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1 message

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COVER LETTER

Title:

Comparative Evaluation of Antimicrobial Toothpastes on Periodontal Bacteria in Orthodontic Patients: A Randomized Controlled Study

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Comparative Evaluation of Antimicrobial Toothpastes on Periodontal Bacteria in Orthodontic Patients: A Randomized Controlled Study

ABSTRACT

Objective: To evaluate the effects of *L. paracasei* probiotic toothpaste, CPC toothpaste, and amyloglucosidase-glucose oxidase toothpaste on the levels of *P. gingivalis*, *A. actinomycetemcomitans*, and plaque index in individuals undergoing fixed orthodontic treatment. **Materials and Methods:** A double-blind randomized controlled clinical trial was conducted using purposive sampling. Participants were randomly assigned to use one of the toothpaste types. Saliva samples were collected at baseline and one month after using the toothpaste. Bacterial levels were quantified using quantitative PCR, and plaque accumulation was assessed using the Orthodontic Plaque Index. **Results:** All groups showed a reduction of *P. gingivalis* and *A. actinomycetemcomitans* following the intervention; however, no significant changes were observed in the plaque index. Statistical analysis using Two-Way Repeated Measures ANOVA with sphericity assumed revealed no significant differences between the groups ($p<0,05$). **Conclusion:** Toothpastes containing *L. paracasei*, CPC, and amyloglucosidase-glucose oxidase enzymes show potential for reducing periodontal pathogens, suggesting a preventive benefit against periodontal complications in patients with fixed orthodontic appliances.

Keywords:

Aggregatibacter actinomycetemcomitans, amyloglucosidase-glucoseoxidase enzyme, antibacterial effect, cetylpyridinium chloride, fixed orthodontic appliances, *L. paracasei*, plaque index, *Porphyromonas gingivalis*

INTRODUCTION

Malocclusion is a common condition with potential impacts on patients' quality of life, psychosocial well-being, and self-confidence.[1] In Indonesia, approximately 80% of the population experiences some form of malocclusion, making it a significant public oral health issue.[2] The increasing public awareness of dental and facial aesthetics has led to a rising demand for orthodontic treatment.[3] Recent studies indicate a rising prevalence of adult patients seeking orthodontic care, with estimates suggesting that adults now represent 20–30% of all orthodontic patients in many countries.[4]

Fixed orthodontic appliances, although effective in correcting malocclusion, create plaque-retentive areas that complicate oral hygiene. This can result in the accumulation of dental biofilm, which shifts the oral microbial balance and promotes colonization by pathogenic species.[5] Clinical signs of periodontal changes, including increased gingival inflammation, bleeding on probing, and periodontal pocketing, are often observed in patients wearing fixed appliances.[6,7]

Two major periodontal pathogens of concern in orthodontic patients are *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*.[8] These organisms are capable of adhering to both tooth surfaces and oral mucosa, contributing to periodontal tissue destruction.[9,10] Conventional plaque control methods such as mechanical brushing may not be sufficient, highlighting the need for adjunctive antimicrobial strategies.[5,11]

Various active agents in toothpaste, such as *Lactobacillus paracasei* probiotics, cetylpyridinium chloride (CPC), and amyloglucosidase-glucose oxidase enzymes, have shown promising antimicrobial activity in previous studies.[12–14] However, most studies have focused on their effects against cariogenic bacteria rather than periodontal pathogens. Therefore, further investigation is warranted to explore the efficacy of these formulations in reducing *P.*

gingivalis, *A. actinomycetemcomitans*, and plaque index in patients undergoing fixed orthodontic treatment.

MATERIALS AND METHODS

This randomized double-blind clinical trial was conducted on orthodontic patients with fixed appliances. Ethical approval for this study (876A/S2/KEPK/FKG/11/2024) was provided by the Research Ethics Committee of the Faculty of Dentistry, Universitas Trisakti, on 11 November 2024. After informed consent was obtained, subjects were screened based on inclusion criteria through anamnesis, intraoral clinical examination, and assessment using the Index of Orthodontic Treatment Need (IOTN) and Gingival Index (GI). Participants with Dental Health Component of IOTN scores ≤3 and GI scores between 0–2.0 were purposively selected. The exclusion criteria in this study were established to minimize potential confounding factors that could influence the outcomes. Participants were excluded if they had a history of probiotic consumption within the preceding three months or were undergoing pharmacological treatment that could interfere with salivary secretion. Individuals receiving systemic or topical antimicrobial therapy were also not considered eligible. In addition, subjects who reported habitual smoking or presented with systemic diseases were excluded from participation, also patients who had undergone professional oral hygiene procedures during the observation period were not included in the study. A total of 32 participants were initially assessed in this study, with 24 participants meeting the inclusion criteria, 16.67% were male and 83.33% were female, with ages ranging from 18 to 23 years. The participants were then randomly assigned to one of three intervention groups: (1) probiotic toothpaste containing *Lactobacillus paracasei*, (2) toothpaste with cetylpyridinium chloride (CPC), or (3) toothpaste with amyloglucosidase-glucose oxidase enzymes. All toothpaste tubes were anonymized, and each participant was given an orthodontic toothbrush and instructed to brush twice daily using the Bass technique for one month.

Saliva samples were collected at baseline (T0) and after one month (T1). Saliva offers a non-invasive, rapid, and reproducible sampling method that reflects the overall microbial

load and oral health status, including the presence of periodontal pathogens such as *P. gingivalis* and *A. actinomycetemcomitans*. Participants were instructed to avoid food, drink, and physical activity one hour before collection. Stimulated saliva was collected via paraffin wax chewing and spitting into sterile tubes. Samples were stored at 2–8 °C temporarily and later frozen at –20 °C to –80 °C. saliva offers a non-invasive, rapid, and reproducible sampling method that reflects the overall microbial load and oral health status, including the presence of periodontal pathogens such as *P. gingivalis* and *A. actinomycetemcomitans*.

DNA extraction from the saliva was performed using heat-shock and centrifugation protocols. Quantification of *P. gingivalis* and *A. actinomycetemcomitans* was conducted using quantitative real-time PCR (qPCR). A total of 10 µL of DNA extraction from saliva was mixed with 90 µL of nuclear free water (NFW). These two mixtures were diluted seven times and produced a concentration of 10^0 µL or equivalent to 1 µL. Homogenization was carried out using a vortex. Every 2 µL of the dilution results were put into a 96-well plate (Nest Biotech, China). Then, mix 10 µL of SYBR green (Thermo Fisher Scientific, Massachusetts, USA), 6 µL of NFW, 1 µL each of the forward and reverse primers (Table 1) into the PCR mix and put into the qPCR plate wells that already contained the previous dilution. The qPCR plate wells were inserted into the qPCR machine at 95^{o}C for 10 minutes for one initiation denaturation cycle, followed by 40 cycles of denaturation at 95^{o}C for 15 seconds per cycle. The expression results of the samples using qPCR were then quantified relative DNA gene expression by calculating using the formula $2^{-\Delta\Delta Ct}$. Plaque levels were assessed using the Orthodontic Plaque Index (OPI) at both T0 and T1.

The normality test on the data uses the Shapiro-Wilk test ($n \leq 50$), if the p-value > 0.05 then the data is normally distributed. The homogeneity test uses Mauchly's Test of Sphericity. Next, a multivariate Two-Way Repeated Measures ANOVA (Analysis of Variance) test will

be conducted with a p-value < 0.05 to see any significant differences and interactions between variables.

RESULTS

A total of 32 individuals were examined in this study, of whom 24 fulfilled the inclusion criteria. With respect to gender, 16.67% were men and 83.33% were women, and the overall age range was 18 to 23 years. The initial assessment consisted of a clinical examination that included evaluation of malocclusion type, jaw relationship, Index of Orthodontic Treatment Need (IOTN), Gingival Index (GI), and Orthodontic Plaque Index (OPI). The most prevalent malocclusion type was Class I, observed in 54.17% of the subjects, while the most frequent jaw relationship was orthognathic, found in 70.83% of participants. The IOTN examination revealed that 41.67% of the subjects were classified in grade 1. All participants (100%) demonstrated mild gingivitis based on the GI and OPI score of 4, corresponding to the poor oral hygiene category.

Based on the type of toothpaste, the *P. gingivalis* count showed a change in $2^{-\Delta\Delta Ct}$ values before (T0) and one month after (T1) treatment. The *L. paracasei* probiotic toothpaste group showed an average decrease of 5.59×10^6 before treatment to 5.03×10^3 after one month using the toothpastes. The CPC toothpaste group showed an average decrease from 3.11×10^3 to 4.79×10^2. The amyloglucosidase-glucoseoxidase enzyme toothpaste group showed a greater average decrease from 1.19×10^7 to 1.92×10^3. The *A. actinomycetemcomitans* count also showed a change in $2^{-\Delta\Delta Ct}$ values before (T0) and one month after (T1) treatment in all three toothpaste groups. The group using L. paracasei probiotic toothpaste showed an average decrease of 9.24 before treatment to 1.31 after treatment. The CPC toothpaste group saw an average decrease from 2.89 to 0.65. The amyloglucosidase-glucoseoxidase enzyme toothpaste group also showed a greater average decrease from 18.62 to 2.82. (Table 2)

The analysis then continued with the evaluation of the mean natural logarithm (NL) values of *P. gingivalis* at baseline (T0) and one month after treatment (T1) across the three toothpaste groups, as presented in Table 3. At baseline, the highest mean NL value was observed in the *L. paracasei* probiotic toothpaste group (7.35 ± 6.63), followed by the amyloglucosidase–glucose oxidase enzyme toothpaste group (6.84 ± 6.42), and the CPC toothpaste group (4.81 ± 2.81). The overall mean NL value of the three groups prior to treatment was 6.33 ± 5.44. After one month of treatment, a reduction in the mean NL values was observed in all groups. The *L. paracasei* probiotic toothpaste group demonstrated a mean NL value of 1.99 ± 4.37, the amyloglucosidase–glucose oxidase enzyme toothpaste group recorded 2.48 ± 4.10, and the CPC toothpaste group showed 2.93 ± 2.98. The combined mean NL value across all groups after treatment was 2.47 ± 3.71. The control of CT values obtained

from the laboratory procedure were 36.25 for *P. gingivalis* ATCC 33277 and 31.48 for *A. Actinomycetemcomitans* ATCC 29522.

Therefore the analysis of the mean values of *A. actinomycetemcomitans* was conducted at baseline (T0) and one month after treatment (T1) across the three toothpaste groups (Table 3). At baseline, the *L. paracasei* probiotic toothpaste group demonstrated a mean value of 9.24 ± 17.79, the CPC toothpaste group recorded 2.89 ± 3.15, and the amyloglucosidase–glucose oxidase enzyme toothpaste group demonstrated the highest value at 18.62 ± 27.62. The overall mean value of the three groups prior to treatment was 10.25 ± 19.37. Following one month of treatment, a reduction in mean values was observed in all groups. The *L. paracasei* probiotic toothpaste group exhibited a mean value of 1.31 ± 2.90, the CPC toothpaste group recorded 0.65 ± 0.89, and the amyloglucosidase–glucose oxidase enzyme toothpaste group demonstrated 2.82 ± 3.26. The combined mean value across all groups after treatment was 1.59 ± 2.63. (Table 3)

The average NL values for the *P. gingivalis* groups and mean values for the *A. actinomycetemcomitans* groups were then tested using Mauchly's Test of Sphericity. The Mauchly's Test yielded a value of 1, indicating that the requirement for homogeneity of covariance for the Two-Way Repeated Measures ANOVA were fully met for those two groups. Overall, there was a significant difference between the *P. gingivalis* groups before (T0) and one month after (T1) treatment. This is evident in the average NL T0 value of *P. gingivalis* of 6.33 ± 5.44, which decreased to 2.47 ± 3.71 at T1. The results of the Assumed Sphericity test for treatment time (Table IV) showed a p-value of 0.021 ($p < 0.05$), which means there was a significant difference between the *A. actinomycetemcomitans* groups before (T0) and one month after (T1) treatment. This can be seen in the average T0 value of *A. actinomycetemcomitans* of 10.25 ± 19.37, which decreased in the average T1 value to 1.59 ± 2.63. To assess the differences among the three toothpaste groups, the Assumed Sphericity test was applied to evaluate the interaction between time and treatment group (Table 4). The analysis yielded a p-value of 0.367 ($p > 0.05$), indicating no statistically significant difference. A decrease in the mean value of *P. gingivalis* was observed from baseline (T0) to one month after treatment (T1) across all three toothpaste groups, namely *L. paracasei* probiotic toothpaste, CPC toothpaste, and amyloglucosidase–glucose oxidase enzyme toothpaste. Similarly, for *A. actinomycetemcomitans*, the assumed sphericity test produced a p-value of 0.298 ($p > 0.05$), demonstrating no significant difference between the three groups. Although reductions in bacterial counts were evident in each group, the extent of decrease did not differ

significantly, suggesting that all three toothpastes produced relatively comparable outcomes in reducing *A. actinomycetemcomitans.*

The results of the Orthodontic Plaque Index (OPI) assessment. At baseline (T0), the mean OPI score in all three toothpaste groups; *L. paracasei* probiotic toothpaste, CPC toothpaste, and amyloglucosidase–glucose oxidase enzyme toothpaste was 4. Similarly, at one month after treatment (T1), the mean OPI score remained unchanged at 4 across all groups.

DISCUSSION

Patients undergoing treatment with fixed orthodontic appliances frequently encounter difficulties in maintaining optimal oral hygiene, as the components of the appliances may hinder effective cleaning. Consequently, these patients are at increased risk of periodontal tissue damage due to plaque accumulation and bacterial colonization.[7] The primary determinant of oral health maintenance is effective plaque control, which includes toothbrushing, interdental cleaning, and the use of mouth rinses.[5,11] Beyond mechanical methods of plaque removal, the selection of toothpaste also plays an essential role in plaque control, aiming to reduce bacterial load within the oral cavity.[16]

Adolescents are an appropriate population for studying periodontal pathogens such as *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* because they commonly undergo fixed orthodontic treatment, which promotes plaque retention and bacterial colonization due to appliance components that hinder cleaning.[8] Poor oral hygiene compliance in this age group further facilitates the proliferation of pathogenic bacteria associated with early periodontal changes. Studies have reported that *A. actinomycetemcomitans* and *P. gingivalis* are frequently detected in adolescents with gingival inflammation or early attachment loss during orthodontic treatment.[17] The prevalence of aggressive or early-onset periodontitis linked to these pathogens among adolescents ranges between 0.3% and 5.9%, emphasizing their importance as a high-risk group for periodontal research.[18]

In this study, saliva was employed as the diagnostic medium owing to its ease, rapidity, and non-invasive nature of collection. Saliva provides valuable insight into the oral environment, including bacterial load and the severity of periodontal disease.[19] Stimulated saliva was chosen because the mechanical action of chewing paraffin wax facilitates the release of bacteria from the gingival sulcus, thereby enhancing the detection of periodontal pathogens.[20] However, while gingival crevicular fluid (GCF) offers higher site specificity for

sampling bacteria and mediators directly from the periodontal pocket, it has drawbacks. GCF collection is technically demanding, requires multiple site-specific samples, prone to contamination with saliva, blood or plaque, and often involves low fluid volume and extensive laboratory processing.[21] Consequently, although GCF may provide more direct information about local periodontal microbiology, for larger scale screening or monitoring purposes saliva remains a more practical and efficient alternative.[21,22]

DNA based detection methods, such as quantitative PCR, are widely used to estimate bacterial load because they offer high sensitivity, specificity, and the ability to identify target species even at low concentrations.[23] Although these techniques cannot distinguish between live and dead bacteria, they provide a reliable measure of total bacterial presence and are less affected by sample handling or bacterial viability compared to culture-based methods.[24] Additionally, many oral pathogens, including *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, are fastidious and difficult to culture, making DNA quantification a practical and efficient alternative for evaluating microbial changes in clinical studies.[25]

Toothpaste is available in several forms, such as paste, gel, powder, and liquid. It generally contains two types of ingredients like non-active and active components. Non-active ingredients do not have therapeutic effects but determine the toothpaste's physical properties, including texture, taste, consistency, and appearance, and usually consist of water, abrasives, humectants, binders, flavours, surfactants, preservatives, and colorants.[26,27] Active ingredients, on the other hand, provide therapeutic benefits such as preventing cavities, reducing plaque, controlling sensitivity, eliminating bad breath, and offering antimicrobial effects. These include enzymes, cetylpyridinium chloride, and probiotics.[26]

The findings demonstrated significant reductions in *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* counts following the use of *Lactobacillus paracasei* probiotic toothpaste, cetylpyridinium chloride (CPC) toothpaste,

and amyloglucosidase–glucoseoxidase enzyme toothpaste among patients with fixed orthodontic appliances. This suggests that all three toothpaste formulations exhibit antibacterial effects.[12–14] However, no statistically significant differences were observed in the degree of bacterial reduction among the three groups, which may be attributed to the distinct mechanisms of action of the active ingredients in each toothpaste in inhibiting bacterial growth.

