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COMPARATIVE EVALUATION OF ANTIMICROBIAL EFFICACY OF NANOCHITOSAN-COATED ORTHODONTIC BRACKETS AGAINST FOUR ORAL MICROORGANISMS: AN IN VITRO STUDY

Running title: Nanochitosan Coatings and Antimicrobial Activity of Orthodontic Brackets

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ABSTRACT

Background and Aim. Fixed orthodontic appliances create plaque-retentive microenvironments that promote colonization by cariogenic and opportunistic microorganisms, increasing the risk of enamel demineralization and white spot lesion (WSL) formation. Conventional compliance-dependent preventive strategies demonstrate limited long-term efficacy. Surface modification of orthodontic brackets with antimicrobial nanocoatings offers a promising compliance-independent approach. This study aimed to comparatively evaluate the antimicrobial efficacy of nanochitosan-coated stainless-steel orthodontic brackets against four clinically relevant oral microorganisms in an in vitro setting.

Methods. A total of 160 pre-adjusted edgewise stainless-steel maxillary premolar brackets (MBT prescription, 0.022″ slot) were divided into two groups of 80 each: Group A (nanochitosan-coated) and Group B (uncoated control). Nanochitosan nanoparticles (molecular weight 3,800–20,000 Da; mean particle size ~50 nm) were deposited on bracket surfaces via a standardized hydrothermal method and confirmed by scanning electron microscopy (SEM) and field emission SEM (FESEM). Four microorganisms *Streptococcus mutans, Lactobacillus acidophilus, Enterococcus faecalis,* and *Staphylococcus aureus* were standardized to 10^5 CFU/mL using a spectrophotometer (OD 600

nm) and inoculated into Mueller–Hinton broth containing bracket samples. Colony-forming unit (CFU) counts were recorded at T0 (1 h), T1 (6 h), T2 (12 h), T3 (24 h), and T4 (72 h). Intragroup and intergroup comparisons were performed using paired t-test and one-way ANOVA, respectively, at a 95% confidence level ($p \leq 0.05$).

Results. No microbial growth was detected in either group at 1 and 6 hours. From 12 hours onward, uncoated brackets demonstrated a progressive and statistically significant increase in CFU counts for all four organisms, reaching mean values of 123.75 ± 2.446, 124.45 ± 2.459, 123.80 ± 2.876, and 124.65 ± 1.954 × 10^5 CFU/mL at 72 h for *S. mutans, L. acidophilus, E. faecalis,* and *S. aureus*, respectively. In contrast, nanochitosan-coated brackets maintained consistently low and stable CFU counts (22–24 × 10^5 CFU/mL) across all time points. Intergroup differences were highly significant for all organisms at all post-baseline intervals (F values 1,025–27,604; $p < 0.001$).

Conclusions. Nanochitosan surface modification significantly enhances the antimicrobial properties of stainless-steel orthodontic brackets, providing broad-spectrum, sustained inhibition of bacterial adhesion and proliferation. This compliance-independent coating strategy holds considerable potential for reducing biofilm accumulation, enamel demineralization, and white spot lesion formation during fixed orthodontic treatment. Further in vivo studies are warranted to confirm clinical durability and biocompatibility.

Keywords: *nanochitosan; orthodontic brackets; antimicrobial activity; white spot lesions; biofilm inhibition; Streptococcus mutans*

1. INTRODUCTION

Fixed orthodontic therapy remains the cornerstone of contemporary dental management of malocclusions, offering precise three-dimensional control of tooth movement. However, the introduction of brackets, archwires, and auxiliaries creates complex retentive surfaces that profoundly alter the oral microenvironment (Featherstone, 2004; Hicks et al., 2003). Surface irregularities on stainless-steel brackets with a mean average surface roughness (Ra) of 0.2 to 0.5 μm and marginal micro-gaps of 20–50 μm at the bracket–adhesive interface together provide an optimal topography for early bacterial adhesion and subsequent biofilm maturation (Eliades & Brantley, 1995; Gwinnett & Ceen, 1979).

Molecular analyses using 16S rRNA sequencing have demonstrated a pronounced ecological shift toward a more cariogenic oral microbiome in patients undergoing fixed orthodontic therapy, characterized by elevated counts of acidogenic and aciduric species (Zheng et al., 2019). Among these, *Streptococcus mutans* constitutes the primary etiological agent of enamel demineralization owing to its ability to synthesize extracellular polysaccharide matrices, lower plaque pH below the critical demineralization threshold of 5.5, and form structured biofilms on bracket surfaces (Marsh, 1994; Stephan, 1940; Lin et al., 2018). *Lactobacillus acidophilus*, as a secondary colonizer, accelerates lesion progression in established cariogenic environments, while *Enterococcus faecalis* and *Staphylococcus aureus* represent clinically relevant opportunistic pathogens linked to oral infections and biofilm-mediated complications (Badet & Thebaud, 2008; Stuart et al., 2006).

