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Probiotics Augment the Effect of Non-Surgical Periodontal Treatment—A Randomised, Double-Blinded, Placebo-Controlled Trial

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ABSTRACT

Aim: To determine the effect of probiotic lozenges containing *Lacticaseibacillus rhamnosus PB01*, *Latilactobacillus curvatus EB10* and xylitol after non-surgical periodontal treatment (NSPT) on changes in microbial composition. The secondary aims were to assess the clinical and immunological impact of probiotic consumption.

Materials and Methods: Eighty adults with stage II or III periodontitis were enrolled and received NSPT at baseline, followed by a 12-week consumption of probiotics or placebo. Microbial sampling and clinical examination were performed at baseline, Week 6 and Week 12. The subgingival microbiota was analysed using 16S sequencing, the salivary microbiota by metagenomic sequencing and selected cytokines and proteases in saliva by bead-based immunoassay.

Results: Sixty-one participants completed the trial (probiotics $n = 32$, placebo $n = 29$). At Week 12, *Treponema socranskii*, *Selenomonas sputigena*, *Dialister pneumosintes*, *Dialister invisus*, *Anaeroglobus geminatus* and *Fusobacterium nucleatum* were significantly associated with the placebo group, while *Streptococcus sanguinis*, *Neisseria elongata* and *Neisseria oralis* were associated with the probiotic group. Bleeding on probing percentage (BoP%) and number of periodontal pockets (PPD) ≥ 5 mm decreased significantly more in the probiotic group compared to the placebo group ($p < 0.05$).

Conclusion: The tested probiotic supplement resulted in an additional short-term decrease in periodontitis-associated species along with greater improvements in BoP% and PPD ≥ 5 mm 12 weeks post-NSPT, compared to the placebo group.

1 | Introduction

Periodontitis is a prevalent, multifactorial inflammatory disease, which, if left untreated, results in loss of tooth-supporting tissues and ultimately tooth loss (Van Dyke et al. 2020; Frencken et al. 2017). Non-surgical periodontal treatment (NSPT) remains the key in treatment of periodontitis (Sanz et al. 2020). However, responses to NSPT vary, which has prompted the exploration of adjunctive strategies, such as probiotics (Sanz et al. 2020).

Probiotics are defined as live microorganisms conferring health benefits to the host when administered in adequate amounts (Hill et al. 2014). The mechanisms of action in oral health are still not fully understood but are believed to be local

by a direct interaction with the oral microbiota and systemic by indirectly promoting anti-inflammatory and inhibiting pro-inflammatory host responses (Plaza-Diaz et al. 2019). Studies reporting microbial compositional changes have employed techniques focusing on selected microbial species, and only a few studies have reported immunological findings (Ho et al. 2020; Hu et al. 2021; Ikram et al. 2018; Li et al. 2023; Martin-Cabezas et al. 2016; Mishra et al. 2021; Ram et al. 2024; Song and Liu 2020; Vives-Soler and Chimenos-Küstner 2020; Lundtorp-Olsen et al. 2024). The application of probiotics in conjunction with NSPT shows equivocal clinical results, with some studies reporting improvements in pocket depth (PPD) and bleeding on probing percentage (BoP%), compared to a placebo group, while other studies fail to reach significance between groups (Ho et al. 2020; Hu et al. 2021; Ikram et al. 2018; Li et al. 2023; Martin-Cabezas et al. 2016; Mishra et al. 2021; Ram et al. 2024; Song and Liu 2020; Vives-Soler and Chimenos-Küstner 2020; Nguyen et al. 2020). To capture the full impact of probiotics as an adjunctive strategy in periodontal treatment, the efficacy of probiotics should ideally be evaluated using compositional microbiological data, clinical parameters and immunological biomarkers.

We have previously tested the effect of a probiotic lozenge containing *Lacticaseibacillus rhamnosus* PB01 DSM14870, *Latilactobacillus curvatus* EB10 DSM32307 and xylitol in conditions of experimental gingivitis and frequent sugar intake in orally healthy young adults in short-term trials. Data showed that this combination of probiotic strains supported microbial resilience after experimental gingivitis and suppressed sugar-induced loss of diversity of the supragingival microbiota (Lundtorp et al. 2023; Lundtorp Olsen, Massarenti, et al. 2023).

In the present study, we aimed to determine whether consumption of probiotics containing *L. rhamnosus* PB01 and *L. curvatus* EB10 was accompanied by a greater decrease in the mean abundance of periodontitis-associated species in the subgingival and salivary microbiota 12 weeks post-NSPT compared to placebo. Secondary outcomes in the study were reduction in mean number of PPD ≥ 5 mm, mean BoP% and mean abundance of proinflammatory cytokines and proteins in saliva.

2 | Materials and Methods

2.1 | Study Design

This double-blinded, randomised, placebo-controlled superiority trial was conducted from 22 October 2022 to 21 June 2023, at the Department of Odontology, University of Copenhagen. It is the third in a series testing the same probiotic consortia on gingivitis, dental caries and periodontitis. All participants provided informed consent, and the trial was conducted per the Helsinki Declaration. Ethical approval was obtained (H-21003295), and the trial was registered at ClinicalTrial.gov (NCT05518747) and reported to the University of Copenhagen's local data authorisation (514-0649/21-3000). Through computerised randomisation (www.randomizer.org, accessed October 1, 2022), 80 participants were allocated 1:1 to probiotic or placebo lozenges by D.B., who was the only person unblinded to the allocation. Lozenges were assigned right after the first two steps of therapy (Sanz et al. 2020) and continued throughout the 12-week study period. Clinical examinations and sample collections were repeated at 6 and 12 weeks (±1 week). The study flow chart is shown in Figure 1, and the CONSORT checklist is given in Appendix 1.