Probiotics are defined as microorganisms that confer health benefits to the host when consumed in adequate amounts. Over the past decade, a growing body of research has highlighted their therapeutic and preventive potential in maintaining oral health. Probiotics are known to modulate both specific and nonspecific immune responses, enhance epithelial barrier function, produce antimicrobial substances, and inhibit the adhesion of pathogenic bacteria within the oral cavity.[28] Among the antimicrobial substances produced by probiotics are bacteriocins and organic acids. Organic acids, particularly acetic acid and lactic acid, play a central role in the inhibitory activity of probiotics against pathogenic species. These acids are able to penetrate bacterial cell membranes, thereby acidifying the intracellular environment, which ultimately leads to bacterial death, especially in Gram-negative organisms.[29]

Chuang et al. reported that oral administration of *Lactobacillus paracasei* GMNL-33 exhibited anticariogenic properties by significantly reducing *Streptococcus mutans* levels in the oral cavity.[30] Similarly, Lee et al. demonstrated in a clinical study that *L. paracasei* GMNL-143–based probiotic toothpaste possesses the ability to co-aggregate with oral pathogens and inhibit their adhesion to gingival tissues.[31] The antibacterial effect of *L. paracasei* is more pronounced under acidic conditions compared with neutral pH environments. This enhanced activity in acidic conditions occurs because peptides are attracted to the phosphate groups of lipopolysaccharide (LPS) molecules, initiating pore formation in the bacterial membrane. Such changes in membrane permeability lead to structural disruption and compromise membrane integrity, ultimately resulting in bacterial cell lysis.[32] These findings are consistent with the

present study, in which *L. paracasei*–containing probiotic toothpaste was shown to effectively reduce bacterial counts in the oral cavity.

Cetylpyridinium chloride (CPC), another active ingredient found in certain toothpaste formulations, is a quaternary ammonium compound with well-established antimicrobial properties. Following use, CPC remains distributed within the oral cavity due to its surfactant chains and cationic charges, which enable sustained absorption onto oral surfaces.[33,34] Structurally, CPC contains hydrophilic and hydrophobic groups. The positively charged hydrophilic groups promote electrostatic binding to the negatively charged surfaces of pathogenic bacteria, while the hydrophobic groups interact with bacterial membranes, facilitating integration into the cytoplasmic membrane. These dual interactions lead to disruption of membrane integrity, impairment of cellular metabolism, cytoplasmic leakage, and eventual bacterial death. In addition, CPC reduces microbial adhesion to oral surfaces, thereby limiting colonization.[33] These mechanisms are consistent with the findings of Vasconcelos et al., who demonstrated that CPC-containing toothpaste significantly reduced bacterial counts in the oral cavity through decreased plaque accumulation and gingival inflammation.[13]

Toothpaste formulations containing the enzymes amyloglucosidase and glucose oxidase are reported to exert antimicrobial effects. The amyloglucosidase enzyme inhibits bacterial proliferation by converting D-glucose into D-glucono-1,5-lactone, thereby reducing the availability of bacterial nutrients in the oral cavity. Meanwhile, glucose oxidase activates the salivary immune defense system, specifically the lactoperoxidase (LPO) pathway, by generating hydrogen peroxide. This hydrogen peroxide interacts with catalase to produce oxygen, reducing the prevalence of anaerobic bacteria. Furthermore, hydrogen peroxide activates the LPO system to generate hypothiocyanite, a compound with antibacterial activity against *P. gingivalis*.[35,36] The findings of this study indicate that toothpaste containing amyloglucosidase and glucose oxidase produced greater reductions in both *P. gingivalis* and *A.*

actinomycetemcomitans compared to the other tested toothpastes. This outcome is consistent with the choice of saliva as a diagnostic tool, as the enzymatic mechanisms are directly linked to salivary immune activity.

As a member of the "red complex," *P. gingivalis* exhibits strong virulence through its capacity to aggregate with other bacterial species, facilitating colonization during later stages of biofilm development and rendering it difficult to eliminate.[9] Likewise, *A. actinomycetemcomitans* produces a wide range of virulence factors to ensure survival within the oral cavity.[37] Both species contribute to robust biofilm formation, aided by antimicrobial-resistant fimbriae and extracellular polysaccharides that hinder immune cell penetration and phagocytosis. These properties allow both pathogens to induce periodontal tissue damage.[32] The present study demonstrates a reduction in the levels of *P. gingivalis* and *A. actinomycetemcomitans*, which may help mitigate the risk of periodontal complications in patients with fixed orthodontic appliances.

The bacterial increase observed in patients with fixed appliances is attributable to the additional niches created by the orthodontic elements. Clinically, the number of oral bacteria has been shown to triple within the first six months following appliance placement.[38] Furthermore, plaque control becomes increasingly difficult in cases of dental misalignment. In this study, no significant changes were observed in plaque index scores before and after the use of probiotic *L. paracasei*, CPC, or amyloglucosidase-glucose oxidase toothpastes. This finding reflects the persistent cycle of plaque formation, as bacterial communities consistently recolonize tooth surfaces. Plaque development begins with pellicle formation initiated by *Streptococcus sanguinis*, followed by the coaggregation of pathogenic species such as *P. gingivalis*, *A. actinomycetemcomitans*, *Fusobacterium nucleatum*, *Treponema denticola*, and *Prevotella intermedia*.[39,40]

Mechanical plaque removal through toothbrushing eliminates only part of the biofilm, as microbial colonization can lead to dysbiosis. *P. gingivalis* plays a central role in this process, functioning as a "keystone pathogen" that manipulates host immune responses and disrupts homeostasis within the oral microbiome. Even at low concentrations, *P. gingivalis* can interact with other microorganisms to promote colonization.[41,42] Consequently, reductions in bacterial counts observed in this study could occur despite relatively unchanged plaque index values. This is explained by the complex biofilm composition of dental plaque, which consists not only of microbial cells but also of extracellular polysaccharides, proteins, and structural molecules that stabilize the biofilm matrix.[40,43]

Additionally, the design and placement of orthodontic appliances contribute significantly to bacterial accumulation and plaque formation. Archwire ligatures serve as additional sites for bacterial colonization, and brackets positioned near the cervical margin can increase the risk of gingivitis.[44,45] The bracket material itself also plays a role: in this study, stainless steel appliances were used, which exhibit higher surface tension and are therefore more prone to plaque retention.[43]

Plaque retention varies among individuals due to differences in plaque formation patterns, oral hygiene practices, and dietary habits.[46] The effectiveness of toothbrushing as a plaque control method is highly dependent on patient compliance, as brushing is a complex and technique-sensitive process. Short-term use of toothpaste has been shown to exert only minimal influence on mechanical plaque removal.[47] Brushing technique plays a critical role in maintaining oral health, particularly for patients with fixed orthodontic appliances, who often experience challenges in adequately cleaning around appliance components. A common error is positioning the toothbrush too coronally, which results in neglect of the cervical region of the teeth and consequently increases plaque accumulation, predisposing patients to gingivitis.[34]

Plaque index was chosen instead of pocket depth or bleeding index because the presence of orthodontic brackets can make periodontal probing difficult and lead to measurement bias. The brackets and archwires hinder probe access and compromise the accuracy of assessing pocket depth and bleeding on probing.[48] Therefore, the plaque index provides a more practical and reliable parameter for evaluating oral hygiene during orthodontic treatment.[49] In addition, the plaque index reflects supragingival plaque accumulation, which is particularly relevant for orthodontic patients who are more prone to plaque retention due to appliance design.[49,50]

Toothbrush selection is also an important factor. The use of orthodontic toothbrushes characterized by a concave bristle arrangement and smaller brush head has been recommended, as these features allow for better adaptation to tooth surfaces and enhance cleaning efficacy around brackets, archwires, and interdental areas.[51] In addition, electric toothbrushes may serve as an effective alternative, as their vibratory action facilitates the removal of both supragingival and subgingival plaque. Professional dental cleaning at each follow-up appointment is likewise essential for patients undergoing fixed orthodontic treatment to further support oral hygiene maintenance.[38]

CONCLUSIONS

The use of probiotic toothpaste containing *Lactobacillus paracasei*, CPC toothpaste, and enzymatic toothpaste containing amyloglucosidase-glucose oxidase was found to reduce the levels of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* but had no effect on the plaque index in patients with fixed orthodontic appliances. There was no significant difference in the reduction of these bacteria among the three types of toothpaste. Therefore, it can be concluded that all three formulations have similar potential in preventing plaque formation and periodontal disease in patients undergoing fixed orthodontic treatment. Further research is expected to include other bacteria than *P. gingivalis* and *A. actinomycetemcomitans* that cause periodontal disease and also longer periods of toothpaste use to provide more comprehensive results.

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AUTHOR CONTRIBUTIONS

JK contributed to conceptualization, investigation, data curation, validation, manuscript review and editing; M contributed to investigation, data curation, original draft preparation; HW contributed to conceptualization, methodology, and validation; BK contributed to validation, manuscript review and editing. All authors critically reviewed and refined the final version of the manuscript. The authors have thoroughly read and granted their approval for its final submission.

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Table 1. Primers of *P. gingivalis* and *A. actinomycetemcomitans* used in qPCR[15]

Primer	Sequence (5'-3')
P. gingivalis Forward	TGC AAC TTG CCT TAC AGA GGG
P. gingivalis Reverse	ACT CGT ATC GCC CGT TAT TC
A. actinomycetemcomitans Forward	CTT ACC TAC TCT TGA CAT CCG AA
A. actinomycetemcomitans Reverse	ATG CAG GAC CTG TCT CAA AGC

Table 2. Minimum, maximum, and average Ct values of *P. gingivalis* and *A. actinomycetemcomitans* bacteria before (T0) and one month after (T1) treatment based on the type of toothpaste group ($2^{-\Delta\Delta Ct}$)

Toothpaste groups	Treatment Time	*P. gingivalis*			*A. actinomycetemcomitans*		
		Minimum Value	Maximum Value	Average Value	Minimum Range	Maximum Range	Average Value
L. paracasei probiotic	T0	2.22	4.22×10^7	5.59×10^6	1.54	53.10	9.24
	T1	0.28	3.89×10^4	5.03×10^3	0.07	8.45	1.31
CPC	T0	6.82	2.11×10^4	3.11×10^3	0.84	9.88	2.89
	T1	1.26	2.56×10^6	4.79×10^2	0.02	2.55	0.65
Amyloglucosidase-glucoseoxidase enzyme	T0	1.33	9.53×10^7	1.19×10^7	2.05	83.34	18.62
	T1	0.43	1.22×10^4	1.92×10^3	0.33	9.49	2.82

Table 3. Analysis of the normal logarithm (LN) values of the average *P. gingivalis* and mean values of *A. actinomycetemcomitans* mean values before (T0) and one month after (T1) treatment in the three toothpaste groups

Toothpaste groups	N	Natural logarithm (NL) values of *P. gingivalis*		Mean values of *A. actinomycetemcomitans*	
		T0	T1	T0	T1
L. paracasei probiotic	8	7.35 ± 6.63	1.99 ± 4.37	9.24 ± 17.79	1.31 ± 2.90
CPC	8	4.81 ± 2.81	2.93 ± 2.98	2.89 ± 3.15	0.65 ± 0.89
Amyloglucosidase-glucoseoxidase enzyme	8	6.84 ± 6.42	2.48 ± 4.10	18.62 ± 27.62	2.82 ± 3.26
Total	24	6.33 ± 5.44	2.47 ± 3.71	10.25 ± 19.37	1.59 ± 2.63

Table 4. Results of the Two Way Repeated ANOVA test with Sphericity Assumed on *P. gingivalis* and *A. actinomycetemcomitans* before (T0) and one month after (T1) treatment in the three toothpaste groups

Assumed Sphericity Test Variable	*P. gingivalis*		*A. actinomycetemcomitans*	
	Mean square	p-value	Mean square	p-value
Treatment time	1.79×10^2	<0.05	8.99×10^2	<0.05
Treatment time*treatment group	12.79	0.367	1.85×10^2	0.298

2. Bukti konfirmasi submit revisi, respon kepada reviewer, dan artikel yang diresubmit

20 November 2025

UNIVERSITAS TRISAKTI

Joko Kusnoto <joko.k@trisakti.ac.id>

jos Manuscript Revision Completed Acknowledgement letter: jos_171_25

1 message

Journal of Orthodontic Science <editors@jorthodsci.org> Thu, Nov 20, 2025 at 12:49 AM
To: joko.k@trisakti.ac.id

Dr Joko Kusnoto,

Journal of Orthodontic Science has received your revised manuscript entitled 'Comparative Evaluation of Antimicrobial Toothpastes on Periodontal Bacteria in Orthodontic Patients: A Randomized Controlled Study'. The manuscript will be re-evaluated by concerned referees for the final decision regarding its suitability for publication. We will get back to you within four weeks.

We thank you for submitting your valuable research work to Journal of Orthodontic Science.

With warm personal regards,

The Editorial Team

Journal of Orthodontic Science

Reviewer Number	Original comments of the reviewer	Reply by the author(s)	Changes done on page number and line number
1	1. Can the author provide the details of double-blind techniques and randomization in the manuscript?	Details of double-blind techniques: In this double-blind clinical trial, blinding procedures were rigorously implemented to minimize performance and assessment bias. All toothpaste formulations were dispensed in identical, unlabeled tubes to prevent participants from recognizing the type of toothpaste they received. Consequently, participants were unaware of their group allocation throughout the study period. Similarly, the investigators responsible for distributing the products, monitoring adherence, and performing clinical evaluations were blinded to the allocation codes. No visual, textual, or sensory cues distinguished one formulation from another. The allocation codes were generated and securely held by an independent third party and were not disclosed to the research team until all data collection, data entry, and preliminary analyses had been completed. This approach ensured that both participants and outcome assessors remained fully blinded, thereby preserving the methodological rigor of the double-blind design. Each participant was also given an orthodontic toothbrush and instructed to brush twice daily using the Bass technique for one month. Details of randomization techniques: Participants were assigned to the study groups using block randomization and the order of	Double blind techniques → Page 5 Line 14-24 & Page 6 Line 1-2 Randomization techniques → Page 5 Line 5-10

		these blocks was further randomized to ensure balanced and unpredictable allocation. The randomization sequence was prepared in advance by an independent third party. Allocation concealment was maintained using sealed opaque envelopes. Throughout the study, both participants and outcome assessors remained blinded to group assignments to preserve the methodological integrity of the double-blind design.	
1 & 2	2. Can the author provide the rationale for the sample size in the study? Advise the authors to provide sample size calculation justification	The sample size for the study was calculated using the following formula: $n = \left[\frac{(Z\alpha + Z\beta)S}{(x1 - x2)}\right]^2$ $n = \left[\frac{(1{,}96 + 0{,}84)1{,}2522}{(1{,}77 - 0{,}06)}\right]^2$ $n \approx 5$ samples per group Zα represents the alpha standard deviation of 1.96 corresponding to a 95% confidence interval, while Zβ refers to the beta standard deviation of 0.84 with the same confidence level. The value S denotes the pooled standard deviation, and x1 – x2 indicates the minimum difference considered statistically significant. The symbol n represents the total number of samples required. The calculated sample size (n) was increased to 8 samples per group. This study consisted of three treatment groups, resulting in a total of 24 research subjects included in the study.	Page 4 Line 17-25 Page 5 Line 1-2

1	3. Can the author clarify the numbers in Table 2? Is it Ct values or 2-ΔΔCt values?	It is 2-ΔΔCt values	Page 24 Line 9
1	4. Can the author provide the evidence of the association of the presence of P. gingivalis and A. Actinomycetemcomitans in the saliva and the gingival sulcus and biofilm?	Several studies have demonstrated a strong association between the presence of *P. gingivalis* and *A. actinomycetemcomitans* in saliva, the gingival sulcus, and dental biofilm. These bacteria are recognized as key periodontal pathogens and have been shown to colonize multiple oral niches simultaneously. A qPCR study by Reddahi et al. found significantly higher levels of *P. gingivalis* and *A. actinomycetemcomitans* in both whole saliva and subgingival plaque from periodontitis patients compared to healthy controls. Moreover, they report a *strong positive correlation* between *A. actinomycetemcomitans* and *P. gingivalis* in the diseased subgingival sites and in saliva. Saliva often serves as a reservoir that reflects the microbial composition of subgingival and supragingival biofilms, including the presence of *P. gingivalis* and *A. actinomycetemcomitans*. Their detection in saliva correlates with their colonization in periodontal pockets and dental biofilm, because these pathogens disseminate through oral fluids and are shed from biofilm communities on tooth surfaces.	Page 15 Line 14-25 Page 16 Line 1-6

		Furthermore, previous research has demonstrated that salivary levels of these bacteria are significantly associated with periodontal inflammation, pocket depth, and microbial loads within the gingival sulcus, supporting the relevance of saliva as a diagnostic medium for monitoring periodontal pathogens.	