The clinical consequence of this dysbiotic shift is the development of white spot lesions (WSLs) the earliest macroscopic manifestation of enamel caries affecting between 50% and 70% of

orthodontic patients in high-risk populations (Tufekci et al., 2011; Julien et al., 2013). WSLs can emerge as early as four weeks after bracket placement, and post-orthodontic restorative management of these lesions adds an estimated 15–20% to total treatment cost (Chapman et al., 2010). Conventional preventive strategies, including fluoride varnish (5% NaF), chlorhexidine (CHX) mouthrinses, and casein phosphopeptide–amorphous calcium phosphate (CPP-ACP) formulations, demonstrate moderate efficacy but are substantially constrained by patient compliance, particularly among adolescents who constitute the majority of the orthodontic population (Stecksén-Blicks et al., 2007; Derks et al., 2004; Scheie et al., 1984).

Nanotechnology-based surface modifications of orthodontic appliances have emerged as a promising compliance-independent strategy to address these limitations. Among available nanomaterials including silver, zinc oxide, titanium dioxide, and copper chitosan-based nanoparticles have attracted particular interest owing to their exceptional biocompatibility, biodegradability, broad-spectrum antimicrobial activity, and low inherent toxicity (Ke et al., 2021; Rinaudo, 2006). Chitosan, a linear polysaccharide derived from chitin via alkaline deacetylation, exerts its antimicrobial effects through electrostatic interaction of its protonated amino groups ($-NH_3^+$) with negatively charged bacterial cell envelope components, including lipopolysaccharides in Gram-negative bacteria and teichoic acids in Gram-positive organisms (Raafat & Sahl, 2009; Helander et al., 2001). This polycationic interaction disrupts membrane integrity, increases permeability, induces leakage of intracellular constituents, and ultimately causes cell lysis.

Transition to the nanoscale confers additional physicochemical advantages: nanochitosan particles (typically 20–100 nm) exhibit three to five times greater surface charge density relative to bulk chitosan, enhanced penetration into biofilm matrices, improved mucoadhesive properties for prolonged surface retention, and controlled-release kinetics (Ke et al., 2021; Kong et al., 2008). Moreover, chitosan's multifactorial mechanism of antibacterial action encompassing membrane disruption, chelation of divalent metal ions essential for microbial enzyme function, and inhibition of DNA transcription minimizes the risk of promoting antimicrobial resistance compared with conventional single-target agents (Rabea et al., 2003; Wang et al., 2023).

Despite growing preclinical evidence supporting the antimicrobial potential of chitosan-modified orthodontic surfaces (Mendes et al., 2019; Butler et al., 2023; Di Palma et al., 2024), studies simultaneously evaluating nanochitosan-coated brackets against multiple clinically representative microorganisms including both cariogenic and opportunistic species across multiple time intervals remain limited. The present in vitro investigation was therefore designed to comparatively evaluate the antimicrobial efficacy of nanochitosan-coated stainless-steel orthodontic brackets against *Streptococcus mutans, Lactobacillus acidophilus, Enterococcus faecalis,* and *Staphylococcus aureus*, with the hypothesis that nanochitosan surface modification would provide statistically significant and sustained inhibition of bacterial growth compared with uncoated control brackets.

2. MATERIALS AND METHODS

2.1 Study Design and Setting

This prospective in vitro experimental study was conducted at the Department of Orthodontics and Dentofacial Orthopaedics, Inderprastha Dental College and Hospital, Sahibabad, Ghaziabad, Uttar Pradesh, India (201010), during the academic year 2023–2026.

2.2 Ethical Approval

The study protocol was reviewed and approved by the Institutional Ethics Committee of Inderprastha Dental College and Hospital (Reference No. IPDC/Th/2024/170B[27]). As this investigation employed extracted teeth and standard bacterial reference strains in an in vitro setting, individual informed consent was not applicable; however, all procedures complied with the Declaration of Helsinki.

2.3 Sample Size and Group Allocation

Sample size was calculated using G*Power Version 3.1.9.6 (Franz Faul, University of Kiel), with a two-tailed formula incorporating a power of 94%, an effect size f = 0.28, and a type I error rate (α) of 0.05. The estimated total sample size was 160 brackets, yielding 80 per group. A pilot study using 16 brackets (10% of total sample) confirmed methodological feasibility and reproducibility. A total of 160 pre-adjusted edgewise stainless-steel maxillary premolar brackets (Mini Diamond, ORMCO; MBT prescription, 0.022″ slot) were randomly assigned to two groups:

- **Group A (n = 80):** Nanochitosan-coated brackets
- **Group B (n = 80):** Uncoated stainless-steel brackets (control)

Each group was subdivided into four subgroups of 20 brackets each (A1–A4 and B1–B4), corresponding to the four tested microorganisms.

2.4 Inclusion and Exclusion Criteria

Inclusion criteria comprised: (i) sound, intact human maxillary premolar teeth exhibiting no cracks, decalcification, carious lesions, or existing restorations; and (ii) brackets demonstrating uniform nanochitosan coating verified by SEM and FESEM. Premolars with enamel defects, existing restorations, or non-uniform coating were excluded.

