2). Quantitative analysis of biofilm biomass obtained using COMSTAT software is presented in Table 2.

3.2 Quantitative evaluation of live/dead biofilm biomass

Using CLSM and COMSTAT analyses, the biofilm biomass and structural characteristics of multispecies dental biofilms composed of *S. mutans*, *L. casei*, and *S. wiggsiae* formed on HAP discs were quantitatively evaluated. Differences in biofilm parameters were observed among the experimental groups following exposure to the tested toothpastes. The fluoride-containing toothpaste (positive control) demonstrated the greatest reduction in live/dead biomass. Probiotic- and postbiotic-containing toothpastes also induced measurable alterations in biofilm structure and viability compared with the control group.

The live/dead biomass ratios of multispecies biofilms following toothpaste exposure are presented in Table 2. A statistically significant difference was observed among the experimental groups in terms of live/dead biomass ratios ($p < 0.001$). The negative control (NC) group exhibited the highest live/dead biomass ratios, whereas the positive control (PC) group showed the lowest ratios.

Intergroup pairwise comparisons of live/dead biomass ratios are presented in Table 3. The NC group exhibited significantly higher live/dead biomass ratios than all toothpaste groups evaluated in this study ($p < 0.05$). The PC group (1450-ppm fluoride-containing children's toothpaste) demonstrated significantly lower live/dead biomass ratios than the P2 ($p = 0.001$), P3 ($p = 0.012$), PO1 ($p < 0.001$), and PO2 ($p = 0.035$) groups, while no statistically significant difference was observed between the PC group and the P1 group ($p = 0.079$). Furthermore, the PO1 group exhibited significantly higher live/dead biomass ratios than the P1 ($p = 0.019$) and PO2 ($p = 0.025$) groups. No statistically significant differences were observed among the remaining group comparisons ($p > 0.05$).

The distribution of live/dead biomass ratios among the experimental groups is presented in Fig. 3. The live/dead biofilm biomass ratios varied across the groups. The NC group exhibited a wider interquartile range (median: 0.78; IQR (interquartile range): 0.65–1.04), indicating greater variability in biofilm viability, whereas the PC group showed a narrower distribution (median: 0.28; IQR: 0.08–0.58), reflecting more consistent antibiofilm effects.

4. Discussion

This *in vitro* study evaluated the effects of probiotic-, postbiotic-, and fluoride-containing toothpastes on the viability and biomass of multispecies dental biofilms composed of *S. mutans*, *L. casei*, and *S. wiggsiae.* The findings demonstrated that all toothpaste formulations altered biofilm viability and structure compared with the control group, as evidenced by both CLSM and COMSTAT analyses. Among the tested formulations, the group PC exhibited the most pronounced reduction in biofilm biomass and live/dead ratios, while probiotic- and postbiotic-containing toothpastes also induced measurable changes in biofilm characteristics. Collectively, these findings suggest that biologically based approaches may contribute to the modulation of cariogenic multispecies biofilms rather than achieving complete biofilm elimination.