Comparative Evaluation of Antimicrobial Toothpastes on Periodontal Bacteria in Orthodontic Patients: A Randomized Controlled Study

ABSTRACT

Objective: To evaluate the effects of *L. paracasei* probiotic toothpaste, CPC toothpaste, and amyloglucosidase-glucose oxidase toothpaste on the levels of *P. gingivalis*, *A. actinomycetemcomitans*, and plaque index in individuals undergoing fixed orthodontic treatment. **Materials and Methods:** A double-blind randomized controlled clinical trial was conducted using purposive sampling. Participants were randomly assigned to use one of the toothpaste types. Saliva samples were collected at baseline and one month after using the toothpaste. Bacterial levels were quantified using quantitative PCR, and plaque accumulation was assessed using the Orthodontic Plaque Index. **Results:** All groups showed a reduction of *P. gingivalis* and *A. actinomycetemcomitans* following the intervention; however, no significant changes were observed in the plaque index. Statistical analysis using Two-Way Repeated Measures ANOVA with sphericity assumed revealed no significant differences between the groups ($p<0,05$). **Conclusion:** Toothpastes containing *L. paracasei*, CPC, and amyloglucosidase-glucose oxidase enzymes show potential for reducing periodontal pathogens, suggesting a preventive benefit against periodontal complications in patients with fixed orthodontic appliances.

Keywords:

Aggregatibacter actinomycetemcomitans, amyloglucosidase-glucoseoxidase enzyme, antibacterial effect, cetylpyridinium chloride, fixed orthodontic appliances, *L. paracasei*, plaque index, *Porphyromonas gingivalis*

INTRODUCTION

Malocclusion is a common condition with potential impacts on patients' quality of life, psychosocial well-being, and self-confidence.[1] In Indonesia, approximately 80% of the population experiences some form of malocclusion, making it a significant public oral health issue.[2] The increasing public awareness of dental and facial aesthetics has led to a rising demand for orthodontic treatment.[3] Recent studies indicate a rising prevalence of adult patients seeking orthodontic care, with estimates suggesting that adults now represent 20–30% of all orthodontic patients in many countries.[4]

Fixed orthodontic appliances, although effective in correcting malocclusion, create plaque-retentive areas that complicate oral hygiene. This can result in the accumulation of dental biofilm, which shifts the oral microbial balance and promotes colonization by pathogenic species.[5] Clinical signs of periodontal changes, including increased gingival inflammation, bleeding on probing, and periodontal pocketing, are often observed in patients wearing fixed appliances.[6,7]

Two major periodontal pathogens of concern in orthodontic patients are *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*.[8] These organisms are capable of adhering to both tooth surfaces and oral mucosa, contributing to periodontal tissue destruction.[9,10] Conventional plaque control methods such as mechanical brushing may not be sufficient, highlighting the need for adjunctive antimicrobial strategies.[5,11]

Various active agents in toothpaste, such as *Lactobacillus paracasei* probiotics, cetylpyridinium chloride (CPC), and amyloglucosidase-glucose oxidase enzymes, have shown promising antimicrobial activity in previous studies.[12–14] However, most studies have focused on their effects against cariogenic bacteria rather than periodontal pathogens. Therefore, further investigation is warranted to explore the efficacy of these formulations in reducing *P.*

gingivalis, *A. actinomycetemcomitans*, and plaque index in patients undergoing fixed orthodontic treatment.

MATERIALS AND METHODS

This randomized double-blind clinical trial was conducted on orthodontic patients with fixed appliances. Ethical approval for this study (876A/S2/KEPK/FKG/11/2024) was provided by the Research Ethics Committee of the Faculty of Dentistry, Universitas Trisakti, on 11 November 2024. After informed consent was obtained, subjects were screened based on inclusion criteria through anamnesis, intraoral clinical examination, and assessment using the Index of Orthodontic Treatment Need (IOTN) and Gingival Index (GI). Participants with Dental Health Component of IOTN scores ≤3 and GI scores between 0–2.0 were purposively selected. The exclusion criteria in this study were established to minimize potential confounding factors that could influence the outcomes. Participants were excluded if they had a history of probiotic consumption within the preceding three months or were undergoing pharmacological treatment that could interfere with salivary secretion. Individuals receiving systemic or topical antimicrobial therapy were also not considered eligible. In addition, subjects who reported habitual smoking or presented with systemic diseases were excluded from participation, also patients who had undergone professional oral hygiene procedures during the observation period were not included in the study.

The sample size for the study was calculated using the following formula:

$$n = \left[\frac{(Z\alpha + Z\beta)S}{(x1 - x2)}\right]^2$$

$$n = \left[\frac{(1{,}96 + 0{,}84)1{,}2522}{(1{,}77 - 0{,}06)}\right]^2$$

$$n \approx 5 \text{ samples per group}$$

Zα represents the alpha standard deviation of 1.96 corresponding to a 95% confidence interval, while Zβ refers to the beta standard deviation of 0.84 with the same confidence level. The value S denotes the pooled standard deviation, and x1 – x2 indicates the minimum difference considered statistically significant. The symbol n represents the total number of samples required. The calculated sample size (n) was increased to 8 samples per group. This

study consisted of three treatment groups, resulting in a total of 24 research subjects included in the study.

From a total of 32 participants were initially assessed in this study, with 24 participants meeting the inclusion criteria, 16.67% were male and 83.33% were female, with ages ranging from 18 to 23 years. Participants were assigned to the study groups using block randomization and the order of these blocks was further randomized to ensure balanced and unpredictable allocation. The randomization sequence was prepared in advance by an independent third party. Allocation concealment was maintained using sealed opaque envelopes. Throughout the study, both participants and outcome assessors remained blinded to group assignments to preserve the methodological integrity of the double-blind design. The participants were then assigned to one of three intervention groups: (1) probiotic toothpaste containing *Lactobacillus paracasei*, (2) toothpaste with cetylpyridinium chloride (CPC), or (3) toothpaste with amyloglucosidase-glucose oxidase enzymes.

In this double-blind clinical trial, blinding procedures were rigorously implemented to minimize performance and assessment bias. All toothpaste formulations were dispensed in identical, unlabeled tubes to prevent participants from recognizing the type of toothpaste they received. Consequently, participants were unaware of their group allocation throughout the study period. Similarly, the investigators responsible for distributing the products, monitoring adherence, and performing clinical evaluations were blinded to the allocation codes. No visual, textual, or sensory cues distinguished one formulation from another. The allocation codes were generated and securely held by an independent third party and were not disclosed to the research team until all data collection, data entry, and preliminary analyses had been completed. This approach ensured that both participants and outcome assessors remained fully blinded, thereby preserving the methodological rigor of the double-blind design. Each participant was

also given an orthodontic toothbrush and instructed to brush twice daily using the Bass technique for one month.

Saliva samples were collected at baseline (T0) and after one month (T1). Saliva offers a non-invasive, rapid, and reproducible sampling method that reflects the overall microbial load and oral health status, including the presence of periodontal pathogens such as *P. gingivalis* and *A. actinomycetemcomitans*. Participants were instructed to avoid food, drink, and physical activity one hour before collection. Stimulated saliva was collected via paraffin wax chewing and spitting into sterile tubes. Samples were stored at 2–8 °C temporarily and later frozen at –20 °C to –80 °C. saliva offers a non-invasive, rapid, and reproducible sampling method that reflects the overall microbial load and oral health status, including the presence of periodontal pathogens such as *P. gingivalis* and *A. actinomycetemcomitans*.

DNA extraction from the saliva was performed using heat-shock and centrifugation protocols. Quantification of *P. gingivalis* and *A. actinomycetemcomitans* was conducted using quantitative real-time PCR (qPCR). A total of 10 µL of DNA extraction from saliva was mixed with 90 µL of nuclear free water (NFW). These two mixtures were diluted seven times and produced a concentration of 10^0 µL or equivalent to 1 µL. Homogenization was carried out using a vortex. Every 2 µL of the dilution results were put into a 96-well plate (Nest Biotech, China). Then, mix 10 µL of SYBR green (Thermo Fisher Scientific, Massachusetts, USA), 6 µL of NFW, 1 µL each of the forward and reverse primers (Table 1) into the PCR mix and put into the qPCR plate wells that already contained the previous dilution. The qPCR plate wells were inserted into the qPCR machine at 95ºC for 10 minutes for one initiation denaturation cycle, followed by 40 cycles of denaturation at 95ºC for 15 seconds per cycle. The expression results of the samples using qPCR were then quantified relative DNA gene expression by calculating using the formula $2^{-\Delta\Delta Ct}$. Plaque levels were assessed using the Orthodontic Plaque Index (OPI) at both T0 and T1.

The normality test on the data uses the Shapiro-Wilk test ($n \leq 50$), if the p-value > 0.05 then the data is normally distributed. The homogeneity test uses Mauchly's Test of Sphericity. Next, a multivariate Two-Way Repeated Measures ANOVA (Analysis of Variance) test will be conducted with a p-value < 0.05 to see any significant differences and interactions between variables.

RESULTS

A total of 32 individuals were examined in this study, of whom 24 fulfilled the inclusion criteria. With respect to gender, 16.67% were men and 83.33% were women, and the overall age range was 18 to 23 years. The initial assessment consisted of a clinical examination that included evaluation of malocclusion type, jaw relationship, Index of Orthodontic Treatment Need (IOTN), Gingival Index (GI), and Orthodontic Plaque Index (OPI). The most prevalent malocclusion type was Class I, observed in 54.17% of the subjects, while the most frequent jaw relationship was orthognathic, found in 70.83% of participants. The IOTN examination revealed that 41.67% of the subjects were classified in grade 1. All participants (100%) demonstrated mild gingivitis based on the GI and OPI score of 4, corresponding to the poor oral hygiene category.

Based on the type of toothpaste, the *P. gingivalis* count showed a change in $2^{-\Delta\Delta Ct}$ values before (T0) and one month after (T1) treatment. The *L. paracasei* probiotic toothpaste group showed an average decrease of 5.59×10^6 before treatment to 5.03×10^3 after one month using the toothpastes. The CPC toothpaste group showed an average decrease from 3.11×10^3 to 4.79×10^2. The amyloglucosidase-glucoseoxidase enzyme toothpaste group showed a greater average decrease from 1.19×10^7 to 1.92×10^3. The *A. actinomycetemcomitans* count also showed a change in $2^{-\Delta\Delta Ct}$ values before (T0) and one month after (T1) treatment in all three toothpaste groups. The group using L. paracasei probiotic toothpaste showed an average decrease of 9.24 before treatment to 1.31 after treatment. The CPC toothpaste group saw an average decrease from 2.89 to 0.65. The amyloglucosidase-glucoseoxidase enzyme toothpaste group also showed a greater average decrease from 18.62 to 2.82. (Table 2)

The analysis then continued with the evaluation of the mean natural logarithm (NL) values of *P. gingivalis* at baseline (T0) and one month after treatment (T1) across the three toothpaste groups, as presented in Table 3. At baseline, the highest mean NL value was observed in the *L. paracasei* probiotic toothpaste group (7.35 ± 6.63), followed by the amyloglucosidase–glucose oxidase enzyme toothpaste group (6.84 ± 6.42), and the CPC toothpaste group (4.81 ± 2.81). The overall mean NL value of the three groups prior to treatment was 6.33 ± 5.44. After one month of treatment, a reduction in the mean NL values was observed in all groups. The *L. paracasei* probiotic toothpaste group demonstrated a mean NL value of 1.99 ± 4.37, the amyloglucosidase–glucose oxidase enzyme toothpaste group recorded 2.48 ± 4.10, and the CPC toothpaste group showed 2.93 ± 2.98. The combined mean NL value across all groups after treatment was 2.47 ± 3.71. The control of CT values obtained

from the laboratory procedure were 36.25 for *P. gingivalis* ATCC 33277 and 31.48 for *A. Actinomycetemcomitans* ATCC 29522.

Therefore the analysis of the mean values of *A. actinomycetemcomitans* was conducted at baseline (T0) and one month after treatment (T1) across the three toothpaste groups (Table 3). At baseline, the *L. paracasei* probiotic toothpaste group demonstrated a mean value of 9.24 ± 17.79, the CPC toothpaste group recorded 2.89 ± 3.15, and the amyloglucosidase–glucose oxidase enzyme toothpaste group demonstrated the highest value at 18.62 ± 27.62. The overall mean value of the three groups prior to treatment was 10.25 ± 19.37. Following one month of treatment, a reduction in mean values was observed in all groups. The *L. paracasei* probiotic toothpaste group exhibited a mean value of 1.31 ± 2.90, the CPC toothpaste group recorded 0.65 ± 0.89, and the amyloglucosidase–glucose oxidase enzyme toothpaste group demonstrated 2.82 ± 3.26. The combined mean value across all groups after treatment was 1.59 ± 2.63. (Table 3)

The average NL values for the *P. gingivalis* groups and mean values for the *A. actinomycetemcomitans* groups were then tested using Mauchly's Test of Sphericity. The Mauchly's Test yielded a value of 1, indicating that the requirement for homogeneity of covariance for the Two-Way Repeated Measures ANOVA were fully met for those two groups. Overall, there was a significant difference between the *P. gingivalis* groups before (T0) and one month after (T1) treatment. This is evident in the average NL T0 value of *P. gingivalis* of 6.33 ± 5.44, which decreased to 2.47 ± 3.71 at T1. The results of the Assumed Sphericity test for treatment time (Table IV) showed a p-value of 0.021 ($p < 0.05$), which means there was a significant difference between the *A. actinomycetemcomitans* groups before (T0) and one month after (T1) treatment. This can be seen in the average T0 value of *A. actinomycetemcomitans* of 10.25 ± 19.37, which decreased in the average T1 value to 1.59 ± 2.63. To assess the differences among the three toothpaste groups, the Assumed Sphericity test was applied to evaluate the interaction between time and treatment group (Table 4). The analysis yielded a p-value of 0.367 ($p > 0.05$), indicating no statistically significant difference. A decrease in the mean value of *P. gingivalis* was observed from baseline (T0) to one month after treatment (T1) across all three toothpaste groups, namely *L. paracasei* probiotic toothpaste, CPC toothpaste, and amyloglucosidase–glucose oxidase enzyme toothpaste. Similarly, for *A. actinomycetemcomitans*, the assumed sphericity test produced a p-value of 0.298 ($p > 0.05$), demonstrating no significant difference between the three groups. Although reductions in bacterial counts were evident in each group, the extent of decrease did not differ

significantly, suggesting that all three toothpastes produced relatively comparable outcomes in reducing *A. actinomycetemcomitans.*

The results of the Orthodontic Plaque Index (OPI) assessment. At baseline (T0), the mean OPI score in all three toothpaste groups; *L. paracasei* probiotic toothpaste, CPC toothpaste, and amyloglucosidase–glucose oxidase enzyme toothpaste was 4. Similarly, at one month after treatment (T1), the mean OPI score remained unchanged at 4 across all groups.

DISCUSSION

Patients undergoing treatment with fixed orthodontic appliances frequently encounter difficulties in maintaining optimal oral hygiene, as the components of the appliances may hinder effective cleaning. Consequently, these patients are at increased risk of periodontal tissue damage due to plaque accumulation and bacterial colonization.[7] The primary determinant of oral health maintenance is effective plaque control, which includes toothbrushing, interdental cleaning, and the use of mouth rinses.[5,11] Beyond mechanical methods of plaque removal, the selection of toothpaste also plays an essential role in plaque control, aiming to reduce bacterial load within the oral cavity.[15]

Adolescents are an appropriate population for studying periodontal pathogens such as *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* because they commonly undergo fixed orthodontic treatment, which promotes plaque retention and bacterial colonization due to appliance components that hinder cleaning.[8] Poor oral hygiene compliance in this age group further facilitates the proliferation of pathogenic bacteria associated with early periodontal changes. Studies have reported that *A. actinomycetemcomitans* and *P. gingivalis* are frequently detected in adolescents with gingival inflammation or early attachment loss during orthodontic treatment.[16] The prevalence of aggressive or early-onset periodontitis linked to these pathogens among adolescents ranges between 0.3% and 5.9%, emphasizing their importance as a high-risk group for periodontal research.[17]

In this study, saliva was employed as the diagnostic medium owing to its ease, rapidity, and non-invasive nature of collection. Saliva provides valuable insight into the oral environment, including bacterial load and the severity of periodontal disease.[18] Stimulated saliva was chosen because the mechanical action of chewing paraffin wax facilitates the release of bacteria from the gingival sulcus, thereby enhancing the detection of periodontal pathogens.[19] However, while gingival crevicular fluid (GCF) offers higher site specificity for

sampling bacteria and mediators directly from the periodontal pocket, it has drawbacks. GCF collection is technically demanding, requires multiple site-specific samples, prone to contamination with saliva, blood or plaque, and often involves low fluid volume and extensive laboratory processing.[20] Consequently, although GCF may provide more direct information about local periodontal microbiology, for larger scale screening or monitoring purposes saliva remains a more practical and efficient alternative.[20,21]

DNA based detection methods, such as quantitative PCR, are widely used to estimate bacterial load because they offer high sensitivity, specificity, and the ability to identify target species even at low concentrations.[22] Although these techniques cannot distinguish between live and dead bacteria, they provide a reliable measure of total bacterial presence and are less affected by sample handling or bacterial viability compared to culture-based methods.[23] Additionally, many oral pathogens, including *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, are fastidious and difficult to culture, making DNA quantification a practical and efficient alternative for evaluating microbial changes in clinical studies.[24]

Toothpaste is available in several forms, such as paste, gel, powder, and liquid. It generally contains two types of ingredients like non-active and active components. Non-active ingredients do not have therapeutic effects but determine the toothpaste's physical properties, including texture, taste, consistency, and appearance, and usually consist of water, abrasives, humectants, binders, flavours, surfactants, preservatives, and colorants.[25,26] Active ingredients, on the other hand, provide therapeutic benefits such as preventing cavities, reducing plaque, controlling sensitivity, eliminating bad breath, and offering antimicrobial effects. These include enzymes, cetylpyridinium chloride, and probiotics.[25]

The findings demonstrated significant reductions in *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* counts following the use of *Lactobacillus paracasei* probiotic toothpaste, cetylpyridinium chloride (CPC) toothpaste,

and amyloglucosidase–glucoseoxidase enzyme toothpaste among patients with fixed orthodontic appliances. This suggests that all three toothpaste formulations exhibit antibacterial effects.[12–14] However, no statistically significant differences were observed in the degree of bacterial reduction among the three groups, which may be attributed to the distinct mechanisms of action of the active ingredients in each toothpaste in inhibiting bacterial growth.