2.5 Nanochitosan Coating Protocol

Brackets were pre-cleaned with deionized water followed by ethanol at 80°C for 30 minutes to remove surface oxide layers. Nanochitosan nanoparticles were synthesized and deposited via a hydrothermal approach (Li et al., 2016). Briefly, 0.5 g of chitosan powder (molecular weight 3,800–20,000 Da) was dispersed in 30 mL of distilled water with continuous stirring for 30 minutes. A 1.5 M acetic acid solution was added dropwise under continuous agitation until the mixture reached pH 10.9, facilitating nanoparticle formation. Brackets and the resultant suspension were transferred to a Teflon-lined stainless-steel autoclave and subjected to hydrothermal treatment at 90°C for 8 hours, during which chitosan nanoparticles were generated and simultaneously deposited onto bracket surfaces. Following gradual cooling to ambient temperature, coated brackets were retrieved, rinsed thoroughly with distilled water, and air-dried. Successful coating was indicated by a characteristic matte surface finish.

2.6 Surface Characterization (SEM and FESEM)

Four randomly selected nanochitosan-coated brackets underwent scanning electron microscopy (SEM) at magnifications of ×42, ×70, ×500, ×1,000, and ×1,500 to verify coating homogeneity. Field emission SEM (FESEM) at ×20,000 and ×30,000 confirmed spherical nanoparticle morphology with a mean particle size of approximately 50 nm and uniform surface distribution. Uncoated brackets were also examined as controls.

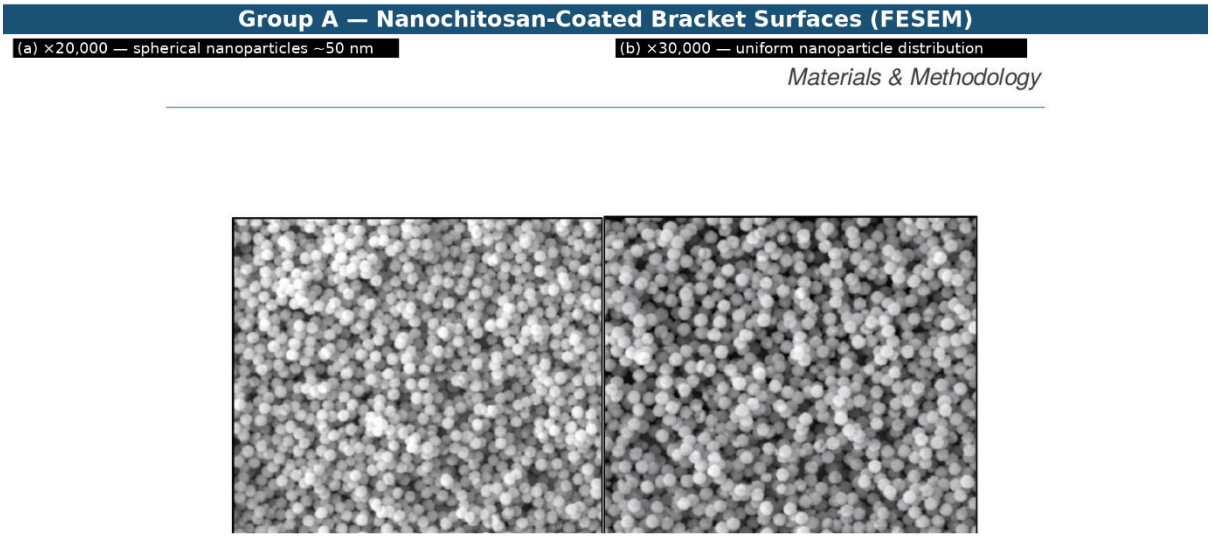


Figure 2. FESEM micrographs of nanochitosan-coated orthodontic bracket surfaces (Group A). Panels (a) and (b) at ×20,000 and ×30,000 magnification reveal a dense, homogeneous distribution of spherical chitosan nanoparticles with a mean diameter of approximately 50 nm, confirming uniform coating coverage achieved by hydrothermal deposition. Scale bar = 1.00 µm.

2.7 Bracket Bonding Procedure

Crowns of 160 extracted maxillary premolars were prepared and randomly allocated into four microorganism groups (20 samples per organism per group). Buccal enamel surfaces were etched with 37% phosphoric acid for 15–30 seconds, rinsed with water for 20 seconds, and dried using oil-free compressed air. A light-cured adhesive primer (ORMCO Enlight) was applied and polymerized for 5–10 seconds. Brackets were positioned on the middle third of the buccal crown, aligned with the tooth long axis, and bonded using light-cured adhesive paste, with excess resin removed and light-cured for 5–10 seconds (Cörekçi et al., 2014).

2.8 Microorganisms and Antimicrobial Assay

Four clinically relevant oral microorganisms were selected: *Streptococcus mutans* (subgroup A1/B1), *Lactobacillus acidophilus* (A2/B2), *Enterococcus faecalis* (A3/B3), and *Staphylococcus aureus* (A4/B4). Pure cultures were grown on Mueller–Hinton Broth (MHB) at 37°C for 24 hours. Bacterial identity was confirmed by Gram staining. Suspensions were standardized to 10^5 CFU/mL using optical density measurement at 600 nm (Wiegand et al., 2008; Andrews, 2001). All bracket samples were autoclaved prior to exposure to eliminate pre-existing contamination.