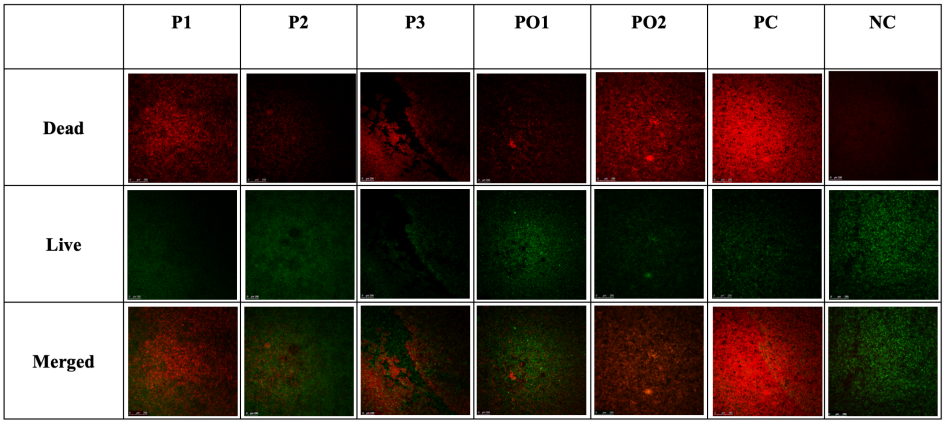

FIGURE 2. CLSM evaluation of live and dead bacterial distribution in multispecies biofilms following toothpaste exposure. Representative confocal laser scanning microscopy (CLSM) images of multispecies biofilms composed of *Streptococcus mutans*, *Lactobacillus casei*, and *Scardovia wiggsiae* formed on hydroxyapatite (HAP) discs after exposure to different toothpaste formulations. The upper row (Dead) shows red fluorescence indicating non-viable bacterial cells; the middle row (Live) shows green fluorescence indicating viable bacterial cells; the lower row (Merged) represents the overlay of live and dead signals. Increased red fluorescence and reduced green fluorescence were observed in the fluoride-containing positive control (PC) group, whereas the negative control (NC) group exhibited predominantly green fluorescence, indicating higher biofilm viability. Scale bar = 250 μm. P1–P3, probiotic-containing toothpastes; PO1–PO2, postbiotic-containing toothpastes; PC, positive control (fluoride-containing toothpaste); NC, negative control.

TABLE 2. Comparison of the ratios of live cell biomass to dead cell biomass after toothpaste application in each group.

Study Groups	Live/Dead Biomass ($\mu m^3/\mu m^2$)	
	Mean ± SD	Median (IQR)
Group P1	0.53 ± 0.38	0.41 (0.29–0.72)^{a,b}
Group P2	0.64 ± 0.21	0.63 (0.52–0.76)^{b,c}
Group P3	0.56 ± 0.28	0.56 (0.28–0.84)^{b,c}
Group PO1	0.67 ± 0.19	0.70 (0.53–0.83)^{c}
Group PO2	0.50 ± 0.25	0.51 (0.28–0.73)^{b,c}
Group PC	0.35 ± 0.28	0.28 (0.08–0.58)^{a}
Group NC	0.87 ± 0.29	0.78 (0.65–1.04)^{d}
*p**	0.0001	

*Data are presented as mean ± standard deviation (SD) and median (interquartile range, IQR). Overall comparisons among groups were performed using the Kruskal-Wallis test; $p < 0.001$. P1, probiotic-containing toothpaste 1; P2, probiotic-containing toothpaste 2; P3, probiotic-containing toothpaste 3; PO1, postbiotic-containing toothpaste 1; PO2, postbiotic-containing toothpaste 2; PC, positive control (fluoride-containing toothpaste); NC, negative control. Different superscript letters (a, b, c, d) indicate statistically significant differences between groups ($p < 0.05$). *Indicates the overall statistical significance obtained from the Kruskal-Wallis test.*

TABLE 3. Intergroup pairwise comparisons of live/dead biomass ratios.

	P1	P2	P3	PO1	PO2	PC
P1	-	-	-	-	-	-
P2	0.071	-	-	-	-	-
P3	0.509	0.440	-	-	-	-
PO1	0.019*	0.542	0.238	-	-	-
PO2	0.837	0.099	0.383	0.025*	-	-
PC	0.079	0.001*	0.012*	0.0001*	0.035*	-
NC	0.0001*	0.008*	0.005*	0.021*	0.0001*	0.0001*

**Dunn's Multiple Comparison Test; significance at $p < 0.05$. P1, probiotic-containing toothpaste 1; P2, probiotic-containing toothpaste 2; P3, probiotic-containing toothpaste 3; PO1, postbiotic-containing toothpaste 1; PO2, postbiotic-containing toothpaste 2; PC, positive control (fluoride-containing toothpaste); NC, negative control.*