Probiotics are defined as microorganisms that confer health benefits to the host when consumed in adequate amounts. Over the past decade, a growing body of research has highlighted their therapeutic and preventive potential in maintaining oral health. Probiotics are known to modulate both specific and nonspecific immune responses, enhance epithelial barrier function, produce antimicrobial substances, and inhibit the adhesion of pathogenic bacteria within the oral cavity.[27] Among the antimicrobial substances produced by probiotics are bacteriocins and organic acids. Organic acids, particularly acetic acid and lactic acid, play a central role in the inhibitory activity of probiotics against pathogenic species. These acids are able to penetrate bacterial cell membranes, thereby acidifying the intracellular environment, which ultimately leads to bacterial death, especially in Gram-negative organisms.[28]

Chuang et al. reported that oral administration of *Lactobacillus paracasei* GMNL-33 exhibited anticariogenic properties by significantly reducing *Streptococcus mutans* levels in the oral cavity.[29] Similarly, Lee et al. demonstrated in a clinical study that *L. paracasei* GMNL-143–based probiotic toothpaste possesses the ability to co-aggregate with oral pathogens and inhibit their adhesion to gingival tissues.[31] The antibacterial effect of *L. paracasei* is more pronounced under acidic conditions compared with neutral pH environments. This enhanced activity in acidic conditions occurs because peptides are attracted to the phosphate groups of lipopolysaccharide (LPS) molecules, initiating pore formation in the bacterial membrane. Such changes in membrane permeability lead to structural disruption and compromise membrane integrity, ultimately resulting in bacterial cell lysis.[31] These findings are consistent with the

present study, in which *L. paracasei*–containing probiotic toothpaste was shown to effectively reduce bacterial counts in the oral cavity.

Cetylpyridinium chloride (CPC), another active ingredient found in certain toothpaste formulations, is a quaternary ammonium compound with well-established antimicrobial properties. Following use, CPC remains distributed within the oral cavity due to its surfactant chains and cationic charges, which enable sustained absorption onto oral surfaces.[32,33] Structurally, CPC contains hydrophilic and hydrophobic groups. The positively charged hydrophilic groups promote electrostatic binding to the negatively charged surfaces of pathogenic bacteria, while the hydrophobic groups interact with bacterial membranes, facilitating integration into the cytoplasmic membrane. These dual interactions lead to disruption of membrane integrity, impairment of cellular metabolism, cytoplasmic leakage, and eventual bacterial death. In addition, CPC reduces microbial adhesion to oral surfaces, thereby limiting colonization.[32] These mechanisms are consistent with the findings of Vasconcelos et al., who demonstrated that CPC-containing toothpaste significantly reduced bacterial counts in the oral cavity through decreased plaque accumulation and gingival inflammation.[13]

Toothpaste formulations containing the enzymes amyloglucosidase and glucose oxidase are reported to exert antimicrobial effects. The amyloglucosidase enzyme inhibits bacterial proliferation by converting D-glucose into D-glucono-1,5-lactone, thereby reducing the availability of bacterial nutrients in the oral cavity. Meanwhile, glucose oxidase activates the salivary immune defense system, specifically the lactoperoxidase (LPO) pathway, by generating hydrogen peroxide. This hydrogen peroxide interacts with catalase to produce oxygen, reducing the prevalence of anaerobic bacteria. Furthermore, hydrogen peroxide activates the LPO system to generate hypothiocyanite, a compound with antibacterial activity against *P. gingivalis*.[34,35] The findings of this study indicate that toothpaste containing amyloglucosidase and glucose oxidase produced greater reductions in both *P. gingivalis* and *A.*

actinomycetemcomitans compared to the other tested toothpastes. This outcome is consistent with the choice of saliva as a diagnostic tool, as the enzymatic mechanisms are directly linked to salivary immune activity.

As a member of the "red complex," *P. gingivalis* exhibits strong virulence through its capacity to aggregate with other bacterial species, facilitating colonization during later stages of biofilm development and rendering it difficult to eliminate.[9] Likewise, *A. actinomycetemcomitans* produces a wide range of virulence factors to ensure survival within the oral cavity.[36] Both species contribute to robust biofilm formation, aided by antimicrobial-resistant fimbriae and extracellular polysaccharides that hinder immune cell penetration and phagocytosis. These properties allow both pathogens to induce periodontal tissue damage.[31] The present study demonstrates a reduction in the levels of *P. gingivalis* and *A. actinomycetemcomitans*, which may help mitigate the risk of periodontal complications in patients with fixed orthodontic appliances.

Several studies have demonstrated a strong association between the presence of *P. gingivalis* and *A. actinomycetemcomitans* in saliva, the gingival sulcus, and dental biofilm. These bacteria are recognized as key periodontal pathogens and have been shown to colonize multiple oral niches simultaneously. A qPCR study by Reddahi et al. found significantly higher levels of *P. gingivalis* and *A. actinomycetemcomitans* in both whole saliva and subgingival plaque from periodontitis patients compared to healthy controls. Moreover, they report a *strong positive correlation* between *A. actinomycetemcomitans* and *P. gingivalis* in the diseased subgingival sites and in saliva.[37] Saliva often serves as a reservoir that reflects the microbial composition of subgingival and supragingival biofilms, including the presence of *P. gingivalis* and *A. actinomycetemcomitans*. Their detection in saliva correlates with their colonization in periodontal pockets and dental biofilm, because these pathogens disseminate through oral fluids and are shed from biofilm communities on tooth surfaces. Furthermore,

previous research has demonstrated that salivary levels of these bacteria are significantly associated with periodontal inflammation, pocket depth, and microbial loads within the gingival sulcus, supporting the relevance of saliva as a diagnostic medium for monitoring periodontal pathogens.[19,20,37] Taken together, the evidence supports that the presence of *P. gingivalis* and *A. actinomycetemcomitans* in saliva corresponds to their presence and activity within the gingival sulcus and dental biofilm.

The bacterial increase observed in patients with fixed appliances is attributable to the additional niches created by the orthodontic elements. Clinically, the number of oral bacteria has been shown to triple within the first six months following appliance placement.[38] Furthermore, plaque control becomes increasingly difficult in cases of dental misalignment. In this study, no significant changes were observed in plaque index scores before and after the use of probiotic *L. paracasei*, CPC, or amyloglucosidase-glucose oxidase toothpastes. This finding reflects the persistent cycle of plaque formation, as bacterial communities consistently recolonize tooth surfaces. Plaque development begins with pellicle formation initiated by *Streptococcus sanguinis*, followed by the coaggregation of pathogenic species such as *P. gingivalis*, *A. actinomycetemcomitans*, *Fusobacterium nucleatum*, *Treponema denticola*, and *Prevotella intermedia*.[39,40]

Mechanical plaque removal through toothbrushing eliminates only part of the biofilm, as microbial colonization can lead to dysbiosis. *P. gingivalis* plays a central role in this process, functioning as a “keystone pathogen” that manipulates host immune responses and disrupts homeostasis within the oral microbiome. Even at low concentrations, *P. gingivalis* can interact with other microorganisms to promote colonization.[41,42] Consequently, reductions in bacterial counts observed in this study could occur despite relatively unchanged plaque index values. This is explained by the complex biofilm composition of dental plaque, which consists not only

of microbial cells but also of extracellular polysaccharides, proteins, and structural molecules that stabilize the biofilm matrix.[40,43]

Additionally, the design and placement of orthodontic appliances contribute significantly to bacterial accumulation and plaque formation. Archwire ligatures serve as additional sites for bacterial colonization, and brackets positioned near the cervical margin can increase the risk of gingivitis.[44,45] The bracket material itself also plays a role: in this study, stainless steel appliances were used, which exhibit higher surface tension and are therefore more prone to plaque retention.[43]

Plaque retention varies among individuals due to differences in plaque formation patterns, oral hygiene practices, and dietary habits.[46] The effectiveness of toothbrushing as a plaque control method is highly dependent on patient compliance, as brushing is a complex and technique-sensitive process. Short-term use of toothpaste has been shown to exert only minimal influence on mechanical plaque removal.[47] Brushing technique plays a critical role in maintaining oral health, particularly for patients with fixed orthodontic appliances, who often experience challenges in adequately cleaning around appliance components. A common error is positioning the toothbrush too coronally, which results in neglect of the cervical region of the teeth and consequently increases plaque accumulation, predisposing patients to gingivitis.[33]

Plaque index was chosen instead of pocket depth or bleeding index because the presence of orthodontic brackets can make periodontal probing difficult and lead to measurement bias. The brackets and archwires hinder probe access and compromise the accuracy of assessing pocket depth and bleeding on probing.[48] Therefore, the plaque index provides a more practical and reliable parameter for evaluating oral hygiene during orthodontic treatment.[49] In addition, the plaque index reflects supragingival plaque accumulation, which is particularly relevant for orthodontic patients who are more prone to plaque retention due to appliance design.[49,50]

Toothbrush selection is also an important factor. The use of orthodontic toothbrushes characterized by a concave bristle arrangement and smaller brush head has been recommended, as these features allow for better adaptation to tooth surfaces and enhance cleaning efficacy around brackets, archwires, and interdental areas.[51] In addition, electric toothbrushes may serve as an effective alternative, as their vibratory action facilitates the removal of both supragingival and subgingival plaque. Professional dental cleaning at each follow-up appointment is likewise essential for patients undergoing fixed orthodontic treatment to further support oral hygiene maintenance.[38]

CONCLUSIONS

The use of probiotic toothpaste containing *Lactobacillus paracasei*, CPC toothpaste, and enzymatic toothpaste containing amyloglucosidase-glucose oxidase was found to reduce the levels of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* but had no effect on the plaque index in patients with fixed orthodontic appliances. There was no significant difference in the reduction of these bacteria among the three types of toothpaste. Therefore, it can be concluded that all three formulations have similar potential in preventing plaque formation and periodontal disease in patients undergoing fixed orthodontic treatment. Further research is expected to include other bacteria than *P. gingivalis* and *A. actinomycetemcomitans* that cause periodontal disease and also longer periods of toothpaste use to provide more comprehensive results.

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AUTHOR CONTRIBUTIONS

JK contributed to conceptualization, investigation, data curation, validation, manuscript review and editing; M contributed to investigation, data curation, original draft preparation; HW contributed to conceptualization, methodology, and validation; BK contributed to validation, manuscript review and editing. All authors critically reviewed and refined the final version of the manuscript. The authors have thoroughly read and granted their approval for its final submission.

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Table 1. Primers of *P. gingivalis* and *A. actinomycetemcomitans* used in qPCR[15]

Primer	Sequence (5'-3')
P. gingivalis Forward	TGC AAC TTG CCT TAC AGA GGG
P. gingivalis Reverse	ACT CGT ATC GCC CGT TAT TC
A. actinomycetemcomitans Forward	CTT ACC TAC TCT TGA CAT CCG AA
A. actinomycetemcomitans Reverse	ATG CAG GAC CTG TCT CAA AGC

Table 2. Minimum, maximum, and average Ct values of *P. gingivalis* and *A. actinomycetemcomitans* bacteria before (T0) and one month after (T1) treatment based on the type of toothpaste group ($2^{-\Delta\Delta Ct}$)

Toothpaste groups	Treatment Time	*P. gingivalis*			*A. actinomycetemcomitans*		
		Minimum Value	Maximum Value	Average Value	Minimum Range	Maximum Range	Average Value
L. paracasei probiotic	T0	2.22	4.22×10^7	5.59×10^6	1.54	53.10	9.24
	T1	0.28	3.89×10^4	5.03×10^3	0.07	8.45	1.31
CPC	T0	6.82	2.11×10^4	3.11×10^3	0.84	9.88	2.89
	T1	1.26	2.56×10^6	4.79×10^2	0.02	2.55	0.65
Amyloglucosidase-glucoseoxidase enzyme	T0	1.33	9.53×10^7	1.19×10^7	2.05	83.34	18.62
	T1	0.43	1.22×10^4	1.92×10^3	0.33	9.49	2.82

Table 3. Analysis of the normal logarithm (LN) values of the average *P. gingivalis* and mean values of *A. actinomycetemcomitans* mean values before (T0) and one month after (T1) treatment in the three toothpaste

Toothpaste groups	N	Natural logarithm (NL) values of *P. gingivalis*		Mean values of *A. actinomycetemcomitans*	
		T0	T1	T0	T1
L. paracasei probiotic	8	7.35 ± 6.63	1.99 ± 4.37	9.24 ± 17.79	1.31 ± 2.90
CPC	8	4.81 ± 2.81	2.93 ± 2.98	2.89 ± 3.15	0.65 ± 0.89
Amyloglucosidase-glucoseoxidase enzyme	8	6.84 ± 6.42	2.48 ± 4.10	18.62 ± 27.62	2.82 ± 3.26
Total	24	6.33 ± 5.44	2.47 ± 3.71	10.25 ± 19.37	1.59 ± 2.63

groups

Table 4. Results of the Two Way Repeated ANOVA test with Sphericity Assumed on *P. gingivalis* and *A. actinomycetemcomitans* before (T0) and one month after (T1) treatment in the three toothpaste groups

Assumed Sphericity Test Variable	*P. gingivalis*		*A. actinomycetemcomitans*	
	Mean square	p-value	Mean square	p-value
Treatment time	1.79×10^2	<0.05	8.99×10^2	<0.05
Treatment time*treatment group	12.79	0.367	1.85×10^2	0.298

3. Bukti konfirmasi artikel accepted

24 November 2025

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Joko Kusnoto <joko.k@trisakti.ac.id>

Author-side fee of your manuscript:jos_171_25

1 message

Journal of Orthodontic Science <editors@jorthodsci.org> Mon, Nov 24, 2025 at 2:34 AM
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We are pleased to inform that your manuscript "Comparative Evaluation of Antimicrobial Toothpastes on Periodontal Bacteria in Orthodontic Patients: A Randomized Controlled Study" is now acceptable after clearing the dues for publication of the manuscript. The details of the same can be found on the journal website under 'Instructions to the Authors' page.

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27 November 2025

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Journal of Orthodontic Science <editors@jorthodsci.org> Thu, Nov 27, 2025 at 6:25 PM
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Dear Dr. Joko Kusnoto,

We have checked and edited your article 'Comparative Evaluation of Antimicrobial Toothpastes on Periodontal Bacteria in Orthodontic Patients: A Randomized Controlled Study' for possibilities of technical and language errors. A revised article is now available online from our site https://review.jow.medknow.com/jos.
The purpose of this step is to check for the queries raised by the technical editors. Please download the latest article file from the site and check it thoroughly. Please make all the changes directly in the article file keeping 'Track Changes' on.

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Journal of Orthodontic Science

5. Bukti penerimaan pemeriksaan teknis dan bahasa serta artikel yang telah dilakukan pemeriksaan

28 November 2025

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1 message

Journal of Orthodontic Science <editors@jorthodsci.org> Fri, Nov 28, 2025 at 1:42 AM
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Dear Dr. Joko Kusnoto,

We have received the corrections of jos_171_25 'Comparative Evaluation of Antimicrobial Toothpastes on Periodontal Bacteria in Orthodontic Patients: A Randomized Controlled Study' which were sent to you for technical and language check.

With warm personal regards,
Yours sincerely,
The Editorial Team
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Technical editing done on 26.11.2025
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Please cite refernence 30 in the text part

<RH>Kusnoto, *et al*.: Running title missing???

Original Article

Comparative evaluation of antimicrobial toothpastes on periodontal bacteria in orthodontic patients: A randomized controlled study

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Abstract

Objective: To evaluate the effects of *Lactobacillus L. paracasei* probiotic toothpaste, cetylpyridinium chloride (CPC) toothpaste, and amyloglucosidase–glucose oxidase toothpaste on the levels of *P. gingivalis*, *Aggregatibacter A. actinomycetemcomitans*, and

plaque index in individuals undergoing fixed orthodontic treatment. **Materials and Methods:** A double-blind randomized controlled clinical trial was conducted using purposive sampling. Participants were randomly assigned to use one of the toothpaste types. Saliva samples were collected at baseline and one month after using the toothpaste. Bacterial levels were quantified using quantitative polymerase chain reaction~~PCR~~, and plaque accumulation was assessed using the Orthodontic Plaque Index. **Results:** All groups showed a reduction ~~of~~ of *P. ~~gingivalis~~ gingivalis* ~~and~~ and *A. ~~actinomycetemcomitans~~ actinomycetemcomitans* following the intervention; however, no significant changes were observed in the plaque index. Statistical analysis using two-way repeated measures analysis of variance ~~ANOVA~~ with sphericity assumed revealed no significant differences between the groups ($~~p~~p$ < 0~~,~~.05). **Conclusion:** Toothpastes containing ~~-~~ *L. paracasei*, CPC, and amyloglucosidase ~~-~~ glucose oxidase enzymes show potential for reducing periodontal pathogens, suggesting a preventive benefit against periodontal complications in patients with fixed orthodontic appliances.