Each bacterial strain was inoculated into 30 mL of MHB containing sterile bracket samples (Group A or Group B) and incubated aerobically at 37°C. Aliquots were serially diluted and plated on Mueller–Hinton Agar (MHA) for CFU enumeration at five predetermined time intervals: T0 (1 h),

T1 (6 h), T2 (12 h), T3 (24 h), and T4 (72 h). This schedule allowed assessment of both immediate and sustained antimicrobial effects.

2.9 Statistical Analysis

Data were recorded in Microsoft Excel 2010 and analyzed using SPSS Version 27.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics were reported as mean ± standard deviation (SD). Data normality was assessed using the Kolmogorov–Smirnov test. Intragroup comparisons of CFU counts at sequential time intervals were performed using the paired t-test. Intergroup comparisons between Group A and Group B at each time interval were analyzed by one-way ANOVA. A confidence interval of 95% and a significance threshold of $p \leq 0.05$ were applied throughout.

3. RESULTS

The antimicrobial assay encompassed 160 stainless-steel orthodontic brackets divided equally between Group A (nanochitosan-coated) and Group B (uncoated control), with each group subdivided into four subgroups corresponding to the four tested microorganisms.

3.1 Baseline and Early Incubation (T0 and T1)

No microbial growth was detected in either group at T0 (1 h) or T1 (6 h) for any of the four organisms, confirming sterile baseline conditions and validating the autoclave sterilization protocol. This finding was consistent across all 20 replicate samples within each subgroup.

3.2 Intragroup Analysis: Group A (Nanochitosan-Coated Brackets)

A highly significant initial increase in CFU counts was observed between T1 and T2 across all subgroups ($p < 0.001$), reflecting the onset of detectable microbial growth at 12 hours. Specifically, for *S. mutans* (A1), the mean CFU count rose to $22.45 \pm 1.877 \times 10^5$ CFU/mL at T2 ($t = -53.485$; $p < 0.001$). For *L. acidophilus* (A2), T2 counts were $23.9 \pm 2.673 \times 10^5$ CFU/mL ($t = -39.980$; $p < 0.001$). For *E. faecalis* (A3), T2 counts were $22.0 \pm 1.891 \times 10^5$ CFU/mL ($t = -52.007$; $p < 0.001$). For *S. aureus* (A4), T2 counts were $21.5 \pm 1.572 \times 10^5$ CFU/mL ($t = -61.134$; $p < 0.001$). Critically, after this initial rise, CFU counts in Group A remained low and largely stable from T2 through T4. For *S. mutans*, counts plateaued at $23.0 \pm 2.000 \times 10^5$ CFU/mL at T3 and T4. For *L. acidophilus*, counts stabilized at approximately $23.6 \pm 2.760 \times 10^5$ CFU/mL. For *E. faecalis* and *S. aureus*, similarly stable values were observed. The negligible change between T3 and T4 was non-significant for *L. acidophilus* ($p = 0.249$), indicating that the nanochitosan coating effectively arrested further microbial proliferation by the 24-hour mark (see Table 1 and Table 2).

3.3 Intragroup Analysis: Group B (Uncoated Brackets)

In contrast, Group B brackets demonstrated a progressive and highly significant increase in CFU counts at every time interval from T2 through T4 for all four microorganisms ($p < 0.001$ at all intervals). For *S. mutans* (B1), counts escalated from $53.9 \pm 2.633 \times 10^5$ CFU/mL at T2 ($t = -91.521$; $p < 0.001$) to $123.75 \pm 2.446 \times 10^5$ CFU/mL by T4 ($t = -226.184$; $p < 0.001$). Comparable patterns were observed for *L. acidophilus* (54.3 to 124.45×10^5 CFU/mL), *E. faecalis* (53.55 to 123.80×10^5 CFU/mL), and *S. aureus* (54.85 to 124.65×10^5 CFU/mL), confirming the absence of any intrinsic antimicrobial activity in uncoated brackets and unimpeded bacterial colonization and proliferation over 72 hours (see Table 3).

3.4 Intergroup Comparison

One-way ANOVA intergroup comparisons revealed highly significant differences between Group A and Group B at all post-baseline time intervals for all four tested organisms ($p < 0.001$) (Table 4). The largest differences were recorded at T4–T0, reflecting cumulative bacterial inhibition. For *S. mutans*, the overall mean reduction in Group A ($23.0 \pm 2.000 \times 10^5$ CFU/mL) versus Group B ($123.75 \pm 2.446 \times 10^5$ CFU/mL) yielded $F = 20{,}327.872$; $p < 0.001$. For *S. aureus*, the corresponding values were 22.55 ± 1.932 versus $124.65 \pm 1.954 \times 10^5$ CFU/mL ($F = 27{,}604.709$; $p < 0.001$). These findings confirm that the null hypothesis that no significant difference in antimicrobial activity exists between nanochitosan-coated and uncoated brackets must be rejected.