2.2 | Study Population

The study population comprised 80 adults recruited at the Department of Odontology, University of Copenhagen. As no prior studies were available to inform a formal sample size calculation for probiotics as an adjunct to periodontal treatment using 16S rRNA sequencing and metagenomic analyses, the study was exploratory/pilot in nature. The sample size was pragmatically informed by our previous study (Belstrøm et al. 2018), which demonstrated significant clinical and microbiological changes. As the additional effect of probiotics was unknown, the sample size was inflated by 40% to account for potential variability ($25 + 25 \times 0.4 = 35$), with a further 10% added to allow for dropouts ($35 + 35 \times 0.1 = 39$), resulting in a final target sample size of $n = 39$ participants per group. Microbiological analyses focused on species previously associated with periodontitis, assessed in an exploratory, data-driven manner using LEfSe and DESeq2. No individual species was pre-specified as the primary outcome. Clinical and immunological outcomes were considered secondary.

The exclusion criteria were treatment requiring diseases in the oral cavity except for periodontitis stage II or III, < 20 teeth, self-reported inflammatory or immunological diseases, dysregulated diabetes (fasting blood sugar outside 4–7 mmol/L and blood sugar 2 h after a meal > 10 mmol/L), cancer within 4 years, corticosteroid and biological medicine intake, antibiotic prescription or subgingival instrumentation within 3 months before trial participation, tooth extraction or surgery within 2 months before trial participation, use of probiotics 1 month before participation and pregnancy. The inclusion criteria were age ≥ 18 years and being diagnosed with periodontitis stage II or III, grade A–C either localised or generalised according to the 2017 Classification (Caton et al. 2018).

2.3 | Clinical Examinations and Periodontal Treatment

Clinical examinations were performed by a Master's student in dentistry (S.V.R.A.) at baseline and repeated 6 and 12 weeks after subgingival instrumentation. Intra- and inter-rater reliability of plaque index (PI), PPD and clinical attachment level (CAL) were assessed. PI (binary) was analysed with Fleiss' κ for intra-rater and unweighted Cohen's κ for inter-rater agreement, while PPD and CAL (continuous) were evaluated using intra-class coefficients (ICC, two-way random, single measures, consistency). Intra-rater reliability was good to excellent (PI, 0.738; PPD, 0.864; CAL, 0.867). Inter-rater reliability was similarly good to excellent (PI, 0.725; PPD, 0.800; CAL, 0.797) (Koo and Li 2016; Landis and Koch 1977). Clinical examinations were performed after the collection of samples and

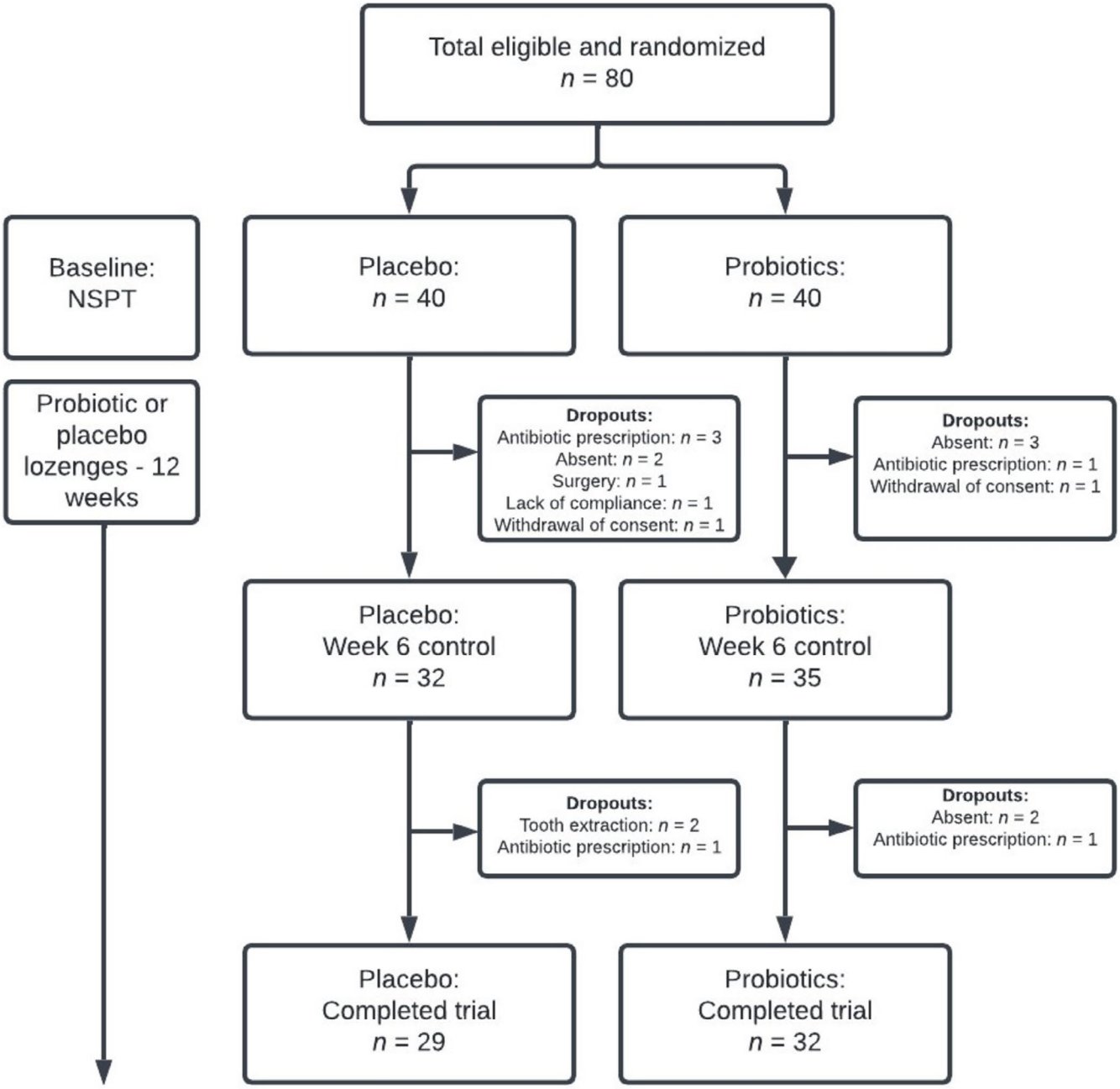

FIGURE 1 | Flowchart of the study.