Keywords:

Aggregatibacter actinomycetemcomitans, amyloglucosidase ~~-~~ glucose oxidase enzyme, antibacterial effect, cetylpyridinium chloride, fixed orthodontic appliances, *L. paracasei*, plaque index, *Porphyromonas gingivalis*

<H1>Introduction

Malocclusion is a common condition with potential impacts on patients' quality of life, psychosocial well-being, and self-confidence.[1] In Indonesia, approximately 80% of the population experiences some form of malocclusion, making it a significant public oral health issue.[2] The increasing public awareness of dental and facial aesthetics has led to a rising demand for orthodontic treatment.[3] Recent studies indicate a rising prevalence ~~of~~ of adult patients seeking orthodontic care, with estimates suggesting that adults now ~~represent~~ represent 20% –30% of all orthodontic ~~patients~~ patients in many countries.[4]

Fixed orthodontic appliances, although effective in correcting malocclusion, create plaque-retentive areas that complicate oral hygiene. This can result in the accumulation of dental biofilm, which shifts the oral microbial balance and promotes colonization by pathogenic species.[5] Clinical signs of periodontal changes, including increased gingival inflammation, bleeding on probing, and periodontal pocketing, are often observed in patients wearing fixed appliances.[6,7]

Two major periodontal pathogens of concern in orthodontic patients ~~are~~ are *Porphyromonas ~~gingivalis~~ gingivalis* ~~and~~ and *Aggregatibacter actinomycetemcomitans*.[8] These organisms are capable of adhering to both tooth surfaces and oral mucosa, contributing to periodontal tissue destruction.[9,10] Conventional plaque control methods, such as mechanical brushing, may not be sufficient, highlighting the need for adjunctive antimicrobial strategies.[5,11]

Various active agents in toothpaste, such ~~as~~ as *Lactobacillus ~~paracasei~~ paracasei* probiotics, cetylpyridinium chloride (CPC), and amyloglucosidase–glucose oxidase enzymes, have shown promising antimicrobial activity in previous studies.[12–14] However, most studies have focused on their effects against cariogenic bacteria rather than periodontal pathogens. Therefore, further investigation is warranted to explore the efficacy of these formulations in ~~reducing~~ reducing *P. gingivalis*, *A. actinomycetemcomitans*, and plaque index in patients

undergoing fixed orthodontic treatment.

<H1>Materials and Methods

This randomized double-blind clinical trial was conducted on orthodontic patients with fixed appliances. Ethical approval for this study (876A/S2/KEPK/FKG/11/2024) was provided by the Research Ethics Committee of the Faculty of Dentistry, Universitas Trisakti, on ~~11~~ November 11, 2024. After informed consent was obtained, subjects were screened based on inclusion criteria through anamnesis, intraoral clinical examination, and assessment using the index of orthodontic treatment need (IOTN) and Gingival Index (GI). Participants with the Dental Health Component of IOTN scores ≤3 and GI scores between 0– and 2.0 were purposively selected. The exclusion criteria in this study were established to minimize potential confounding factors that could influence the outcomes. Participants were excluded if they had a history of probiotic consumption within the preceding three months or were undergoing pharmacological treatment that could interfere with salivary secretion. Individuals receiving systemic or topical antimicrobial therapy were also not considered eligible. In addition, subjects who reported habitual smoking or presented with systemic diseases were excluded from participation, also patients who had undergone professional oral hygiene procedures during the observation period were not included in the study.

The sample size for the study was calculated using the following formula:

$$n = \left[\frac{(Z\alpha + Z\beta)S}{(x1 - x2)}\right]^2$$

$$n = \left[\frac{(1{,}96 + 0{,}84)1{,}2522}{(1{,}77 - 0{,}06)}\right]^2$$

$$n \approx 5 \text{ samples per group}$$

Zα represents the alpha standard deviation of 1.96 corresponding to a 95% confidence interval, while Zβ refers to the beta standard deviation of 0.84 with the same confidence level. The value S denotes the pooled standard deviation, and x1 – x2 indicates the minimum difference considered statistically significant. The symbol *n* represents the total number of samples

required. The calculated sample size (*n*) was increased to ~~8~~eight samples per group. This study consisted of three treatment groups, resulting in a total of 24 research subjects included in the study.

From a total of 32 participants who were initially assessed in this study, with 24 participants meeting the inclusion criteria, 16.67% were male and 83.33% were female, with ages ranging from 18 to 23 years. Participants were assigned to the study groups using block randomization, and the order of these blocks was further randomized to ensure balanced and unpredictable allocation. The randomization sequence was prepared in advance by an independent third party. Allocation concealment was maintained using sealed opaque envelopes. Throughout the study, both participants and outcome assessors remained blinded to group assignments to preserve the methodological integrity of the double-blind design. The participants were then assigned to one of three intervention groups: (1) probiotic toothpaste ~~containing~~ containing ~~*Lactobacillus*~~ *L. paracasei*, (2) toothpaste with ~~cetylpyridinium chloride (~~CPC~~)~~, or (3) toothpaste with amyloglucosidase~~ ~~~~–~~glucose oxidase enzymes.

In this double-blind clinical trial, blinding procedures were rigorously implemented to minimize performance and assessment bias. All toothpaste formulations were dispensed in identical, unlabeled tubes to prevent participants from recognizing the type of toothpaste they received. Consequently, participants were unaware of their group allocation throughout the study period. Similarly, the investigators responsible for distributing the products, monitoring adherence, and performing clinical evaluations were blinded to the allocation codes. No visual, textual, or sensory cues distinguished one formulation from another. The allocation codes were generated and securely held by an independent third party and were not disclosed to the research team until all data collection, data entry, and preliminary analyses had been completed. This approach ensured that both participants and outcome assessors remained fully blinded, thereby preserving the methodological rigor of the double-blind design. Each participant was

also given an orthodontic toothbrush and instructed to brush twice daily using the Bass technique for one month.

Saliva samples were collected at baseline (T0) and after one month (T1). Saliva offers a non-invasive, rapid, and reproducible sampling method that reflects the overall microbial load and oral health status, including the presence of periodontal pathogens, such ~~as~~ as *P. ~~gingivalis~~ gingivalis* ~~and~~ and *A. actinomycetemcomitans*. Participants were instructed to avoid food, drink, and physical activity one hour before collection. Stimulated saliva was collected via paraffin wax chewing and spitting into sterile tubes. Samples were stored at 2°C–8° ~~°~~C temporarily and later frozen at –20 °C to – –80 °C. ~~s~~Saliva offers a non-invasive, rapid, and reproducible sampling method that reflects the overall microbial load and oral health status, including the presence of periodontal pathogens such ~~as~~ as *P. ~~gingivalis~~ gingivalis* ~~and~~ and *A. actinomycetemcomitans*.

DNA extraction from the saliva was performed using heat-shock and centrifugation protocols. Quantification ~~of~~ of *P. gingivalis* and *A. actinomycetemcomitans* was conducted using quantitative real-time polymerase chain reaction~~PCR~~ (qPCR). A total of 10 µL of DNA extraction from saliva was mixed with 90 µL of nuclear free water (NFW). These two mixtures were diluted seven times and produced a concentration of 10^0 µL or equivalent to 1 µL. Homogenization was carried out using a vortex. Every 2 µL of the dilution results ~~were~~ was put into a 96-well plate (Nest Biotech, China). Then, mix 10 µL of SYBR green (Thermo Fisher Scientific, Massachusetts, USA), 6 µL of NFW, 1 µL each of the forward and reverse primers ~~(~~[Table 1~~)~~][15] into the PCR mix and put into the qPCR plate wells that already contained the previous dilution. The qPCR plate wells were inserted into the qPCR machine at 95°~~°~~C for 10 minutes for one initiation denaturation cycle, followed by 40 cycles of denaturation at 95°~~°~~C for 15 seconds per cycle. The expression results of the samples using qPCR were then quantified relative DNA gene expression by calculating using the formula $2^{-\Delta\Delta Ct}$. Plaque levels

were assessed using the Orthodontic Plaque Index (OPI) at both T0 and T1.

The normality test on the data uses the Shapiro–Wilk test ($n \leq 50$), if the *p*-value > 0.05 then the data is are normally distributed. The homogeneity test uses Mauchly's test of Sphericity. Next, a multivariate two-way repeated measures analysis of variance (ANOVA) (analysis of variance) test will be conducted with a *p*-value < 0.05 to see any significant differences and interactions between variables.

<H1>

Results

A total of 32 individuals were examined in this study, of whom 24 fulfilled the inclusion criteria. With respect to gender, 16.67% were men and 83.33% were women, and the overall age range was 18 to 23 years. The initial assessment consisted of a clinical examination that included evaluation of malocclusion type, jaw relationship, ~~Index of Orthodontic Treatment Need (~~IOTN~~)~~, ~~Gingival Index (~~GI~~)~~, and ~~Orthodontic Plaque Index (~~OPI~~)~~. The most prevalent malocclusion type was Class I, observed in 54.17% of the subjects, while the most frequent jaw relationship was orthognathic, found in 70.83% of participants. The IOTN examination revealed that 41.67% of the subjects were classified in grade 1. All participants (100%) demonstrated mild gingivitis based on the GI and OPI score of 4, corresponding to the poor oral hygiene category.

Based on the type of toothpaste, the *P. gingivalis* count showed a change in $2^{-\Delta\Delta Ct}$ values before (T0) and one month after (T1) treatment. The *L. paracasei* probiotic toothpaste group showed an average decrease of 5.59 ×~~x~~ 10^6 before treatment to 5.03 ×~~x~~ 10^3 after one month using the toothpastes. The CPC toothpaste group showed an average decrease from 3.11 ×~~x~~ 10^3 to 4.79 ×~~x~~ 10^2. The amyloglucosidase~~-~~ glucose oxidase enzyme toothpaste group showed a greater average decrease from 1.19 ×~~x~~ 10^7 to 1.92 ×~~x~~ 10^3. The *A. actinomycetemcomitans* count also showed a change in $2^{-\Delta\Delta Ct}$ values before (T0) and one month after (T1) treatment in all three toothpaste groups. The group using *L. paracasei* probiotic toothpaste showed an average decrease of 9.24 before treatment to 1.31 after treatment. The CPC toothpaste group saw an average decrease from 2.89 to 0.65. The amyloglucosidase~~-~~ glucose oxidase enzyme toothpaste group also showed a greater average decrease from 18.62 to 2.82~~.~~ ~~(~~[Table 2~~)~~].

The analysis then continued with the evaluation of the mean natural logarithm (NL) values of *P. gingivalis* at baseline (T0) and one month after treatment (T1) across the three toothpaste groups, as presented in Table 3. At baseline, the highest mean NL value was observed in the *L.*

paracasei probiotic toothpaste group (7.35 ± 6.63), followed by the amyloglucosidase–glucose oxidase enzyme toothpaste group (6.84 ± 6.42), and the CPC toothpaste group (4.81 ± 2.81). The overall mean NL value of the three groups ~~prior to~~before treatment was 6.33 ± 5.44. After one month of treatment, a reduction in the mean NL values was observed in all groups. The *L. paracasei* probiotic toothpaste group demonstrated a mean NL value of 1.99 ± 4.37, the amyloglucosidase–glucose oxidase enzyme toothpaste group recorded 2.48 ± 4.10, and the CPC toothpaste group showed 2.93 ± 2.98. The combined mean NL value across all groups after treatment was 2.47 ± 3.71. The control of ~~CT~~ Ct values obtained from the laboratory procedure ~~were~~ was 36.25 for *P. gingivalis* ATCC 33277 and 31.48 for *A. actinomycetemcomitans* ATCC 29522.

Therefore, the analysis of the mean values of *A. actinomycetemcomitans* was conducted at baseline (T0) and one month after treatment (T1) across the three toothpaste groups ~~(~~[Table 3~~).~~]. At baseline, the *L. paracasei* probiotic toothpaste group demonstrated a mean value of 9.24 ± 17.79, the CPC toothpaste group recorded 2.89 ± 3.15, and the amyloglucosidase–glucose oxidase enzyme toothpaste group demonstrated the highest value at 18.62 ± 27.62. The overall mean value of the three groups ~~prior to~~before treatment was 10.25 ± 19.37. Following one month of treatment, a reduction in mean values was observed in all groups. The *L. paracasei* probiotic toothpaste group exhibited a mean value of 1.31 ± 2.90, the CPC toothpaste group recorded 0.65 ± 0.89, and the amyloglucosidase–glucose oxidase enzyme toothpaste group demonstrated 2.82 ± 3.26. The combined mean value across all groups after treatment was 1.59 ± 2.63~~.~~ ~~(~~[Table 3]~~).~~

The average NL values for the *P. gingivalis* groups and mean values for the *A. actinomycetemcomitans*~~-~~ groups were then tested using Mauchly~~'~~'s ~~T~~test of ~~S~~sphericity. The Mauchly~~'~~'s ~~T~~test yielded a value of 1, indicating that the requirement for homogeneity of covariance for the two-way repeated measures ANOVA ~~were~~ was fully met for those two

groups. Overall, there was a significant difference between the *P. gingivalis* groups before (T0) and one month after (T1) treatment. This is evident in the average NL T0 value of *P. gingivalis* of 6.33 ± 5.44, which decreased to 2.47 ± 3.71 at T1. The results of the Aassumed Ssphericity test for treatment time ([Table IV4)] showed a *pp*-value of 0.021 (*p* < 0.05), which means that there was a significant difference between the *A. actinomycetemcomitans* groups before (T0) and one month after (T1) treatment. This can be seen in the average T0 value of *A. actinomycetemcomitans* of 10.25 ± 19.37, which decreased in the average T1 value to 1.59 ± 2.63. To assess the differences among the three toothpaste groups, the Aassumed Ssphericity test was applied to evaluate the interaction between time and treatment group [(Table 4).]. The analysis yielded a *p*-value of 0.367 (*p* > 0.05), indicating no statistically significant difference. A decrease in the mean value of *P. gingivalis* was observed from baseline (T0) to one month after treatment (T1) across all three toothpaste groups, namely, *L. paracasei* probiotic toothpaste, CPC toothpaste, and amyloglucosidase–glucose oxidase enzyme toothpaste. Similarly, for *A. actinomycetemcomitans*, the assumed sphericity test produced a *p*-value of 0.298 (*p* > 0.05), demonstrating no significant difference between the three groups. Although reductions in bacterial counts were evident in each group, the extent of decrease did not differ significantly, suggesting that all three toothpastes produced relatively comparable outcomes in reducing *A. actinomycetemcomitans.*

The results of the Orthodontic Plaque Index (OPI) assessment. At baseline (T0), the mean OPI score in all three toothpaste groups;, namely, *L. paracasei paracasei* probiotic toothpaste, CPC toothpaste, and amyloglucosidase–glucose oxidase enzyme toothpaste, was 4. Similarly, at one month after treatment (T1), the mean OPI score remained unchanged at 4 across all groups.