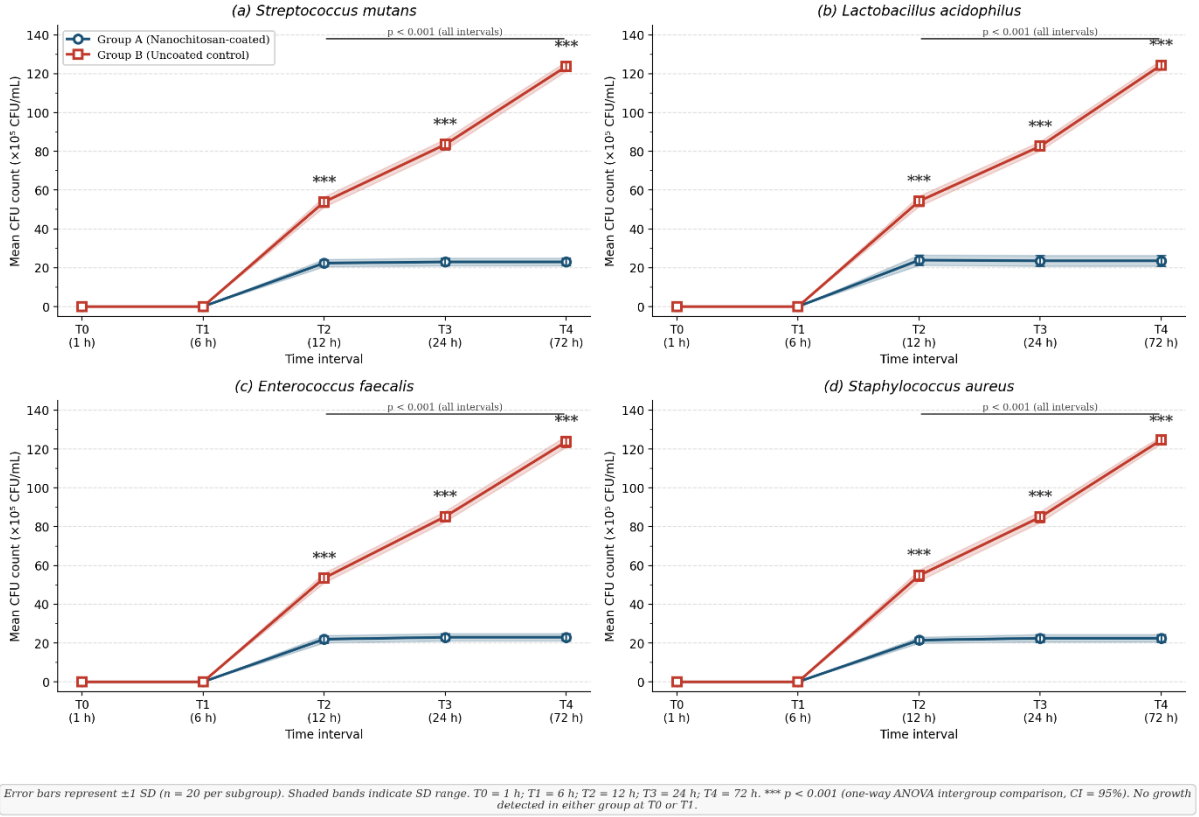
Table 1. Mean CFU counts ($\times 10^5$ CFU/mL ± SD) for Group A and Group B across all time intervals and microorganisms.

Microorganism	Time	Group A Mean	Group A SD	Group B Mean	Group B SD	Interpretation
S. mutans	T0 (1h)	0.00	0.000	0.00	0.000	No growth
	T1 (6h)	0.00	0.000	0.00	0.000	No growth
	T2 (12h)	22.4	1.877	53.9	2.633	Significant inhibition
	T3 (24h)	23.0	2.000	83.5	2.724	Sustained inhibition
	T4 (72h)	23.0	2.000	123.7	2.446	Sustained inhibition
L. acidophilus	T0 (1h)	0.00	0.000	0.00	0.000	No growth
	T1 (6h)	0.00	0.000	0.00	0.000	No growth
	T2 (12h)	23.9	2.673	54.3	2.677	Significant inhibition
	T3 (24h)	23.6	2.760	82.6	2.393	Sustained inhibition
	T4 (72h)	23.6	2.760	124.4	2.459	Sustained inhibition

Microorganism	Time	Group A Mean	Group A SD	Group B Mean	Group B SD	Interpretation
E. faecalis	T0 (1h)	0.00	0.000	0.00	0.000	No growth
	T1 (6h)	0.00	0.000	0.00	0.000	No growth
	T2 (12h)	22.0	1.891	53.5	2.502	Significant inhibition
	T3 (24h)	23.0	1.919	85.2	2.647	Sustained inhibition
	T4 (72h)	23.0	1.919	123.8	2.876	Sustained inhibition
S. aureus	T0 (1h)	0.00	0.000	0.00	0.000	No growth
	T1 (6h)	0.00	0.000	0.00	0.000	No growth
	T2 (12h)	21.5	1.572	54.8	2.777	Significant inhibition
	T3 (24h)	22.5	1.932	84.9	2.781	Sustained inhibition
	T4 (72h)	22.5	1.932	124.6	1.954	Sustained inhibition

SD = standard deviation; CFU = colony-forming units. All values expressed as mean × 10^5 CFU/mL.