after participants performed routine oral hygiene procedures. At baseline and week 12, PI, PPD, BoP and CAL were measured, while only PI and BoP were registered at Week 6. PI was registered by using SUNSTAR G·U·M RED-COTE disclosing tablets. At all three time points, all measurements were performed at six sites (disto-facial, mid-facial, mesio-facial, disto-oral, mid-oral and mesio-oral) on each tooth (third molars excluded). Periodontal therapy consisted of the first two steps of therapy (oral hygiene instructions, elimination of local retentive factors, professional mechanical removal of supragingival plaque and calculus and subgingival instrumentation) according to the EFP guideline (Sanz et al. 2020) (adjunctive therapies, host-modulation and chemical agents and antimicrobials were avoided), and was delivered simultaneously by C.M.L.O. within 4 weeks of the baseline registrations. Subgingival instrumentation was performed with curettes and ultrasonic instruments in combination. Oral hygiene instructions were repeated at week 6 by S.V.R.A.

2.4 | Probiotic and Placebo Lozenges

The probiotic and placebo lozenges were identical in appearance, both lemon-flavoured, and prepared at ADM Denmark as previously described (Lundtorp et al. 2023; Lundtorp Olsen, Massarenti, et al. 2023). Each lozenge contained a minimum of 1×10^8 CFU of *L. rhamnosus* PB01 DSM14870 and *L. curvatus* EB10 DSM32307 and 491 mg xylitol. Participants were instructed verbally and in writing to slowly dissolve one lozenge and swallow the saliva in the morning and in the evening after oral hygiene procedure, avoiding food and drinks for the subsequent 30 min.

2.5 | Collection of Samples

Paraffin, chew-stimulated saliva and subgingival plaque samples were collected as described (Lundtorp et al. 2023; Lundtorp Olsen, Massarenti, et al. 2023; Belstrøm et al. 2018; Lundtorp-Olsen, Enevold, Juel Jensen, et al. 2021; Lundtorp-Olsen, Enevold, Twetman, and Belstrøm 2021). In brief, participants refrained from food and drink 2 h before sampling. Saliva was collected first, followed by subgingival plaque from the two deepest periodontal pockets (excluding third molars and distal sites on second molars with impacted wisdom teeth). Samples were collected before any instrumentation, with oral hygiene postponed until after sampling. All samples were collected between 8.00 AM and 3.00 PM, immediately stored at −18°C, transferred to −80°C within 6 h and kept until analysis. Sampling times were standardised across visits.

2.6 | Sample DNA Extraction, Library Preparation and DNA Sequencing

DNA extraction, sequencing library preparation and 16S sequencing were performed as previously described (Lundtorp-Olsen, Enevold, Juel Jensen, et al. 2021; Lundtorp-Olsen, Enevold, Twetman, and Belstrøm 2021). In brief, the V1–V3 region of the 16S gene was targeted using MiSeq (Illumina, San Diego, CA, USA).

Metagenomic libraries were prepared with the Nextera XT Library kit according to the manufacturer's instructions (Illumina). DNA was simultaneously fragmented and tagged with dual index sequencing adapters. Library quality control was ensured using the HSD5000 kit in the TapeStation 4200 equipment (Genomic DNA and D1000 screentapes, Agilent, USA). NovaSeq 6000 sequencing platform in a 150 paired-end reads configuration generated *.bcl files as primary sequencing output (NovaSeq Control Software (NCS) v1.6). Bcl2fastq 2.20 program was used to translate the sequencing reads from bcl (Base Calling) to FASTQ format and remove sequencing adapters.

2.7 | Cytokine, Protein and Enzyme Analysis

Analyses of cytokines were performed following manufacturer's protocol as previously described (Lundtorp et al. 2023; Lundtorp Olsen, Massarenti, et al. 2023; Yilmaz et al. 2023). In brief, samples were centrifuged for 5 min at 9300*g*, and levels of interleukin (IL)-8, IL-1β, monocyte chemoattractant protein-1 (MCP-1) and macrophage migration inhibitory factor (MIF) were measured from salivary supernatants by bead-based immunoassay. The limit of detection (LOD) was 0.36 pg/mL for IL-8, 0.24 pg/mL for IL-1β, 0.44 pg/mL for MCP-1 and 2.45 pg/mL for MIF. For details, see Appendix 2.

The analyses of the proteins, amylase activity, total protease activity (TPA), chitinase activity and albumin were performed as previously described (Lundtorp et al. 2023; Lundtorp Olsen, Massarenti, et al. 2023; Morquecho-Campos et al. 2020; Bikker et al. 2019; Prodan et al. 2015). Briefly, samples were centrifuged (10.000*g*, 10 min) and the supernatant was aliquoted. Total protein concentration was analysed using Pierce BCA Protein Assay Kit (ThermoFisher, CAT#23227) according to manufacturer's instructions (Lundtorp et al. 2023; Lundtorp Olsen, Massarenti, et al. 2023; Morquecho-Campos et al. 2020). Albumin was quantified on rabbit anti-human albumin–coated microplates with bovine serum albumin (BSA) standards (25–1500 μg/mL) (Bikker et al. 2019; Prodan et al. 2015). Samples were diluted 1:2000 and incubated with horseradish peroxidase (HRP)-conjugated anti-human albumin, with *o*-phenylenediamine dihydrochloride as substrate (Bikker et al. 2019; Prodan et al. 2015). Amylase activity was determined by diluting the samples 1:100 in MILLI-Q and mixing with 2-chloro-4-nitrophenyl α-D-maltotrioside (Morquecho-Campos et al. 2020). TPA was assessed by mixing 50 μL PEK-54 substrate with 50 μL saliva, while chitinase activity was measured mixing 50 μL 4-methylumbelliferyl β-D-*N*,*N*′,*N*″-triacetyl chitotriose substrate with 50 μL saliva. TPA and chitinase activity were measured fluorometrically at 37°C for about 1 h at 5-min intervals. For details, see Appendix 3.