<H1>Discussion

Patients undergoing treatment with fixed orthodontic appliances frequently encounter difficulties in maintaining optimal oral hygiene, as the components of the appliances may hinder effective cleaning. Consequently, these patients are at increased risk of periodontal tissue damage due to plaque accumulation and bacterial colonization.[7] The primary determinant of oral health maintenance is effective plaque control, which includes toothbrushing, interdental cleaning, and the use of mouth rinses.[5,11] Beyond mechanical methods of plaque removal, the selection of toothpaste also plays an essential role in plaque control, aiming to reduce bacterial load within the oral cavity.[15]

Adolescents are an appropriate population for studying periodontal pathogens, such ~~as~~ as ~~*Aggregatibacter*~~ *A. ~~actinomycetemcomitans~~ actinomycetemcomitans* ~~and~~ and ~~*Porphyromonas*~~ *P. ~~gingivalis~~ gingivalis*, because they commonly undergo fixed orthodontic treatment, which promotes plaque retention and bacterial colonization due to appliance components that hinder cleaning.[8] Poor oral hygiene compliance in this age group further facilitates the proliferation of pathogenic bacteria associated with early periodontal changes. Studies have reported ~~that~~ that *A. ~~actinomycetemcomitans~~ actinomycetemcomitans* ~~and~~ and *P. ~~gingivalis~~ gingivalis* are frequently detected in adolescents with gingival inflammation or early attachment loss during orthodontic treatment.[16] The prevalence of aggressive or early-onset periodontitis linked to these pathogens among adolescents ranges between 0.3% and 5.9%, emphasizing their importance as a high-risk group for periodontal research.[17]

In this study, saliva was employed as the diagnostic medium owing to its ease, rapidity, and non-invasive nature of collection. Saliva provides valuable insight into the oral environment, including bacterial load and the severity of periodontal disease.[18] Stimulated saliva was chosen because the mechanical action of chewing paraffin wax facilitates the release of bacteria from the gingival sulcus, thereby enhancing the detection of periodontal pathogens.[19]

However, while gingival crevicular fluid (GCF) offers higher site specificity for sampling bacteria and mediators directly from the periodontal pocket, it has drawbacks. GCF collection is technically demanding, requires multiple site-specific samples, prone to contamination with saliva, blood or plaque, and often involves low fluid volume and extensive laboratory processing.[20] Consequently, although GCF may provide more direct information about local periodontal microbiology, for larger scale screening or monitoring purposes saliva remains a more practical and efficient alternative.[20,21]

DNA-~~-~~based detection methods, such as ~~quantitative~~ qPCR, are widely used to estimate bacterial load because they offer high sensitivity, specificity, and the ability to identify target species even at low concentrations.[22] Although these techniques cannot distinguish between live and dead bacteria, they provide a reliable measure of total bacterial presence and are less affected by sample handling or bacterial viability compared to culture-based methods.[23] Additionally, many oral pathogens, ~~including~~ including *~~Porphyromonas~~ P. ~~gingivalis~~ gingivalis* ~~and~~ and *~~Aggregatibacter~~ A. actinomycetemcomitans*, are fastidious and difficult to culture, making DNA quantification a practical and efficient alternative for evaluating microbial changes in clinical studies.[24]

Toothpaste is available in several forms, such as paste, gel, powder, and liquid. It generally contains two types of ingredients, like non-active and active components. Non-active ingredients do not have therapeutic effects but determine the toothpaste's physical properties, including texture, taste, consistency, and appearance, and usually consist of water, abrasives, humectants, binders, flavo~~u~~rs, surfactants, preservatives, and colorants.[25,26] Active ingredients, ~~on the other hand~~however, provide therapeutic benefits, such as preventing cavities, reducing plaque, controlling sensitivity, eliminating bad breath, and offering antimicrobial effects. These include enzymes, ~~cetylpyridinium chloride~~CPC, and probiotics.[25]

The findings demonstrated significant reductions ~~in~~ in *~~Porphyromonas~~ P. ~~gingivalis~~ gingivalis*

and and *Aggregatibacter A. actinomycetemcomitans actinomycetemcomitans* counts following the use of of *Lactobacillus L. paracasei paracasei* probiotic toothpaste, cetylpyridinium chloride (CPC) toothpaste, and amyloglucosidase–glucose oxidase enzyme toothpaste among patients with fixed orthodontic appliances. This suggests that all three toothpaste formulations exhibit antibacterial effects.[12–14] However, no statistically significant differences were observed in the degree of bacterial reduction among the three groups, which may be attributed to the distinct mechanisms of action of the active ingredients in each toothpaste in inhibiting bacterial growth.

Probiotics are defined as microorganisms that confer health benefits to the host when consumed in adequate amounts. Over the past decade, a growing body of research has highlighted their therapeutic and preventive potential in maintaining oral health. Probiotics are known to modulate both specific and nonspecific immune responses, enhance epithelial barrier function, produce antimicrobial substances, and inhibit the adhesion of pathogenic bacteria within the oral cavity.[27] Among the antimicrobial substances produced by probiotics are bacteriocins and organic acids. Organic acids, particularly acetic acid and lactic acid, play a central role in the inhibitory activity of probiotics against pathogenic species. These acids are able to penetrate bacterial cell membranes, thereby acidifying the intracellular environment, which ultimately leads to bacterial death, especially in Gram-negative organisms.[28]

Chuang *et al*. reported that oral administration of of *Lactobacillus L. paracasei paracasei* GMNL-33 exhibited anticariogenic properties by significantly reducing reducing *Streptococcus mutans mutans* levels in the oral cavity.[29] Similarly, Lee *et al*. demonstrated in a clinical study that that *L. paracasei paracasei* GMNL-143–based probiotic toothpaste possesses the ability to co-aggregate with oral pathogens and inhibit their adhesion to gingival tissues.[30] The antibacterial effect of of *L. paracasei paracasei* is more pronounced under acidic conditions compared with neutral pH environments. This enhanced activity in acidic

conditions occurs because peptides are attracted to the phosphate groups of lipopolysaccharide ~~(LPS)~~ molecules, initiating pore formation in the bacterial membrane. Such changes in membrane permeability ~~lead~~led to structural disruption and compromise membrane integrity, ultimately resulting in bacterial cell lysis.[31] These findings are consistent with the present study, in ~~which~~ which *L. paracasei* ~~-~~-containing probiotic toothpaste was shown to effectively reduce bacterial counts in the oral cavity.

~~Cetylpyridinium chloride (~~CPC~~)~~, another active ingredient found in certain toothpaste formulations, is a quaternary ammonium compound with well-established antimicrobial properties. Following use, CPC remains distributed within the oral cavity due to its surfactant chains and cationic charges, which enable sustained absorption onto oral surfaces.[32,33] Structurally, CPC contains hydrophilic and hydrophobic groups. The positively charged hydrophilic groups promote electrostatic binding to the negatively charged surfaces of pathogenic bacteria, while the hydrophobic groups interact with bacterial membranes, facilitating integration into the cytoplasmic membrane. These dual interactions lead to disruption of membrane integrity, impairment of cellular metabolism, cytoplasmic leakage, and eventual bacterial death. In addition, CPC reduces microbial adhesion to oral surfaces, thereby limiting colonization.[32] These mechanisms are consistent with the findings of Vasconcelos *et al*., who demonstrated that CPC-containing toothpaste significantly reduced bacterial counts in the oral cavity through decreased plaque accumulation and gingival inflammation.[13]

Toothpaste formulations containing the enzymes amyloglucosidase and glucose oxidase are reported to exert antimicrobial effects. The amyloglucosidase enzyme inhibits bacterial proliferation by converting D-glucose into D-glucono-1,5-lactone, thereby reducing the availability of bacterial nutrients in the oral cavity. Meanwhile, glucose oxidase activates the salivary immune defense system, specifically the lactoperoxidase (LPO) pathway, by generating hydrogen peroxide. This hydrogen peroxide interacts with catalase to produce

oxygen, reducing the prevalence of anaerobic bacteria. Furthermore, hydrogen peroxide activates the LPO system to generate hypothiocyanite, a compound with antibacterial activity ~~against~~ against *P. gingivalis*.[34,35] The findings of this study indicate that toothpaste containing amyloglucosidase and glucose oxidase produced greater reductions in ~~both~~ both *P. ~~gingivalis~~ gingivalis* ~~and~~ and *A. actinomycetemcomitans* compared to the other tested toothpastes. This outcome is consistent with the choice of saliva as a diagnostic tool, as the enzymatic mechanisms are directly linked to salivary immune activity.

As a member of the "red complex~~,"~~," *P. ~~gingivalis~~ gingivalis* exhibits strong virulence through its capacity to aggregate with other bacterial species, facilitating colonization during later stages of biofilm development and rendering it difficult to eliminate.[9] Likewise~~,~~, *A. ~~actinomycetemcomitans~~ actinomycetemcomitans* produces a wide range of virulence factors to ensure survival within the oral cavity.[36] Both species contribute to robust biofilm formation, aided by antimicrobial-resistant fimbriae and extracellular polysaccharides that hinder immune cell penetration and phagocytosis. These properties allow both pathogens to induce periodontal tissue damage.[31] The present study demonstrates a reduction in the levels ~~of~~ of *P. ~~gingivalis~~ gingivalis* ~~and~~ and *A. actinomycetemcomitans*, which may help mitigate the risk of periodontal complications in patients with fixed orthodontic appliances.

Several studies have demonstrated a strong association between the presence ~~of~~ of *P. ~~gingivalis~~ gingivalis* ~~and~~ and *A. ~~actinomycetemcomitans~~ actinomycetemcomitans* in saliva, the gingival sulcus, and dental biofilm. These bacteria are recognized as key periodontal pathogens and have been shown to colonize multiple oral niches simultaneously. A qPCR study by Reddahi *et al*. found significantly higher levels ~~of~~ of *P. ~~gingivalis~~ gingivalis* ~~and~~ and *A. actinomycetemcomitans* in both whole saliva and subgingival plaque from periodontitis patients compared to healthy controls. Moreover, they report ~~a~~ a *strong positive ~~correlation~~ correlation* ~~between~~ between *A. ~~actinomycetemcomitans~~ actinomycetemcomitans*

~~and~~ and *P. ~~gingivalis~~ gingivalis* in the diseased subgingival sites and in saliva.[37] Saliva often serves as a reservoir that reflects the microbial composition of subgingival and supragingival biofilms, including the presence ~~of~~ of *P. ~~gingivalis~~ gingivalis* ~~and~~ and *A. actinomycetemcomitans*. Their detection in saliva correlates with their colonization in periodontal pockets and dental biofilm, because these pathogens disseminate through oral fluids and are shed from biofilm communities on tooth surfaces. Furthermore, previous research has demonstrated that salivary levels of these bacteria are significantly associated with periodontal inflammation, pocket depth, and microbial loads within the gingival sulcus, supporting the relevance of saliva as a diagnostic medium for monitoring periodontal pathogens.[19,20,37] Taken together, the evidence supports that the presence ~~of~~ of *P. ~~gingivalis~~ gingivalis* ~~and~~ and *A. ~~actinomycetemcomitans~~ actinomycetemcomitans* in saliva corresponds to their presence and activity within the gingival sulcus and dental biofilm.

The bacterial increase observed in patients with fixed appliances is attributable to the additional niches created by the orthodontic elements. Clinically, the number of oral bacteria has been shown to triple within the first six months following appliance placement.[38] Furthermore, plaque control becomes increasingly difficult in cases of dental misalignment. In this study, no significant changes were observed in plaque index scores before and after the use of probiotic *L. paracasei*, CPC, or amyloglucosidase~~-~~ glucose oxidase toothpastes. This finding reflects the persistent cycle of plaque formation, as bacterial communities consistently recolonize tooth surfaces. Plaque development begins with pellicle formation initiated ~~by~~ by *Streptococcus sanguinis*, followed by the coaggregation of pathogenic species such ~~as~~ as *P. gingivalis*~~,~~ , *A. actinomycetemcomitans*~~,~~ , *Fusobacterium nucleatum*~~,~~ , *Treponema denticola*, ~~and~~ and *Prevotella intermedia*.[39,40]

Mechanical plaque removal through toothbrushing eliminates only part of the biofilm, as microbial colonization can lead to dysbiosis~~.~~ . *P. ~~gingivalis~~ gingivalis* plays a central role in

this process, functioning as a “keystone pathogen” that manipulates host immune responses and disrupts homeostasis within the oral microbiome. Even at low concentrations~~,~~ , *P. ~~gingivalis~~ gingivalis* can interact with other microorganisms to promote colonization.[41,42] Consequently, reductions in bacterial counts observed in this study could occur despite relatively unchanged plaque index values. This is explained by the complex biofilm composition of dental plaque, which consists not only of microbial cells but also of extracellular polysaccharides, proteins, and structural molecules that stabilize the biofilm matrix.[40,43]

Additionally, the design and placement of orthodontic appliances contribute significantly to bacterial accumulation and plaque formation. Archwire ligatures serve as additional sites for bacterial colonization, and brackets positioned near the cervical margin can increase the risk of gingivitis.[44,45] The bracket material itself also plays a role: ~~i~~In this study, stainless steel appliances were used, which exhibit higher surface tension and are therefore more prone to plaque retention.[43]

Plaque retention varies among individuals due to differences in plaque formation patterns, oral hygiene practices, and dietary habits.[46] The effectiveness of toothbrushing as a plaque control method is highly dependent on patient compliance, as brushing is a complex and technique-sensitive process. Short-term use of toothpaste has been shown to exert only minimal influence on mechanical plaque removal.[47] Brushing technique plays a critical role in maintaining oral health, particularly for patients with fixed orthodontic appliances, who often experience challenges in adequately cleaning around appliance components. A common error is positioning the toothbrush too coronally, which results in neglect of the cervical region of the teeth and consequently increases plaque accumulation, predisposing patients to gingivitis.[33]

Plaque index was chosen instead of pocket depth or bleeding index because the presence of orthodontic brackets can make periodontal probing difficult and lead to measurement bias. The

brackets and archwires hinder probe access and compromise the accuracy of assessing pocket depth and bleeding on probing.[48] Therefore, the plaque index provides a more practical and reliable parameter for evaluating oral hygiene during orthodontic treatment.[49] In addition, the plaque index reflects supragingival plaque accumulation, which is particularly relevant for orthodontic patients who are more prone to plaque retention due to appliance design.[49,50]

Toothbrush selection is also an important factor. The use of orthodontic toothbrushes characterized by a concave bristle arrangement and smaller brush head has been recommended, as these features allow for better adaptation to tooth surfaces and enhance cleaning efficacy around brackets, archwires, and interdental areas.[51] In addition, electric toothbrushes may serve as an effective alternative, as their vibratory action facilitates the removal of both supragingival and subgingival plaque. Professional dental cleaning at each follow-up appointment is likewise essential for patients undergoing fixed orthodontic treatment to further support oral hygiene maintenance.[38]

<H1>Conclusions

The use of probiotic toothpaste containing containing *Lactobacillus L. paracasei*, CPC toothpaste, and enzymatic toothpaste containing amyloglucosidase glucose oxidase was found to reduce the levels of of *Porphyromonas P. gingivalis gingivalis* and and *Aggregatibacter A. actinomycetemcomitans* but had no effect on the plaque index in patients with fixed orthodontic appliances. There was no significant difference in the reduction of these bacteria among the three types of toothpaste. Therefore, it can be concluded that all three formulations have similar potential in preventing plaque formation and periodontal disease in patients undergoing fixed orthodontic treatment. Further research is expected to include other bacteria than *P. gingivalis* and *A. actinomycetemcomitans* that cause periodontal disease and also longer periods of toothpaste use to provide more comprehensive results.

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The authors would like to thank the Department of Orthodontics and the Department of ~~Periodontics~~Periodontics~~tis~~, Faculty of Dentistry, Universitas Trisakti, for their valuable support throughout this study. Special appreciation is extended to all study participants and clinical staff involved in data collection and laboratory analysis.

~~AUTHOR CONTRIBUTIONS~~

~~JK contributed to conceptualization, investigation, data curation, validation, manuscript review and editing; M contributed to investigation, data curation, original draft preparation; HW contributed to conceptualization, methodology, and validation; BK contributed to validation, manuscript review and editing. All authors critically reviewed and refined the final version of the manuscript. The authors have thoroughly read and granted their approval for its final submission.~~

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This study received leading faculty research grant from Universitas Trisakti.

<H2>**Conflicts of interest**

There are no conflicts of interest.

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Tables

Table 1. Primers of *P. gingivalis* and *A. actinomycetemcomitans* used in qPCR[15]

Table 1. Primers of *Porphyromonas* gingivalis and *Aggregatibacter* actinomycetemcomitans used in quantitative real-time polymerase chain reaction.[15]	
Primer	**Sequence (5'–3')**
P. gingivalis forward	TGC AAC TTG CCT TAC AGA GGG
P. gingivalis reverse	ACT CGT ATC GCC CGT TAT TC
A. actinomycetemcomitans forward	CTT ACC TAC TCT TGA CAT CCG AA
A. actinomycetemcomitans reverse	ATG CAG GAC CTG TCT CAA AGC
P. gingivalis = Porphyromonas gingivalis, *A. actinomycetemcomitans = Aggregatibacter actinomycetemcomitans*	

Table 2. Minimum, maximum, and average Ct values of *P. gingivalis* and *A. actinomycetemcomitans* bacteria before (T0) and one month after (T1) treatment based on the type of toothpaste group ($2^{-\Delta\Delta Ct}$)

Table 2: Minimum, maximum, and average Ct values of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* bacteria before (T0) and one month after (T1) treatment based on the type of toothpaste group ($2^{-\Delta\Delta Ct}$).			
Toothpaste	**Treatment**	***P. gingivalis***	***A. actinomycetemcomitans***

groups	time	Minimum value	Maximum value	Average value	Minimum range	Maximum range	Average value
L. paracasei probiotic	T0	2.22	4.22 x 10^7	5.59 x 10^6	1.54	53.10	9.24
	T1	0.28	3.89 x 10^4	5.03 x 10^3	0.07	8.45	1.31
CPC	T0	6.82	2.11 x 10^4	3.11 x 10^3	0.84	9.88	2.89
	T1	1.26	2.56 x 10^6	4.79 x 10^2	0.02	2.55	0.65
Amyloglucosidase–glucose oxidase enzyme	T0	1.33	9.53 x 10^7	1.19 x 10^7	2.05	83.34	18.62
	T1	0.43	1.22 x 10^4	1.92 x 10^3	0.33	9.49	2.82

P. gingivalis = *Porphyromonas gingivalis*, *A. actinomycetemcomitans* = *Aggregatibacter actinomycetemcomitans*, *L. paracasei* = *Lactobacillus paracasei*, CPC = Cetylpyridinium chloride

~~Table 3. Analysis of the normal logarithm (LN) values of the average *P. gingivalis* and mean values of *A. actinomycetemcomitans* mean values before (T0) and one month after (T1) treatment in the three toothpaste groups~~

Table 3: Analysis of the normal logarithm (~~L~~LN~~N~~) values of the average *Porphyromonas gingivalis* and mean values of *Aggregatibacter actinomycetemcomitans* mean values before (T0) and one month after (T1) treatment in the three toothpaste groups.