Figure 3. Mean CFU counts (×10⁵ CFU/mL) over time for Group A (nanochitosan-coated) and Group B (uncoated) orthodontic brackets against four tested microorganisms



*Error bars represent ±1 SD (n = 20 per subgroup). Shaded bands indicate SD range. T0 = 1 h; T1 = 6 h; T2 = 12 h; T3 = 24 h; T4 = 72 h. *** p < 0.001 (one-way ANOVA intergroup comparison, CI = 95%). No growth detected in either group at T0 or T1.*

Table 2. Intergroup (ANOVA) comparison of antimicrobial activity between Group A and Group B at key time intervals.

Microorganism	Interval	Group A Mean ± SD	Group B Mean ± SD	F-value	p-value	Significance
S. mutans	T2–T1	22.45 ± 1.877	53.9 ± 2.633	1,891.114	<0.001	**Significant**
S. mutans	T3–T2	0.55 ± 0.998	29.6 ± 2.798	1,911.672	<0.001	**Significant**
S. mutans	T4–T0	23.0 ± 2.000	123.75 ± 2.446	20,327.872	<0.001	**Significant**
L. acidophilus	T2–T1	23.9 ± 2.673	54.30 ± 2.677	1,291.106	<0.001	**Significant**
L. acidophilus	T3–T2	0.30 ± 1.128	28.30 ± 3.310	1,337.456	<0.001	**Significant**

Microorganism	Interval	Group A Mean ± SD	Group B Mean ± SD	F-value	p-value	Significance
L. acidophilus	T4–T0	23.6 ± 2.760	124.45 ± 2.459	14,879.209	<0.001	**Significant**
E. faecalis	T2–T1	22.0 ± 1.891	53.55 ± 2.502	2,023.284	<0.001	**Significant**
E. faecalis	T3–T2	1.00 ± 1.297	31.65 ± 3.842	1,142.155	<0.001	**Significant**
E. faecalis	T4–T0	23.0 ± 1.919	123.80 ± 2.876	16,994.028	<0.001	**Significant**
S. aureus	T2–T1	21.5 ± 1.572	54.85 ± 2.777	2,183.645	<0.001	**Significant**
S. aureus	T3–T2	1.05 ± 1.316	30.10 ± 3.837	1,025.365	<0.001	**Significant**
S. aureus	T4–T0	22.55 ± 1.932	124.65 ± 1.954	27,604.709	<0.001	**Significant**

S = statistically significant ($p \leq 0.05$); CI = 95%; ANOVA = one-way analysis of variance. All CFU values × 10^5 CFU/mL.

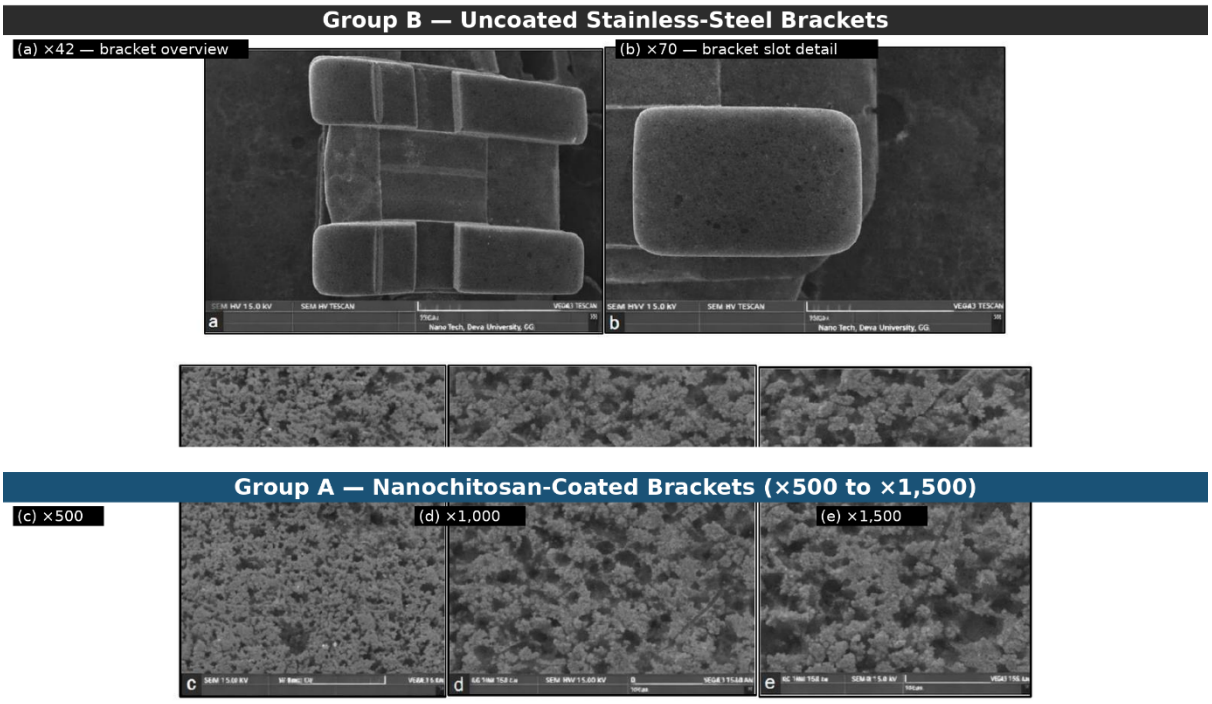


Figure 12: SEM images of nanochitosan-coated orthodontic braces at different magnifications