2.8 | Bioinformatic Processing and Statistics

Background information is presented by descriptive statistics. Clinical data and data on cytokines, enzymes and albumin were checked for normal distribution by Q–Q plots. Immunological data were log-transformed. Comparisons within groups were performed by ANOVA adjusted with Tukey's corrections for multiple testing and ANCOVA between groups with baseline measurements as covariates. Nicotine usage was considered a confounder. An intention-to-treat (IIT) analysis was performed to assess clinical effectiveness, as well as a dichotomous analysis for clinical interpretation of periodontal therapy outcomes. To evaluate the relationship between microbial shifts and clinical or immunological improvements, principal component analysis (PCA) and Spearman correlation were performed. A p value < 0.05 was considered significant for all analyses.

Bioinformatic processing of 16S samples was performed as previously described (Lundtorp et al. 2023; Lundtorp Olsen, Massarenti, et al. 2023; Lundtorp-Olsen, Enevold, Juel Jensen, et al. 2021; Lundtorp-Olsen, Enevold, Twetman, and Belstrøm 2021; Lundtorp Olsen, Markvart, et al. 2023) by matching the demultiplexed Illumina reads against the 16S rRNA Human Oral Microbiome RefSeq database (HOMD) v. 15.2 (Escapa et al. 2018). The subgingival microbiota was characterised and compared according to relative abundance adjusted for multiple testing by Benjamini–Hochberg's correction (Hochberg and Benjamini 1990). Linear discriminant analysis effect size (LEfSe) was used to identify differences between groups (Segata et al. 2011) with significant differences identified by the combined criteria of Kruskal–Wallis $p < 0.05$, Wilcoxon p-adjusted < 0.05 and LDAscore > 2. α-Diversity was compared between groups by ANCOVA with baseline adjustment.

Metagenomic data were processed as previously described, with minor modifications (Rodenes-Gavidia et al. 2023). In brief, reads were standardised and assigned using Metaphlan (Blanco-Míguez et al. 2023) to eHOMD (Escapa et al. 2018). Metagenomes were annotated by Kyoto Encyclopaedia of Genes and Genomes (KEGG) annotation (Kanehisa et al. 2016). Data were normalised (McMurdie and Holmes 2013) and α-diversity was tested by the Wilcoxon test. β-Diversity was analysed by Bray–Curtis dissimilarity matrix and PERMANOVA analysis (Vegan et al., 2019).

DESeq2 (Love et al. 2014) was used to identify differentially abundant taxa and genes ($p < 0.05$ and if present in at least 50% of the samples in one group). A gene set enrichment analysis (GSEA) was conducted on KEGG modules based on the results from DESeq2 (Korotkevich et al. 2019). All analyses were performed using R and R Studio. For details, see Appendix 4.

3 | Results

3.1 | Background Data and Compliance

Nineteen participants dropped out due to antibiotic prescription ($n = 6$), absence ($n = 7$), withdrawal of consent ($n = 2$), tooth extraction ($n = 2$), surgery ($n = 1$) and non-compliance ($n = 1$). These were excluded from further analysis. Groups were comparable at baseline regarding diagnoses, age, sex, nicotine use, well-regulated diabetes (fasting blood sugar levels: 4–7 mmol/L) and previous periodontal treatment ($p > 0.05$, Table S1). Compliance was similar between groups, $p > 0.05$ (mean forgotten lozenges; placebo, $n = 25.6$, probiotics, $n = 18.6$, Figure S1).

3.2 | Sequencing Metadata

DNA extraction and library preparation succeeded for 180/183 subgingival samples (98.4%), yielding between 13.418 and 136.109 DNA reads after quality check (QC) and bioinformatic processing. In total, 126 genera and 461 bacterial species were identified, covering 96.42% and 61.96% of sequences, respectively. For saliva, sequencing succeeded in all 183 samples, yielding 5.7–51.2 million reads after QC and bioinformatic processing and identifying 604 species.

3.3 | Subgingival Microbiota

PCA showed modest separation between groups 6 weeks post-NSPT, but no clustering at 12 weeks. α-diversity decreased significantly more in the probiotic group at 12 weeks (Shannon $p = 0.01$; Simpson $p = 0.03$). Relative abundance analysis of the 20 most abundant species showed significantly higher abundance of *Capnocytophaga* and *Lautropia* at Week 6 and *Neisseria* at both 6 and 12 weeks in the probiotic group compared to placebo. *Fusobacterium* was significantly more abundant in the placebo group at Week 12 (Figure 2). LefSe identified 30 species differing between groups (Figure 3), with *Treponema socranskii, Selenomonas sputigena, Dialister pneumosintes, Dialister invisus, Anaeroglobus geminatus* and *Fusobacterium nucleatum* enriched in the placebo group, while *Streptococcus sanguinis, Neisseria elongata* and *Neisseria oralis* were enriched in probiotics group after 12 weeks.

3.4 | Salivary Microbiota

In saliva, α-diversity decreased more in the probiotic group from baseline to Week 6 (Richness $p = 0.043$; Shannon $p = 0.034$) and to Week 12 (Richness $p = 0.007$). PERMANOVA attributed 77.4% of variance to individual effects ($p = 0.001$), 1% to NSPT ($p = 0.002$) and 2% to the probiotic intervention ($p < 0.001$).