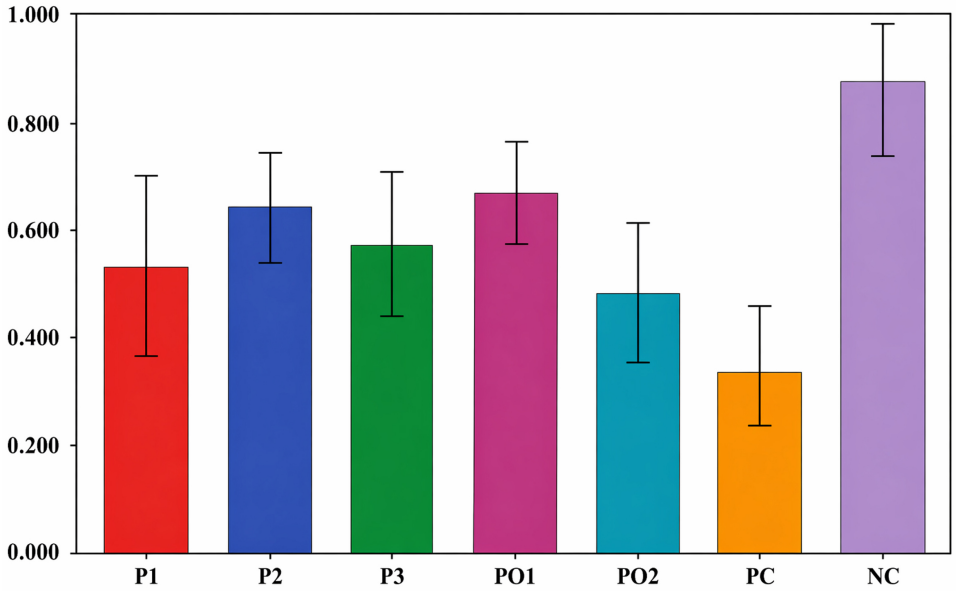

FIGURE 3. Diagram illustrating the ratio of the average biomass of live bacteria to dead bacteria after the toothpaste application. P1, probiotic-containing toothpaste 1; P2, probiotic-containing toothpaste 2; P3, probiotic-containing toothpaste 3; PO1, postbiotic-containing toothpaste 1; PO2, postbiotic-containing toothpaste 2; PC, positive control (fluoride-containing toothpaste); NC, negative control.

Single-species biofilm models, although useful for investigating the behavior of individual bacterial species under controlled conditions, are insufficient to reflect the ecological dynamics and interspecies interactions characteristic of oral biofilms. In contrast, multispecies biofilm models allow the simultaneous evaluation of metabolic interactions, nutrient competition, acid production and tolerance, and stress response mechanisms within a complex microbial community [17, 20]. Previous studies have demonstrated that interspecies interactions can substantially alter biofilm behavior and antimicrobial susceptibility. In particular, differences in acid production and reduced sensitivity to antimicrobial agents have been reported in dual- and multispecies biofilm models compared with single-species systems, highlighting the limitations of simplified models in predicting treatment responses [21, 22]. In the present study, *S. mutans*, *L. casei*, and *S. wiggsiae* were selected to represent cariogenic bacteria with complementary ecological roles in caries development. While *S. mutans* is primarily associated with caries initiation due to its acidogenic potential, aciduric species such as *S. wiggsiae* and *L. casei* contribute to biofilm persistence and progression under low pH conditions [7, 8, 23, 24]. Interactions among these species are therefore critical determinants of biofilm structure, viability, and resistance to antimicrobial interventions. Accordingly, the use of a multispecies biofilm model in the present study enabled a more clinically relevant evaluation of the effects of probiotic- and postbiotic-containing toothpastes by capturing treatment-induced changes within a complex biofilm ecosystem rather than isolated bacterial responses.

A wide range of microscopic techniques is available for the evaluation of *in vitro* oral biofilms. Among these, CLSM, combined with COMSTAT analysis, enables three-dimensional assessment of biofilm structure and bacterial viability and is widely used in dental biofilm research [25]. Previous studies have demonstrated that CLSM-COMSTAT analysis allows sensitive detection of treatment-induced changes in biofilm biomass and live/dead bacterial distribution in biofilms formed on saliva-coated HAP discs [26–28]. In the present study, multispecies biofilms composed of *S. mutans*, *L. casei*, and *S. wiggsiae* were evaluated using CLSM and COMSTAT following exposure to toothpaste slurries. Analysis of live and dead bacterial biomass enabled assessment of treatment-induced modulation of biofilm viability and structural organization rather than complete biofilm eradication. This approach is particularly relevant in multispecies biofilm models, where interbacterial interactions and ecological adaptation mechanisms can influence antimicrobial responses and lead to shifts in viability distribution instead of total biofilm removal [20].

Within this framework, probiotic- and postbiotic-containing toothpastes significantly altered live/dead biomass distribution compared with the negative control group, indicating that these formulations exert measurable effects on multispecies biofilm dynamics. However, their effects were less pronounced than those of the fluoride-containing toothpaste, suggesting a modulatory rather than strong bactericidal profile. The intermediate positioning of these groups between the negative and positive controls supports the concept that probiotic and postbiotic strategies regulate biofilm viability balance instead of non-selectively eliminating bacterial communities. Mechanistically, probiotic and postbiotic strategies are thought to suppress pathogenic dominance while supporting microbial balance within the oral biofilm. Postbiotics may influence biofilm formation and metabolism through biologically active compounds such as organic acids, short-chain fatty acids, bacteriocins, and cell wall components [29, 30]. Probiotic formulations have been reported to affect biofilm behavior via competitive exclusion, inhibition of co-aggregation, and modulation of quorum sensing pathways [31, 32]. In line

with these mechanisms, the changes in live/dead biomass ratios observed in the present study can be interpreted as indicators of ecological modulation within complex biofilm communities rather than simple bactericidal effects.