Figure 1. SEM micrographs of nanochitosan-coated (Group A) and uncoated (Group B) stainless-steel orthodontic brackets. Top row (Group B): panels (a) and (b) show a smooth, unmodified bracket surface. Bottom row (Group A): panels (c–e) reveal a uniformly distributed nanochitosan layer at increasing magnifications (×500–×1,500), confirming successful nanoparticle deposition via hydrothermal synthesis.

4. DISCUSSION

The present in vitro study provides robust experimental evidence supporting the superior antimicrobial efficacy of nanochitosan-coated orthodontic brackets over uncoated controls, with statistically significant differences observed for all four tested microorganisms across all post-baseline time intervals. The findings support rejection of the null hypothesis and are consistent with and extend the current body of literature on chitosan-based antimicrobial coatings in dentistry (Mendes et al., 2019; Butler et al., 2023; Di Palma et al., 2024).

The inhibition of *S. mutans* is of primary clinical relevance. This organism, recognized as the principal etiological agent of enamel demineralization and WSL formation, demonstrated consistently low CFU counts in Group A (approximately 22–23 × 10^5 CFU/mL) from T2 through T4, in stark contrast to the exponential proliferation in Group B (reaching 123.75 × 10^5 CFU/mL at T4; $F = 20{,}327.872$; $p < 0.001$). Ahn et al. (2005) previously demonstrated that metallic orthodontic brackets serve as favorable niches for *S. mutans* adhesion owing to surface roughness and retentive morphology, conferring heightened cariogenic risk. The present findings corroborate the work of Mendes et al. (2019), who reported over 80% reduction in *S. mutans* adhesion on

chitosan nanoparticle-coated brackets compared with uncoated controls, and align with the observations of Ke et al. (2021), who confirmed the potent anti-biofilm properties of chitosan derivatives against this organism.

The sustained suppression of *L. acidophilus* in nanochitosan-coated brackets is also clinically significant. Lactobacilli are secondary colonizers that thrive in established low-pH microenvironments beneath orthodontic appliances and contribute substantially to the deepening of incipient enamel lesions (Badet & Thebaud, 2008). The stable CFU plateau observed in Group A from T2 onward suggests that the polycationic nanochitosan layer effectively disrupts the aciduric biofilm ecology, consistent with the membrane-disruption mechanisms elucidated by Raafat & Sahl (2009) and Helander et al. (2001), and the broader antimicrobial frameworks described by Yan et al. (2021) and Muthu et al. (2023).

The broad-spectrum antibacterial activity of nanochitosan was further demonstrated by significant inhibition of *E. faecalis* and *S. aureus*. Enterococcus faecalis is a resilient opportunistic pathogen exhibiting exceptional resistance to environmental stressors and conventional antimicrobials, and has been associated with persistent peri-implant and endodontic infections; its ability to adhere strongly to metallic and resin surfaces is well documented (Stuart et al., 2006). The substantial reduction in E. faecalis CFUs in Group A (23.0 vs. 123.80×10^5 CFU/mL at T4; $F = 16{,}994.028$; $p < 0.001$) aligns with the findings of Costa et al. (2012), who demonstrated that chitosan nanoparticles induce structural deformation and lysis of Gram-positive cocci through membrane permeabilization and essential ion chelation. Similarly, the inhibition of *S. aureus* a pathogen associated with nosocomial infections and oral opportunistic colonization supports the utility of nanochitosan for broad-spectrum biofilm management in the oral environment.

From a mechanistic standpoint, the superior antimicrobial performance of nanochitosan is attributable to the polycationic character of chitosan at physiological pH. The protonated amino groups ($–NH_3^+$) on the chitosan backbone interact electrostatically with the negatively charged phospholipid heads of bacterial cell membranes, leading to membrane disruption, efflux of cytoplasmic contents, inhibition of nutrient transport, and ultimately cell death (Kong et al., 2008; Rabea et al., 2003). At the nanoscale, these effects are potentiated by greater charge density, higher surface area-to-volume ratios, and enhanced penetration into biofilm extracellular matrix compared with bulk chitosan, as comprehensively reviewed by Wu et al. (2024) and Hemmingsen et al. (2022). The multifactorial nature of this mechanism renders it unlikely to induce conventional resistance pathways, an important consideration in the context of global antimicrobial resistance (Allaker & Memarzadeh, 2014; Huh & Kwon, 2011).