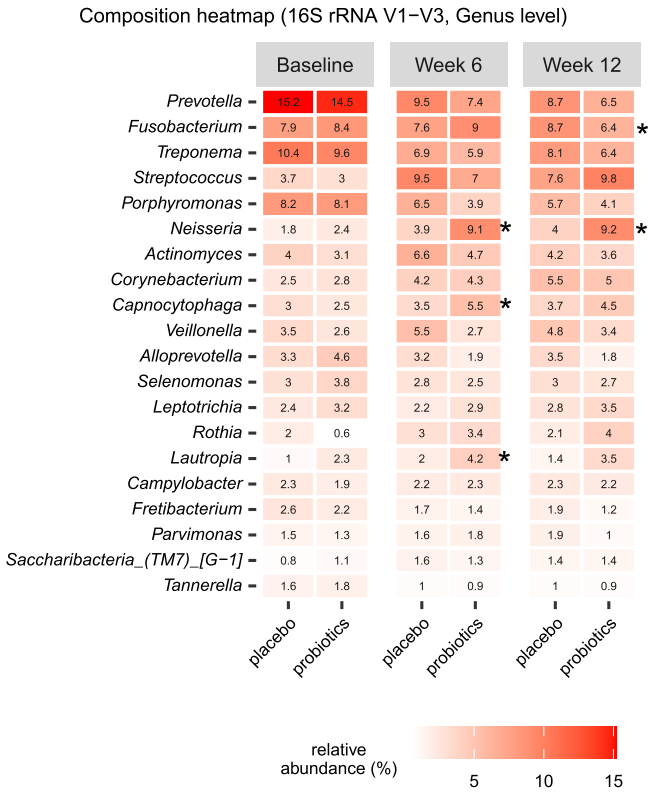

FIGURE 2 | Heatmap of 15 predominant genera. Impact of NSPT on the subgingival microbiota for the placebo and probiotic group presented as mean values of relative abundances of the 15 predominant genera. *Significant differences between groups.

DESeq2 identified 23 differentially abundant species. Several species decreased in the probiotic group, while the microbiota in the placebo group remained relatively stable throughout the trial. Specifically, *Tanerella forsythia* and *Campolybacter rectus* decreased more after Week 6, and *Prevotella nigrescens* and *C. rectus* decreased more after Week 12 in the probiotic group (Figure 4). Functionally, the probiotic group showed greater decrease in abundance of genes related to oxidative phosphorylation, fatty acid biosynthesis, alanine, aspartate and glutamate metabolism, valine, leucine and isoleucine degradation, arginine and proline metabolism and citrate cycle and increased genes related to B6 vitamin metabolism and ascorbate and aldarate metabolism compared to the placebo group ($p < 0.05$; Figure S2).

3.5 | Clinical Changes

All clinical parameters improved significantly within both groups ($p < 0.05$, Table S2A,B). From baseline to week 12, reductions in BoP% and sites ≥ 5 mm were greater in the probiotic group ($p = 0.036$, $p = 0.03$), remaining significant after adjustment for smoking ($p = 0.049$, $p = 0.016$). Mean PPD reduction was significant before but not after smoking adjustment ($p = 0.07$ vs. $p = 0.03$). At week 12, 5 placebo and 14 probiotic participants had BoP < 10% ($p = 0.03$), while 3 probiotic and 0 placebo participants achieved periodontal health (no PPD ≥ 4 mm with BoP and total BoP < 10%; $p = 0.24$).