Toothpaste groups	*n*	~~Natural logarithm (~~NL~~)~~	Mean values of *A.*

		values of *P. gingivalis*		*actinomycetemcomitans*	
		T0	**T1**	**T0**	**T1**
L. paracasei probiotic	8	7.35 ± 6.63	1.99 ± 4.37	9.24 ± 17.79	1.31 ± 2.90
CPC	8	4.81 ± 2.81	2.93 ± 2.98	2.89 ± 3.15	0.65 ± 0.89
Amyloglucosidase – glucose oxidase enzyme	8	6.84 ± 6.42	2.48 ± 4.10	18.62 ± 27.62	2.82 ± 3.26
Total	24	6.33 ± 5.44	2.47 ± 3.71	10.25 ± 19.37	1.59 ± 2.63

NL = Natural logarithm, *P. gingivalis* = *Porphyromonas gingivalis*, *A. actinomycetemcomitans* = *Aggregatibacter actinomycetemcomitans*, *L. paracasei* = *Lactobacillus paracasei*, CPC = Cetylpyridinium chloride

~~Table 4. Results of the Two-way repeated ANOVA test with Sphericity assumed on *P. gingivalis* and *A. actinomycetemcomitans* before (T0) and one month after (T1) treatment in the three toothpaste groups~~

Table 4: Results of the ~~T~~two-way repeated analysis of variance test with ~~S~~sphericity assumed on *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* before (T0) and one month after (T1) treatment in the three toothpaste groups.

Assumed sphericity test variable	***P. gingivalis***		***A. actinomycetemcomitans***	
	Mean square	***p-valuepP***	**Mean square**	***p-valuepP***

Treatment time	1.79 x × 10^2	<0.05	8.99 x × 10^2	<0.05
Treatment time * treatment group	12.79	0.367	1.85 x × 10^2	0.298

P. gingivalis = Porphyromonas gingivalis, *A. actinomycetemcomitans = Aggregatibacter actinomycetemcomitans*, $p < 0.05$

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Comparative evaluation of antimicrobial toothpastes on periodontal bacteria in orthodontic patients: A randomized controlled study

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Abstract

OBJECTIVE: To evaluate the effects of *Lactobacillus paracasei* probiotic toothpaste, cetylpyridinium chloride (CPC) toothpaste, and amyloglucosidase–glucose oxidase toothpaste on the levels of *Porphyromonas. gingivalis*, *Aggregatibacter actinomycetemcomitans*, and plaque index in individuals undergoing fixed orthodontic treatment.

MATERIALS AND METHODS: A double-blind randomized controlled clinical trial was conducted using purposive sampling. Participants were randomly assigned to use one of the toothpaste types. Saliva samples were collected at baseline and one month after using the toothpaste. Bacterial levels were quantified using quantitative polymerase chain reaction, and plaque accumulation was assessed using the Orthodontic Plaque Index.

RESULTS: All groups showed a reduction of *P. gingivalis* and *A. actinomycetemcomitans* following the intervention; however, no significant changes were observed in the plaque index. Statistical analysis using two-way repeated measures analysis of variance with sphericity assumed revealed no significant differences between the groups ($p < 0.05$).

CONCLUSION: Toothpastes containing *L. paracasei*, CPC, and amyloglucosidase–glucose oxidase enzymes show potential for reducing periodontal pathogens, suggesting a preventive benefit against periodontal complications in patients with fixed orthodontic appliances.

Keywords:

Aggregatibacter actinomycetemcomitans, amyloglucosidase–glucose oxidase enzyme, antibacterial effect, cetylpyridinium chloride, fixed orthodontic appliances, *L. paracasei*, plaque index, *Porphyromonas gingivalis*

Introduction

Malocclusion is a common condition with potential impacts on patients' quality of life, psychosocial well-being, and self-confidence.[1] In Indonesia, approximately 80% of the population experiences some form of malocclusion, making it a significant public oral health issue.[2] The increasing public awareness of dental and facial aesthetics has led to a rising demand for orthodontic treatment.[3] Recent studies indicate a rising prevalence of adult patients seeking orthodontic care, with estimates suggesting that adults now represent 20%–30% of all orthodontic patients in many countries.[4]

Fixed orthodontic appliances, although effective in correcting malocclusion, create plaque-retentive areas that complicate oral

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hygiene. This can result in the accumulation of dental biofilm, which shifts the oral microbial balance and promotes colonization by pathogenic species.[5] Clinical signs of periodontal changes, including increased gingival inflammation, bleeding on probing, and periodontal pocketing, are often observed in patients wearing fixed appliances.[6,7]

Two major periodontal pathogens of concern in orthodontic patients are *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*.[8] These organisms are capable of adhering to both tooth surfaces and oral mucosa, contributing to periodontal tissue destruction.[9,10] Conventional plaque control methods, such as mechanical brushing, may not be sufficient, highlighting the need for adjunctive antimicrobial strategies.[5,11]

Various active agents in toothpaste, such as *Lactobacillus paracasei* probiotics, cetylpyridinium chloride (CPC), and amyloglucosidase–glucose oxidase enzyme, have shown promising antimicrobial activity in previous studies.[12-14] However, most studies have focused on their effects against cariogenic bacteria rather than periodontal pathogens. Therefore, further investigation is warranted to explore the efficacy of these formulations in reducing *P. gingivalis*, *A. actinomycetemcomitans*, and plaque index in patients undergoing fixed orthodontic treatment.

Materials and Methods

This randomized double-blind clinical trial was conducted on orthodontic patients with fixed appliances. Ethical approval for this study (876A/S2/KEPK/FKG/11/2024) was provided by the Research Ethics Committee of the Faculty of Dentistry, Universitas Trisakti, on November 11, 2024. After informed consent was obtained, subjects were screened based on inclusion criteria through anamnesis, intraoral clinical examination, and assessment using the index of orthodontic treatment need (IOTN) and Gingival Index (GI). Participants with the Dental Health Component of IOTN scores ≤3 and GI scores between 0 and 2.0 were purposively selected. The exclusion criteria in this study were established to minimize potential confounding factors that could influence the outcomes. Participants were excluded if they had a history of probiotic consumption within the preceding three months or were undergoing pharmacological treatment that could interfere with salivary secretion. Individuals receiving systemic or topical antimicrobial therapy were also not considered eligible. In addition, subjects who reported habitual smoking or presented with systemic diseases were excluded from participation, also patients who had undergone professional oral hygiene procedures during the observation period were not included in the study.

The sample size for the study was calculated using the following formula:

$$n = \left[\frac{(Z\alpha + Z\beta)S}{(x1 - x2)} \right]^2$$

$$n = \left[\frac{(1.96 + 0.84)1.2522}{(1.77 - 0.06)} \right]^2$$

$n \approx 5$ samples per group

Zα represents the alpha standard deviation of 1.96 corresponding to a 95% confidence interval, while Zβ refers to the beta standard deviation of 0.84 with the same confidence level. The value S denotes the pooled standard deviation, and x1 – x2 indicates the minimum difference considered statistically significant. The symbol *n* represents the total number of samples required. The calculated sample size (*n*) was increased to eight samples per group. This study consisted of three treatment groups, resulting in a total of 24 research subjects included in the study.

From a total of 32 participants who were initially assessed in this study, with 24 participants meeting the inclusion criteria, 16.67% were male and 83.33% were female, with ages ranging from 18 to 23 years. Participants were assigned to the study groups using block randomization, and the order of these blocks was further randomized to ensure balanced and unpredictable allocation. The randomization sequence was prepared in advance by an independent third party. Allocation concealment was maintained using sealed opaque envelopes. Throughout the study, both participants and outcome assessors remained blinded to group assignments to preserve the methodological integrity of the double-blind design. The participants were then assigned to one of three intervention groups: (1) probiotic toothpaste containing *L. paracasei*, (2) toothpaste with CPC, or (3) toothpaste with amyloglucosidase–glucose oxidase enzymes.

In this double-blind clinical trial, blinding procedures were rigorously implemented to minimize performance and assessment bias. All toothpaste formulations were dispensed in identical, unlabeled tubes to prevent participants from recognizing the type of toothpaste they received. Consequently, participants were unaware of their group allocation throughout the study period. Similarly, the investigators responsible for distributing the products, monitoring adherence, and performing clinical evaluations were blinded to the allocation codes. No visual, textual, or sensory cues distinguished one formulation from another. The allocation codes were generated and securely held by an independent third party and were not disclosed to the research team

until all data collection, data entry, and preliminary analyses had been completed. This approach ensured that both participants and outcome assessors remained fully blinded, thereby preserving the methodological rigor of the double-blind design. Each participant was also given an orthodontic toothbrush and instructed to brush twice daily using the Bass technique for one month.

Saliva samples were collected at baseline (T0) and after one month (T1). Saliva offers a noninvasive, rapid, and reproducible sampling method that reflects the overall microbial load and oral health status, including the presence of periodontal pathogens, such as *P. gingivalis* and *A. actinomycetemcomitans*. Participants were instructed to avoid food, drink, and physical activity one hour before collection. Stimulated saliva was collected via paraffin wax chewing and spitting into sterile tubes. Samples were stored at 2°C–8°C temporarily and later frozen at – 20°C to – 80°C.

DNA extraction from the saliva was performed using heat-shock and centrifugation protocols. Quantification of *P. gingivalis* and *A. actinomycetemcomitans* was conducted using quantitative real-time polymerase chain reaction (qPCR). A total of 10 µL of DNA extraction from saliva was mixed with 90 µL of nuclear free water (NFW). These two mixtures were diluted seven times and produced a concentration of 10^0 µL or equivalent to 1 µL. Homogenization was carried out using a vortex. Every 2 µL of the dilution results was put into a 96-well plate (Nest Biotech, China). Then, mix 10 µL of SYBR green (Thermo Fisher Scientific, Massachusetts, USA), 6 µL of NFW, 1 µL each of the forward and reverse primers [Table 1][15] into the PCR mix and put into the qPCR plate wells that already contained the previous dilution. The qPCR plate wells were inserted into the qPCR machine at 95°C for 10 minutes for one initiation denaturation cycle, followed by 40 cycles of denaturation at 95°C for 15 seconds per cycle. The expression results of the samples using qPCR were then quantified relative DNA gene expression by calculating using the formula $2^{-\Delta\Delta Ct}$. Plaque levels were assessed using the Orthodontic Plaque Index (OPI) at both T0 and T1.

Table 1: Primers of *Porphyromonas* gingivalis and *Aggregatibacter* actinomycetemcomitans used in quantitative real-time polymerase chain reaction

Primer	Sequence (5'–3')
P. gingivalis forward	TGC AAC TTG CCT TAC AGA GGG
P. gingivalis reverse	ACT CGT ATC GCC CGT TAT TC
A. actinomycetemcomitans forward	CTT ACC TAC TCT TGA CAT CCG AA
A. actinomycetemcomitans reverse	ATG CAG GAC CTG TCT CAA AGC

P. gingivalis=Porphyromonas gingivalis, A. actinomycetemcomitans=Aggregatibacter actinomycetemcomitans

The normality test on the data uses the Shapiro–Wilk test ($n \leq 50$), if the $P > 0.05$ then the data are normally distributed. The homogeneity test uses Mauchly's test of Sphericity. Next, a multivariate two-way repeated measures analysis of variance (ANOVA) test will be conducted with a $P < 0.05$ to see any significant differences and interactions between variables.

Results

A total of 32 individuals were examined in this study, of whom 24 fulfilled the inclusion criteria. With respect to gender, 16.67% were men and 83.33% were women, and the overall age range was 18 to 23 years. The initial assessment consisted of a clinical examination that included evaluation of malocclusion type, jaw relationship, IOTN, GI, and OPI. The most prevalent malocclusion type was Class I, observed in 54.17% of the subjects, while the most frequent jaw relationship was orthognathic, found in 70.83% of participants. The IOTN examination revealed that 41.67% of the subjects were classified in grade 1. All participants (100%) demonstrated mild gingivitis based on the GI and OPI score of 4, corresponding to the poor oral hygiene category.

Based on the type of toothpaste, the *P. gingivalis* count showed a change in $2^{-\Delta\Delta Ct}$ values before (T0) and one month after (T1) treatment. The *L. paracasei* probiotic toothpaste group showed an average decrease of 5.59×10^6 before treatment to 5.03×10^3 after one month using the toothpastes. The CPC toothpaste group showed an average decrease from 3.11×10^3 to 4.79×10^2. The amyloglucosidase–glucose oxidase enzyme toothpaste group showed a greater average decrease from 1.19×10^7 to 1.92×10^3. The *A. actinomycetemcomitans* count also showed a change in $2^{-\Delta\Delta Ct}$ values before (T0) and one month after (T1) treatment in all three toothpaste groups. The group using *L. paracasei* probiotic toothpaste showed an average decrease of 9.24 before treatment to 1.31 after treatment. The CPC toothpaste group saw an average decrease from 2.89 to 0.65. The amyloglucosidase–glucose oxidase enzyme toothpaste group also showed a greater average decrease from 18.62 to 2.82 [Table 2].

The analysis then continued with the evaluation of the mean natural logarithm (NL) values of *P. gingivalis* at baseline (T0) and one month after treatment (T1) across the three toothpaste groups, as presented in Table 3. At baseline, the highest mean NL value was observed in the *L. paracasei* probiotic toothpaste group (7.35 ± 6.63), followed by the amyloglucosidase–glucose oxidase enzyme toothpaste group (6.84 ± 6.42), and the CPC toothpaste group (4.81 ± 2.81). The overall mean NL value of the three groups before treatment was 6.33 ± 5.44. After one month of treatment, a reduction

Table 2: Minimum, maximum, and average Ct values of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* bacteria before (T0) and one month after (T1) treatment based on the type of toothpaste group ($2^{-\Delta\Delta Ct}$)

Toothpaste groups	Treatment time	*P. gingivalis*			*A. actinomycetemcomitans*		
		Minimum value	Maximum value	Average value	Minimum range	Maximum range	Average value
L. paracasei probiotic	T0	2.22	4.22×10^{7}	5.59×10^{6}	1.54	53.10	9.24
	T1	0.28	3.89×10^{4}	5.03×10^{3}	0.07	8.45	1.31
CPC	T0	6.82	2.11×10^{4}	3.11×10^{3}	0.84	9.88	2.89
	T1	1.26	2.56×10^{6}	4.79×10^{2}	0.02	2.55	0.65
Amyloglucosidase–glucose oxidase enzyme	T0	1.33	9.53×10^{7}	1.19×10^{7}	2.05	83.34	18.62
	T1	0.43	1.22×10^{4}	1.92×10^{3}	0.33	9.49	2.82

P. gingivalis=*Porphyromonas gingivalis*, *A. actinomycetemcomitans*=*Aggregatibacter actinomycetemcomitans*, *L. paracasei*=*Lactobacillus paracasei*, CPC=Cetylpyridinium chloride

Table 3: Analysis of the normal logarithm (NL) values of the average *Porphyromonas gingivalis* and mean values of *Aggregatibacter actinomycetemcomitans* mean values before (T0) and one month after (T1) treatment in the three toothpaste groups

Toothpaste groups	*n*	NL values of *P. gingivalis*		Mean values of *A. actinomycetemcomitans*	
		T0	T1	T0	T1
L. paracasei probiotic	8	7.35±6.63	1.99±4.37	9.24±17.79	1.31±2.90
CPC	8	4.81±2.81	2.93±2.98	2.89±3.15	0.65±0.89
Amyloglucosidase–glucose oxidase enzyme	8	6.84±6.42	2.48±4.10	18.62±27.62	2.82±3.26
Total	24	6.33±5.44	2.47±3.71	10.25±19.37	1.59±2.63

NL=Natural logarithm, *P. gingivalis*=*Porphyromonas gingivalis*, *A. actinomycetemcomitans*=*Aggregatibacter actinomycetemcomitans*, *L. paracasei*=*Lactobacillus paracasei*, CPC=Cetylpyridinium chloride

in the mean NL values was observed in all groups. The *L. paracasei* probiotic toothpaste group demonstrated a mean NL value of 1.99 ± 4.37, the amyloglucosidase–glucose oxidase enzyme toothpaste group recorded 2.48 ± 4.10, and the CPC toothpaste group showed 2.93 ± 2.98. The combined mean NL value across all groups after treatment was 2.47 ± 3.71. The control of Ct values obtained from the laboratory procedure was 36.25 for *P. gingivalis* ATCC 33277 and 31.48 for *A. actinomycetemcomitans* ATCC 29522.

The analysis of the mean values of *A. actinomycetemcomitans* was conducted at baseline (T0) and one month after treatment (T1) across the three toothpaste groups [Table 3]. At baseline, the *L. paracasei* probiotic toothpaste group demonstrated a mean value of 9.24 ± 17.79, the CPC toothpaste group recorded 2.89 ± 3.15, and the amyloglucosidase–glucose oxidase enzyme toothpaste group demonstrated the highest value at 18.62 ± 27.62. The overall mean value of the three groups before treatment was 10.25 ± 19.37. Following one month of treatment, a reduction in mean values was observed in all groups. The *L. paracasei* probiotic toothpaste group exhibited a mean value of 1.31 ± 2.90, the CPC toothpaste group recorded 0.65 ± 0.89, and the amyloglucosidase–glucose oxidase enzyme toothpaste group demonstrated 2.82 ± 3.26. The combined mean value across all groups after treatment was 1.59 ± 2.63 [Table 3].