The progressive and unimpeded increase in CFU counts in Group B at every time interval is consistent with established understanding of uncoated orthodontic appliance surfaces as favorable substrata for bacterial colonization. Gwinnett & Ceen (1979) demonstrated through early SEM studies that plaque accumulation is most pronounced at the gingival margin of bracket bases, and Eliades & Brantley (1995) emphasized that bracket material properties and surface energy significantly modulate early microbial attachment. The exponential proliferation from T2 to T4 in Group B (more than doubling from T2 to T4 for all organisms) is consistent with typical biofilm

growth kinetics reported in previous in vitro orthodontic studies (Lima et al., 2019; Wang et al., 2023).

The hydrothermal synthesis approach employed in the present study produced a homogeneous, stable nanochitosan coating with a mean particle size of ~50 nm, confirmed by FESEM. This uniform coating, corroborated by Li et al. (2016), is critical for ensuring consistent antimicrobial surface coverage and avoiding localized areas of reduced activity. The stability of CFU counts in Group A from T2 to T4 further supports the prolonged contact-dependent antimicrobial action of the coating, a finding consistent with Butler et al. (2023), who emphasized that long-term retention of the antimicrobial effect is a key determinant of clinical utility.

From a clinical perspective, these results are highly pertinent. WSLs can develop within four weeks of appliance placement (Tufekci et al., 2011; Julien et al., 2013), and existing preventive modalities fluoride varnishes and CHX mouthrinses are associated with variable compliance, adverse effects (staining, taste alterations), and requirement for repeated application (Stecksén-Blicks et al., 2007; Derks et al., 2004). A passive, compliance-independent antimicrobial surface modification of orthodontic brackets, as investigated in the present study, could therefore offer a clinically meaningful and patient-friendly strategy to reduce biofilm-mediated complications throughout fixed orthodontic treatment, without reliance on patient behavior. This aligns with the broader vision for nanotechnology-enhanced dental biomaterials articulated by Di Palma et al. (2024), Besinis et al. (2015), and Al Kahtani (2018).

4.1 Strengths

The study employed a well-powered design (94% power), standardized microbiological protocols including OD-calibrated inocula, a pilot study for method validation, and multi-time-point assessment over 72 hours. SEM and FESEM verification of coating uniformity adds methodological rigor. The use of four microorganisms representing different ecological niches and pathogenic roles enhances the clinical relevance of the findings.

4.2 Limitations

As an in vitro investigation, the study cannot fully replicate the complexity of the oral environment, including salivary flow, masticatory forces, pH fluctuations, temperature cycling, and polymicrobial interactions. The evaluation was restricted to four organisms and a short-term follow-up of 72 hours, without assessment of long-term coating durability, wear resistance under clinical conditions, or potential effects on bracket–adhesive bond strength and frictional properties. Biocompatibility of the nanochitosan coating with oral soft tissues was not assessed in this study. Furthermore, the CFU counts observed in Group A, while significantly lower than in Group B, were not zero, indicating partial rather than complete inhibition of microbial growth; the clinical significance of residual CFU counts at these levels requires clarification in future in vivo investigations.

4.3 Future Directions

Future research should include randomized controlled in vivo clinical trials evaluating the effect of nanochitosan-coated brackets on WSL incidence, quantitative plaque indices, and salivary cariogenic bacterial counts in orthodontic patients over full treatment periods (18–24 months).

Long-term evaluation of coating durability, bond strength, and friction characteristics is also warranted. Comparative studies against alternative antimicrobial nanocoatings (silver, zinc oxide, titanium dioxide) would help establish the relative efficacy and safety profile of nanochitosan. Biocompatibility assessment using cytotoxicity assays and oral tissue models should be incorporated into preclinical pipelines before clinical translation.

5. CONCLUSION

This in vitro study demonstrates that nanochitosan surface modification of stainless-steel orthodontic brackets confers significantly superior and sustained broad-spectrum antimicrobial activity against *Streptococcus mutans, Lactobacillus acidophilus, Enterococcus faecalis,* and *Staphylococcus aureus* compared with uncoated control brackets over a 72-hour period. Nanochitosan-coated brackets maintained consistently low colony-forming unit counts at 12, 24, and 72 hours, while uncoated brackets demonstrated progressive and exponential bacterial proliferation. Intergroup differences were statistically highly significant at all post-baseline intervals ($p < 0.001$), leading to rejection of the null hypothesis.

Nanochitosan surface modification provides an effective, compliance-independent, and broad-spectrum antimicrobial strategy applicable to orthodontic bracket surfaces. This approach holds clinical promise for reducing plaque-mediated biofilm accumulation and minimizing the risk of enamel demineralization and white spot lesion formation during fixed orthodontic therapy. Validation through long-term in vivo randomized controlled trials, coupled with assessment of coating durability, biocompatibility, and mechanical performance, is recommended before clinical implementation.

DECLARATIONS

Ethical Approval: Approved by the Institutional Ethics Committee, Inderprastha Dental College and Hospital, Ghaziabad, India (Ref. No. IPDC/Th/2024/170B[27]).

Informed Consent: Not applicable (in vitro study).

Conflict of Interest: The authors declare no conflicts of interest.

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

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