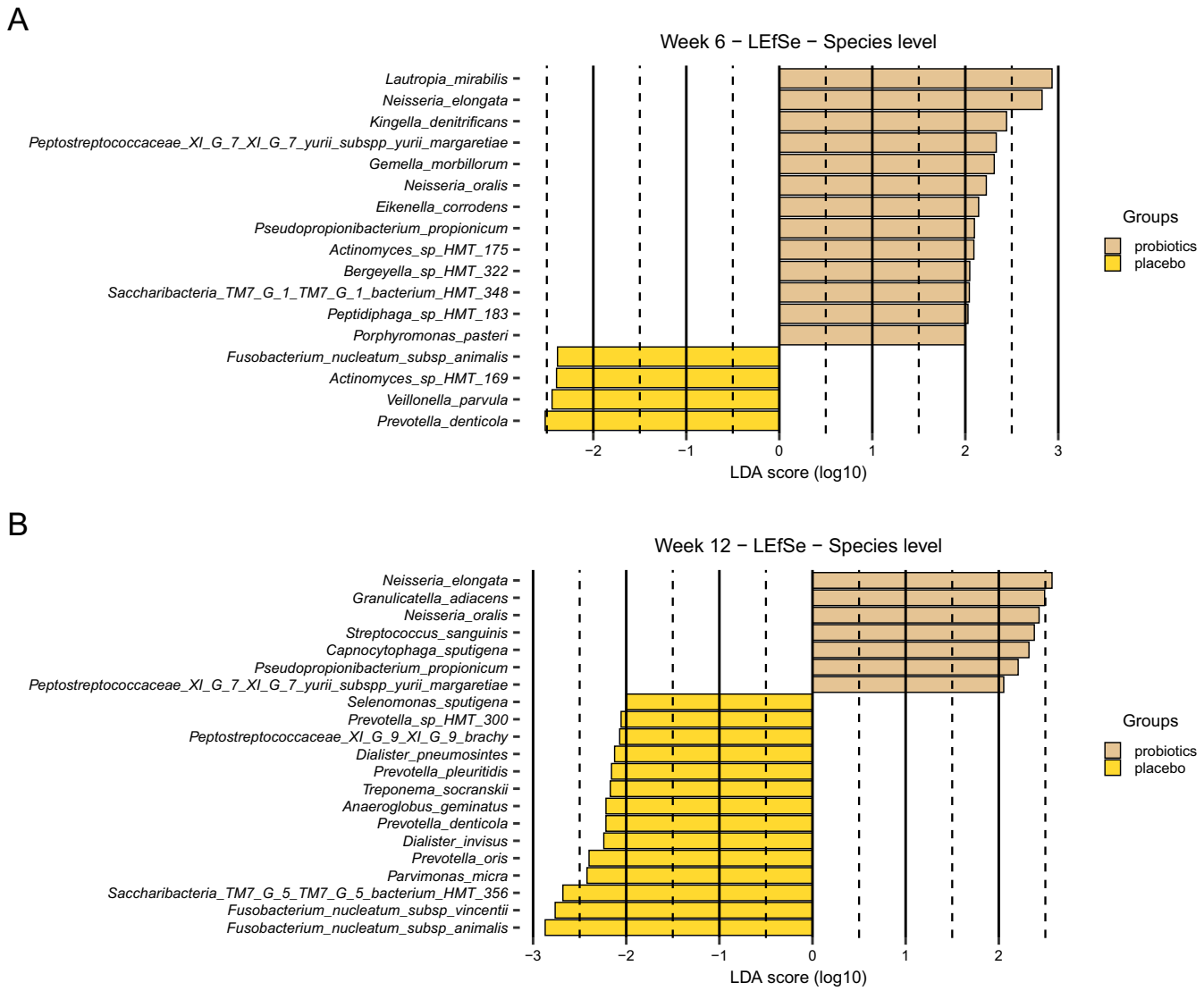

FIGURE 3 | Subgingival differentially expressed species. LEfSe analysis is expressed by significant species at weeks 6 (A) and 12 (B).

An ITT analysis (including all dropouts) showed significant group differences only for BoP% ($p = 0.014$).

3.6 | Salivary Cytokines and Proteases

Six MIF samples below LOD were replaced with LOD/2. No significant differences were observed between groups for any cytokines, and the changes within groups were modest. From baseline to 12 weeks, albumin increased more in the placebo group ($p = 0.008$), while chitinase decreased more in the probiotics group ($p = 0.009$; Figure 5, Table S3A–C).

3.7 | Sub-Analyses

Sub-analyses excluding smokers/diabetics confirmed significant differences in PPD < 5 mm and PPD ≥ 5 mm but not BoP%. Microbiologically, fewer species were significantly different between the groups. Immunological findings were almost identical to the original results (Table S4A–D, Figure S3A,B).

3.8 | Correlation Analyses

Subgingival PCA revealed that BoP% and plaque positively correlated with PC1, while PPD and sites ≥ 5 mm correlated with PC1 and PC2. Sites < 5 mm were negatively associated with PC1 and PC2. Immunological correlations were weak, but albumin, amylase and chitinase correlated with both PC1 and PC2, while IL-1β correlated with PC1 (Figure S4A,B). Salivary PCA showed that the mean PPD, sites ≥ 5 mm, BoP%, MCP-1, albumin and IL-1β positively correlated with both PC1 and PC2, while sites < 5 mm correlated negatively (Figure S4C,D). Spearman correlations indicated strong correlation between the 20 most abundant subgingival species and clinical parameters, while correlations with immune parameters and for the salivary microbiota were weaker (Figure S5A–D).

4 | Discussion

The primary outcome supported our hypothesis because the placebo group showed significantly higher subgingival abundance

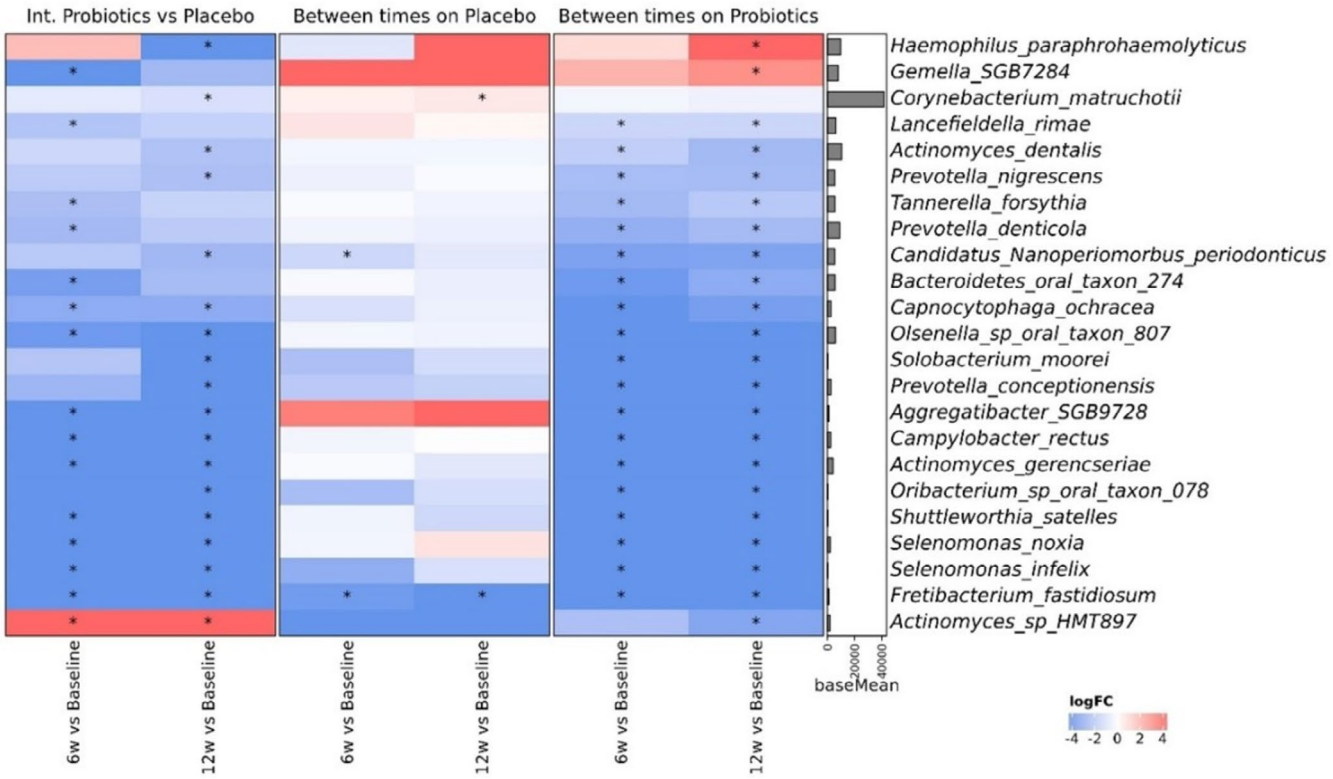

FIGURE 4 | Differentially expressed species in the saliva presented by DESeq2. Heatmaps show the changes from baseline versus week 6 and baseline versus week 12 between the placebo and probiotic groups and within the groups. Red indicates an increase, while blue indicates a decrease. The figure only presents species with significantly different abundance between groups and species represented in ≥ 50% of samples in either the placebo or probiotic group. *Significant differences.