The average NL values for the *P. gingivalis* groups and mean values for the *A. actinomycetemcomitans* groups were then tested using Mauchly's test of sphericity. The Mauchly's test yielded a value of 1, indicating that the requirement for homogeneity of covariance for the two-way repeated measures ANOVA was fully met for those two groups. Overall, there was a significant difference between the *P. gingivalis* groups before (T0) and one month after (T1) treatment. This is evident in the average NL T0 value of *P. gingivalis* of 6.33 ± 5.44, which decreased to 2.47 ± 3.71 at T1. The results of the assumed sphericity test for treatment time [Table 4] showed a p value of 0.021 (p <0.05), which means that there was a significant difference between the *A. actinomycetemcomitans* groups before (T0) and one month after (T1) treatment. This can be seen in the average T0 value of *A. actinomycetemcomitans* of 10.25 ± 19.37, which decreased in the average T1 value to 1.59 ± 2.63. To assess the differences among the three toothpaste groups, the assumed sphericity test was applied to evaluate the interaction between time and treatment group [Table 4]. The analysis yielded a p value of 0.367 (p > 0.05), indicating no statistically significant difference. A decrease in the mean value of *P. gingivalis* was observed from baseline (T0) to one month after treatment (T1) across all three toothpaste groups, namely, *L. paracasei* probiotic toothpaste, CPC toothpaste, and amyloglucosidase–glucose oxidase enzyme toothpaste. Similarly, for *A. actinomycetemcomitans*, the assumed sphericity test produced a p value of 0.298 (p > 0.05), demonstrating no significant difference between the three groups. Although reductions in bacterial counts were evident in each group, the extent of decrease did not

Table 4: Results of the two-way repeated analysis of variance test with sphericity assumed on *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* before (T0) and one month after (T1) treatment in the three toothpaste groups

Assumed sphericity test variable	*P. gingivalis*		*A. actinomycetemcomitans*	
	Mean square	*p*	Mean square	*p*
Treatment time	1.79×10^2	<0.05	8.99×10^2	<0.05
Treatment time * treatment group	12.79	0.367	1.85×10^2	0.298

P. gingivalis=*Porphyromonas gingivalis*, *A. actinomycetemcomitans*=*Aggregatibacter actinomycetemcomitans*, $p<0.05$

differ significantly, suggesting that all three toothpastes produced relatively comparable outcomes in reducing *A. actinomycetemcomitans*.

The results of the OPI assessment. At baseline (T0), the mean OPI score in all three toothpaste groups, namely, *L. paracasei* probiotic toothpaste, CPC toothpaste, and amyloglucosidase–glucose oxidase enzyme toothpaste, was 4. Similarly, at one month after treatment (T1), the mean OPI score remained unchanged at 4 across all groups.

Discussion

Patients undergoing treatment with fixed orthodontic appliances frequently encounter difficulties in maintaining optimal oral hygiene, as the components of the appliances may hinder effective cleaning. Consequently, these patients are at increased risk of periodontal tissue damage due to plaque accumulation and bacterial colonization.[7] The primary determinant of oral health maintenance is effective plaque control, which includes toothbrushing, interdental cleaning, and the use of mouth rinses.[5,11] Beyond mechanical methods of plaque removal, the selection of toothpaste also plays an essential role in plaque control, aiming to reduce bacterial load within the oral cavity.[15]

Adolescents are an appropriate population for studying periodontal pathogens, such as *A. actinomycetemcomitans* and *P. gingivalis*, because they commonly undergo fixed orthodontic treatment, which promotes plaque retention and bacterial colonization due to appliance components that hinder cleaning.[8] Poor oral hygiene compliance in this age group further facilitates the proliferation of pathogenic bacteria associated with early periodontal changes. Studies have reported that *A. actinomycetemcomitans* and *P. gingivalis* are frequently detected in adolescents with gingival inflammation or early attachment loss during orthodontic treatment.[16] The prevalence of aggressive or early-onset periodontitis linked to these pathogens among adolescents ranges between 0.3% and 5.9%, emphasizing their importance as a high-risk group for periodontal research.[17]

In this study, saliva was employed as the diagnostic medium owing to its ease, rapidity, and noninvasive nature of collection. Saliva provides valuable insight into the oral environment, including bacterial load and the severity of periodontal disease.[18] Stimulated saliva was chosen because the mechanical action of chewing paraffin wax facilitates the release of bacteria from the gingival sulcus, thereby enhancing the detection of periodontal pathogens.[19] However, while gingival crevicular fluid (GCF) offers higher site specificity for sampling bacteria and mediators directly from the periodontal pocket, it has drawbacks. GCF collection is technically demanding, requires multiple site‐specific samples, prone to contamination with saliva, blood or plaque, and often involves low fluid volume and extensive laboratory processing.[20] Consequently, although GCF may provide more direct information about local periodontal microbiology, for larger scale screening or monitoring purposes saliva remains a more practical and efficient alternative.[20,21]

DNA-based detection methods, such as qPCR, are widely used to estimate bacterial load because they offer high sensitivity, specificity, and the ability to identify target species even at low concentrations.[22] Although these techniques cannot distinguish between live and dead bacteria, they provide a reliable measure of total bacterial presence and are less affected by sample handling or bacterial viability compared to culture-based methods.[23] Additionally, many oral pathogens, including *P. gingivalis* and *A. actinomycetemcomitans*, are fastidious and difficult to culture, making DNA quantification a practical and efficient alternative for evaluating microbial changes in clinical studies.[24]

Toothpaste is available in several forms, such as paste, gel, powder, and liquid. It generally contains two types of ingredients, like non-active and active components. Non-active ingredients do not have therapeutic effects but determine the toothpaste's physical properties, including texture, taste, consistency, and appearance, and usually consist of water, abrasives, humectants, binders, flavors, surfactants, preservatives, and colorants.[25,26] Active ingredients, however, provide therapeutic benefits, such as preventing cavities, reducing plaque, controlling sensitivity, eliminating bad breath, and offering antimicrobial effects. These include enzymes, CPC, and probiotics.[25]

The findings demonstrated significant reductions in *P. gingivalis* and *A. actinomycetemcomitans* counts

following the use of *L. paracasei* probiotic toothpaste, CPC toothpaste, and amyloglucosidase–glucose oxidase enzyme toothpaste among patients with fixed orthodontic appliances. This suggests that all three toothpaste formulations exhibit antibacterial effects.[12-14] However, no statistically significant differences were observed in the degree of bacterial reduction among the three groups, which may be attributed to the distinct mechanisms of action of the active ingredients in each toothpaste in inhibiting bacterial growth.

Probiotics are defined as microorganisms that confer health benefits to the host when consumed in adequate amounts. Over the past decade, a growing body of research has highlighted their therapeutic and preventive potential in maintaining oral health. Probiotics are known to modulate both specific and nonspecific immune responses, enhance epithelial barrier function, produce antimicrobial substances, and inhibit the adhesion of pathogenic bacteria within the oral cavity.[27] Among the antimicrobial substances produced by probiotics are bacteriocins and organic acids. Organic acids, particularly acetic acid and lactic acid, play a central role in the inhibitory activity of probiotics against pathogenic species. These acids are able to penetrate bacterial cell membranes, thereby acidifying the intracellular environment, which ultimately leads to bacterial death, especially in Gram-negative organisms.[28]

Chuang *et al*. reported that oral administration of *L. paracasei* GMNL-33 exhibited anticariogenic properties by significantly reducing *Streptococcus mutans* levels in the oral cavity.[29] Similarly, Lee *et al*. demonstrated in a clinical study that *L. paracasei* GMNL-143-based probiotic toothpaste possesses the ability to co-aggregate with oral pathogens and inhibit their adhesion to gingival tissues.[30] The antibacterial effect of *L. paracasei* is more pronounced under acidic conditions compared with neutral pH environments. This enhanced activity in acidic conditions occurs because peptides are attracted to the phosphate groups of lipopolysaccharide molecules, initiating pore formation in the bacterial membrane. Such changes in membrane permeability led to structural disruption and compromise membrane integrity, ultimately resulting in bacterial cell lysis.[31] These findings are consistent with the present study, in which *L. paracasei*-containing probiotic toothpaste was shown to effectively reduce bacterial counts in the oral cavity.

CPC, another active ingredient found in certain toothpaste formulations, is a quaternary ammonium compound with well-established antimicrobial properties. Following use, CPC remains distributed within the oral cavity due to its surfactant chains and cationic charges, which enable sustained absorption onto oral surfaces.[32,33] Structurally, CPC contains hydrophilic and hydrophobic groups. The positively charged hydrophilic groups promote electrostatic binding to the negatively charged surfaces of pathogenic bacteria, while the hydrophobic groups interact with bacterial membranes, facilitating integration into the cytoplasmic membrane. These dual interactions lead to disruption of membrane integrity, impairment of cellular metabolism, cytoplasmic leakage, and eventual bacterial death. In addition, CPC reduces microbial adhesion to oral surfaces, thereby limiting colonization.[32] These mechanisms are consistent with the findings of Vasconcelos *et al*., who demonstrated that CPC-containing toothpaste significantly reduced bacterial counts in the oral cavity through decreased plaque accumulation and gingival inflammation.[13]

Toothpaste formulations containing the enzymes amyloglucosidase and glucose oxidase are reported to exert antimicrobial effects. The amyloglucosidase enzyme inhibits bacterial proliferation by converting D-glucose into D-glucono-1,5-lactone, thereby reducing the availability of bacterial nutrients in the oral cavity. Meanwhile, glucose oxidase activates the salivary immune defense system, specifically the lactoperoxidase (LPO) pathway, by generating hydrogen peroxide. This hydrogen peroxide interacts with catalase to produce oxygen, reducing the prevalence of anaerobic bacteria. Furthermore, hydrogen peroxide activates the LPO system to generate hypothiocyanite, a compound with antibacterial activity against *P. gingivalis*.[34,35] The findings of this study indicate that toothpaste containing amyloglucosidase and glucose oxidase produced greater reductions in both *P. gingivalis* and *A. actinomycetemcomitans* compared to the other tested toothpastes. This outcome is consistent with the choice of saliva as a diagnostic tool, as the enzymatic mechanisms are directly linked to salivary immune activity.

As a member of the "red complex," *P. gingivalis* exhibits strong virulence through its capacity to aggregate with other bacterial species, facilitating colonization during later stages of biofilm development and rendering it difficult to eliminate.[9] Likewise, *A. actinomycetemcomitans* produces a wide range of virulence factors to ensure survival within the oral cavity.[36] Both species contribute to robust biofilm formation, aided by antimicrobial-resistant fimbriae and extracellular polysaccharides that hinder immune cell penetration and phagocytosis. These properties allow both pathogens to induce periodontal tissue damage.[31] The present study demonstrates a reduction in the levels of *P. gingivalis* and *A. actinomycetemcomitans*, which may help mitigate the risk of periodontal complications in patients with fixed orthodontic appliances.

Several studies have demonstrated a strong association between the presence of *P. gingivalis* and *A. actinomycetemcomitans* in saliva, the gingival sulcus,

and dental biofilm. These bacteria are recognized as key periodontal pathogens and have been shown to colonize multiple oral niches simultaneously. A qPCR study by Reddahi *et al*. found significantly higher levels of *P. gingivalis* and *A. actinomycetemcomitans* in both whole saliva and subgingival plaque from periodontitis patients compared to healthy controls. Moreover, they report a *strong positive correlation* between *A. actinomycetemcomitans* and *P. gingivalis* in the diseased subgingival sites and in saliva.[37] Saliva often serves as a reservoir that reflects the microbial composition of subgingival and supragingival biofilms, including the presence of *P. gingivalis* and *A. actinomycetemcomitans*. Their detection in saliva correlates with their colonization in periodontal pockets and dental biofilm, because these pathogens disseminate through oral fluids and are shed from biofilm communities on tooth surfaces. Furthermore, previous research has demonstrated that salivary levels of these bacteria are significantly associated with periodontal inflammation, pocket depth, and microbial loads within the gingival sulcus, supporting the relevance of saliva as a diagnostic medium for monitoring periodontal pathogens.[19,20,37] Taken together, the evidence supports that the presence of *P. gingivalis* and *A. actinomycetemcomitans* in saliva corresponds to their presence and activity within the gingival sulcus and dental biofilm.

The bacterial increase observed in patients with fixed appliances is attributable to the additional niches created by the orthodontic elements. Clinically, the number of oral bacteria has been shown to triple within the first six months following appliance placement.[38] Furthermore, plaque control becomes increasingly difficult in cases of dental misalignment. In this study, no significant changes were observed in plaque index scores before and after the use of probiotic *L. paracasei*, CPC, or amyloglucosidase–glucose oxidase toothpastes. This finding reflects the persistent cycle of plaque formation, as bacterial communities consistently recolonize tooth surfaces. Plaque development begins with pellicle formation initiated by *Streptococcus sanguinis*, followed by the coaggregation of pathogenic species such as *P. gingivalis*, *A. actinomycetemcomitans*, *Fusobacterium nucleatum*, *Treponema denticola*, and *Prevotella intermedia*.[39,40]

Mechanical plaque removal through toothbrushing eliminates only part of the biofilm, as microbial colonization can lead to dysbiosis. *P. gingivalis* plays a central role in this process, functioning as a "keystone pathogen" that manipulates host immune responses and disrupts homeostasis within the oral microbiome. Even at low concentrations, *P. gingivalis* can interact with other microorganisms to promote colonization.[41,42] Consequently, reductions in bacterial counts observed in this study could occur despite relatively unchanged plaque index values. This is explained by the complex biofilm composition of dental plaque, which consists not only of microbial cells but also of extracellular polysaccharides, proteins, and structural molecules that stabilize the biofilm matrix.[40,43]

Additionally, the design and placement of orthodontic appliances contribute significantly to bacterial accumulation and plaque formation. Archwire ligatures serve as additional sites for bacterial colonization, and brackets positioned near the cervical margin can increase the risk of gingivitis.[44,45] The bracket material itself also plays a role: In this study, stainless steel appliances were used, which exhibit higher surface tension and are therefore more prone to plaque retention.[43]

Plaque retention varies among individuals due to differences in plaque formation patterns, oral hygiene practices, and dietary habits.[46] The effectiveness of toothbrushing as a plaque control method is highly dependent on patient compliance, as brushing is a complex and technique-sensitive process. Short-term use of toothpaste has been shown to exert only minimal influence on mechanical plaque removal.[47] Brushing technique plays a critical role in maintaining oral health, particularly for patients with fixed orthodontic appliances, who often experience challenges in adequately cleaning around appliance components. A common error is positioning the toothbrush too coronally, which results in neglect of the cervical region of the teeth and consequently increases plaque accumulation, predisposing patients to gingivitis.[33]

Plaque index was chosen instead of pocket depth or bleeding index because the presence of orthodontic brackets can make periodontal probing difficult and lead to measurement bias. The brackets and archwires hinder probe access and compromise the accuracy of assessing pocket depth and bleeding on probing.[48] Therefore, the plaque index provides a more practical and reliable parameter for evaluating oral hygiene during orthodontic treatment.[49] In addition, the plaque index reflects supragingival plaque accumulation, which is particularly relevant for orthodontic patients who are more prone to plaque retention due to appliance design.[49,50]

Toothbrush selection is also an important factor. The use of orthodontic toothbrushes characterized by a concave bristle arrangement and smaller brush head has been recommended, as these features allow for better adaptation to tooth surfaces and enhance cleaning efficacy around brackets, archwires, and interdental areas.[51] In addition, electric toothbrushes may serve as an effective alternative, as their vibratory action

facilitates the removal of both supragingival and subgingival plaque. Professional dental cleaning at each follow-up appointment is likewise essential for patients undergoing fixed orthodontic treatment to further support oral hygiene maintenance.[38]

Conclusions

The use of probiotic toothpaste containing *L. paracasei*, CPC toothpaste, and enzymatic toothpaste containing amyloglucosidase–glucose oxidase was found to reduce the levels of *P. gingivalis* and *A. actinomycetemcomitans* but had no effect on the plaque index in patients with fixed orthodontic appliances. There was no significant difference in the reduction of these bacteria among the three types of toothpaste. Therefore, it can be concluded that all three formulations have similar potential in preventing plaque formation and periodontal disease in patients undergoing fixed orthodontic treatment. Further research is expected to include other bacteria than *P. gingivalis* and *A. actinomycetemcomitans* that cause periodontal disease and also longer periods of toothpaste use to provide more comprehensive results.

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Conflicts of interest

There are no conflicts of interest.

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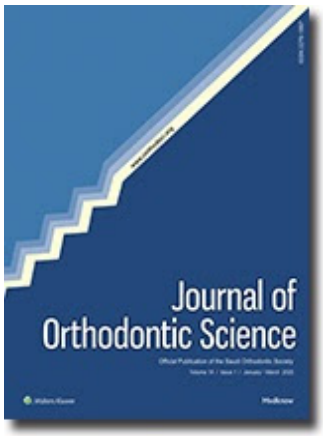

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