Previous studies support the ability of probiotics and postbiotics to modulate cariogenic biofilms rather than eliminate them entirely. Probiotic use has been associated with reduced *S. mutans* levels in dental plaque and saliva and improved oral microbial balance [33, 34]. In multispecies biofilm models, viable probiotics have demonstrated the capacity to reduce cariogenic bacterial counts and enamel mineral loss, whereas cell-free supernatants showed more limited effects [35]. Similarly, postbiotics derived from lactic acid bacteria have been reported to suppress pathogenic biofilms by interfering with adhesion, aggregation, virulence factor expression, and quorum sensing mechanisms [36, 37]. Studies evaluating multispecies biofilms formed with *S. mutans* and clinical plaque samples have shown that probiotic lactobacilli reduce the proportion of cariogenic bacteria without complete biofilm removal, supporting an ecologically based preventive strategy [38]. This perspective is consistent with the Ecological Plaque Hypothesis, which emphasizes microbial dysbiosis rather than the presence of a single pathogen as the primary driver of dental caries [11].

Pairwise comparisons further revealed a graded response across formulations, suggesting that different toothpaste compositions may shift the ecological balance of multispecies biofilms to varying extents. In a complex biofilm environment, interbacterial interactions, metabolic cooperation, and stress adaptation mechanisms may modulate how formulations affect overall viability distribution. Therefore, the observed differences likely reflect not only direct antimicrobial activity but also ecological reshaping within the biofilm structure.

The PC group exhibited the lowest live/dead biomass ratio, which may be attributed to its higher fluoride concentration (1450 ppm) and well-documented non-selective antibacterial properties. In contrast, the more moderate reduction observed in the PO2 group (1000 ppm fluoride) suggests a potential dose-dependent relationship between fluoride concentration and biofilm viability suppression. Importantly, the absence of a significant difference between the PC group and the probiotic-containing toothpaste P1 highlights heterogeneity among probiotic formulations. Variations in probiotic strain viability, concentration, formulation matrix, and additional bioactive components may influence how effectively ecological modulation occurs in a multispecies biofilm setting.

From a pediatric perspective, long-term daily exposure to highly potent antibacterial agents may alter the developing oral microbiome. Accordingly, probiotic- and postbiotic-containing toothpastes may represent a complementary strategy that supports microbial homeostasis while maintaining antibacterial efficacy. Nevertheless, given the inherent limitations of *in vitro* biofilm models, further clinical studies are required to determine whether these ecological effects translate into sustained caries-preventive outcomes *in vivo*.

Despite the strengths of this study, several limitations should be considered. This study was conducted using an *in vitro* multispecies oral biofilm model, which, although providing a controlled environment, cannot fully reflect the complex biological and environmental conditions of the oral cavity. In addition, since the tested toothpastes differed in terms of composition and target age groups, the study was not designed to directly compare probiotic and postbiotic approaches.

In this context, although the fluoride-containing toothpaste demonstrated the most pronounced antibiofilm effect, the magnitude of the differences observed between formulations should be interpreted with caution. While fluoride-based products provide strong and immediate antibacterial activity, probiotic- and postbiotic-containing formulations may offer additional long-term benefits by promoting a balanced oral microbiome rather than inducing non-selective bacterial reduction. This distinction may be particularly relevant in pediatric patients and individuals with orthodontic appliances, where maintaining microbial homeostasis is essential for preventing dysbiosis-associated oral diseases. Therefore, probiotic and postbiotic toothpastes should be considered as complementary approaches to conventional fluoride therapies rather than direct alternatives. Accordingly, the null hypothesis of the present study was rejected.

5. Conclusions

The findings of this study revealed that probiotic and postbiotic formulations may contribute to reducing pathogenic bacterial activity and biofilm development in children's oral environments under simulated *in vitro* experimental conditions.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

LBM, GDB and NT—designed the research study and wrote the manuscript. LBM and NT—performed the experiments. MYÜ—contributed to the interpretation of the data and critically revised the manuscript. Data analysis was conducted by all authors. All authors contributed to editorial revisions and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The authors declare that there are no financial or commercial conflicts of interest related to this study. The products used in this research were commercially available and were not provided or funded by any manufacturer.

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