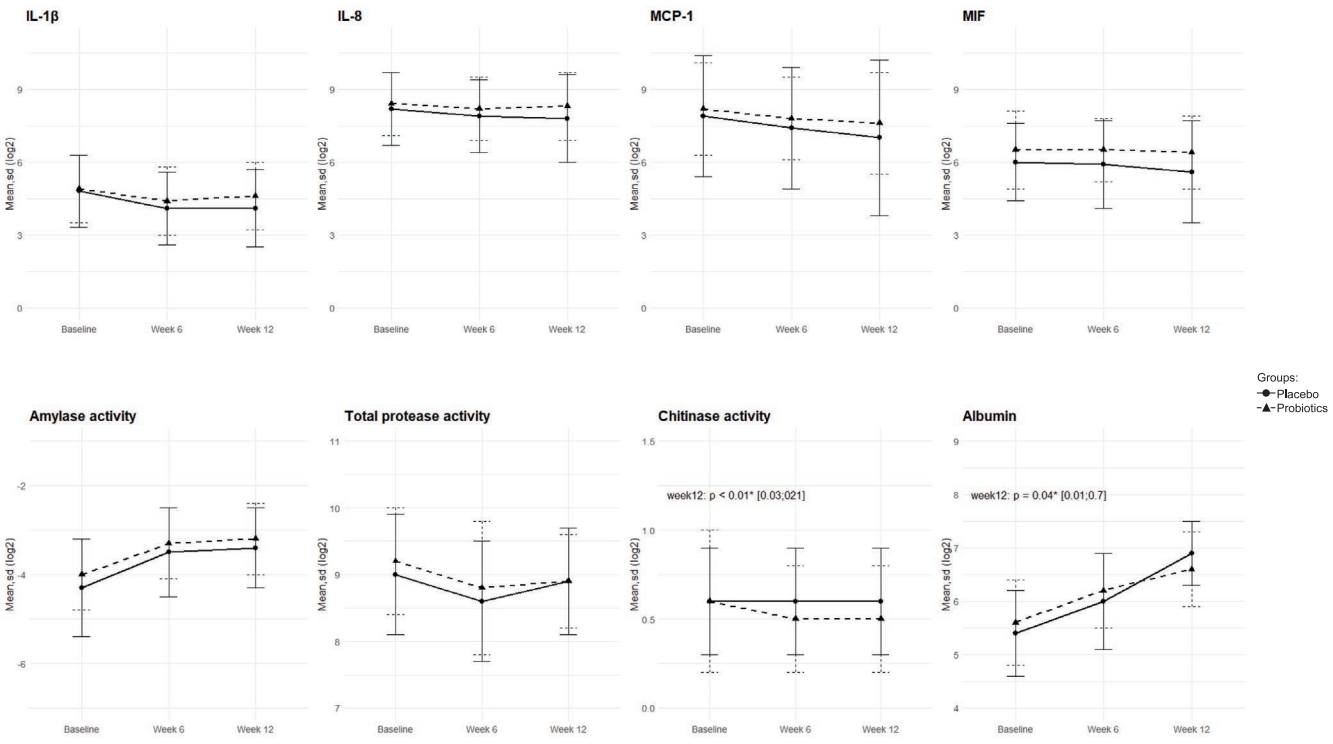

FIGURE 5 | Salivary levels of IL-1β, IL-8, MIF and MCP-1 expressed in pg/mL and amylase activity (slope/min), chitinase activity (dF/dT), total protease activity (U/mL) and albumin concentration (μg/mL). Data are $\log_2$-transformed and presented by means and standard deviation (SD) for the probiotic and placebo groups. Significant p-values and confidence intervals (CIs) are presented from comparison between groups by ANCOVA, with baseline values as covariates.

of *T. socranski, S. sputigena, D. pneumosintes, D. invisus, A. geminatur* and *F. nucleatum* at Week 12, all strongly associated with periodontitis (Antezack et al. 2023). In contrast, the probiotic group was enriched with *S. sanguinis, N. elongata* and *N. oralis*. In the saliva, probiotic consumption led to greater reductions in periodontitis-associated species, including *T. forsythia*, *C. rectus* and *P. nigrescens* (Antezack et al. 2023; Socransky et al. 1998; Dabdoub et al. 2016), together with a greater reduction in α-diversity, which is associated with periodontal health (Dabdoub et al. 2016; Kumar et al. 2021) ($p < 0.05$). Notably, *T. forsythia* belongs to Socransky's red complex, *F. nucleatum* and *C. rectus* belong to the orange complex and *S. sanguinis* belongs to the green complex (Socransky et al. 1998). Previous probiotic studies using qPCR, DNA–DNA checkerboard and culturing have reported significant reductions in subgingival abundance of *T. forsythia, P. gingivalis, P. intermedia*, *T. denticola* and *F. nucleatum* (Laleman et al. 2015; El-Bagoory et al. 2021; Invernici et al. 2018; Tapashetti et al. 2022; Teughels et al. 2013) as well as total counts of obligate anaerobes (Tekce et al. 2015) following NSPT with probiotic supplementation. However, other studies found no significant differences (Teughels et al. 2013; Mayanagi et al. 2009; Morales et al. 2018; de Oliveira et al. 2022; Pudgar et al. 2021).

The secondary clinical outcome supported the hypothesis because the probiotic group showed significantly lower BoP% and fewer sites with PPD ≥5 mm at 12 weeks post-NSPT compared with placebo (smoking-adjusted *p*-values: $p = 0.049$, $p = 0.01$, Table 1). These results are supported by the dichotomous analysis and consistent with most prior probiotic studies reporting reductions in PPD and BoP (Ho et al. 2020; Hu et al. 2021; Ikram et al. 2018; Li et al. 2023; Martin-Cabezas et al. 2016; Mishra et al. 2021; Ram et al. 2024; Song and Liu 2020; Vives-Soler and Chimenos-Küstner 2020). Some studies also reported superior improvements in CAL and PI (Ho et al. 2020; Hu et al. 2021; Ikram et al. 2018; Li et al. 2023; Martin-Cabezas et al. 2016; Mishra et al. 2021; Ram et al. 2024; Song and Liu 2020; Vives-Soler and Chimenos-Küstner 2020), which were not observed in the present study.

The immunological secondary outcome did not confirm the hypothesis, because NSPT had only a minimal impact on cytokines, enzymes and albumin levels in both groups (Figure 5). This is surprising given the central role of inflammation in periodontitis (Page and Schroeder 1976; Papapanou et al. 2018), particularly the increase in albumin despite decreased BoP%. One explanation is that BoP reflects immediate clinical gingival inflammation, whereas salivary albumin, a plasma-derived protein, has a slower biological response and is influenced by systemic and biological factors (Fazekas et al. 1992; Persson 2024; Curtis et al. 1988; Rantonen and Meurman 2000; Anura 2014). The smaller albumin increases and lower chitinase levels in the probiotic group may indicate reduced vascular leakage and tissue breakdown (Bikker et al. 2019). Previous studies based on gingival crevicular fluid (GCF) reported reduced pro-inflammatory markers (IL-1β, IL-8, IL-17, TNF-α, MMP-8) and increased anti-inflammatory markers (IL-10, TIMP-1) with probiotics following NSPT (Invernici et al. 2018; Szkaradkiewicz et al. 2014; İnce et al. 2015). Comparisons are limited by the small heterogeneous studies and by our use of saliva instead of GCF. Saliva was chosen to avoid site-specific bias, but our results suggest that immune markers are diluted in saliva. The sample size was calculated from microbiological data, and although the microbiological and clinical outcomes indicate sufficient power, the immunological results are most likely underpowered. A post hoc analysis duplicating the dataset yielded similar findings. Future studies should consider GCF, a broader panel of cytokines or metaproteomic analysis to capture host immune responses better.

A key limitation of the present study is its short-term study design, as significant clinical and microbial changes often occur over 6–12 months (Ausenda et al. 2023; Socransky et al. 2013;

TABLE 1 | Clinical results.

	Baseline		Week 12			
	Placebo	**Probiotics**	**Placebo**	**Probiotics**	***p* [CI]**	**Nicotine adjustment**
PI % Mean (SD)	84.4 (14)	83.3 (15.2)	35.1 (16.2)	37.4 (19.4)	0.65 [−12.04; 7.53]	0.51 [−13.5; 6.68]
BoP% Mean (SD)	29.2 (10.8)	32.7 (14.7)	18.7 (8.4)	15.6 (10.8)	0.04[a] [03; 9.55]	0.03[a] [0.41; 9.75]
PPD Mean (SD)	3 (0.5)	3 (0.3)	2.9 (0.4)	2.7 (0.3)	0.03[a] [−0.25; −0.01]	0.07 [−0.23; 0.01]
PPD < 5 mm Mean *n* (SD)	136.9 (18.4)	138.8 (18.2)	144.5 (18.4)	150.3 (13.6)	0.03[a] [0.41; 8.32]	0.02[a] [0.99; 9.17]
PPD ≥ 5 mm Mean *n* (SD)	22.4 (17.4)	20.4 (14.6)	14.8 (15.6)	8.9 (9.1)	0.01[a] [−8.17; −1.09]	0.01[a] [−8.59; −1.19]
CAL Mean (SD)	1.57 (1.1)	1.65 (0.9)	1.49 (1.1)	1.58 (0.9)	0.8 [−0.17; 0.22]	0.94 [−0.19; 0.21]

Note: PI%, BoP%, CAL and mean PD are reported as means and standard deviations (SD), with PD < 5 mm, and PD ≥ 5 mm quantified for placebo and probiotics at baseline and Week 12. *p*-values and confidence intervals are shown from comparisons between groups at baseline versus Week 12 using ANCOVA, incorporating baseline values as covariates with and without adjustments for smoking.
[a]Significant differences between groups.

Krajewski et al. 2025). The observed clinical changes were relatively small, especially in the placebo group, and the mild but diverse disease severity in our population may have made minor improvements statistically significant. The inclusion of smokers and individuals with diabetes is also a limitation, as these factors act as confounders. However, post hoc analyses excluding these participants yielded similar results despite being underpowered, and their inclusion reflects real-world conditions. Selection bias is another concern, as participants were recruited from the Department of Odontology, where patients generally have fewer resources, which also affects generalisability. Nevertheless, attrition, performance and detection bias were minimised, and the intervention was realistic, with at-home probiotic consumption and high compliance. It should be noted that probiotics involve an additional economic burden, and adherence might be higher among better resourced individuals. Future studies would benefit from longer follow-up, a broader population with solely generalised periodontitis and larger sample sizes stratified for confounders—ideally in a multi-centre design.

The major strength of the present study is the comprehensive analysis of both clinical and immunological parameters of subgingival and salivary microbiota. The differences observed between subgingival and salivary results likely reflect the distinct ecological niches: the periodontal pocket as a localised environment versus saliva representing a composite of all oral niches (Escapa et al. 2018; Belstrøm 2020). The consistent direction of changes in saliva and subgingival plaque further supports this interpretation. The use of high-throughput, open-ended 16S rRNA sequencing and metagenomics allowed detailed characterisation of microbial communities compared to previous use of qPCR, DNA–DNA checkerboard and culturing (Teughels et al. 2013; Mayanagi et al. 2009; Morales et al. 2018; de Oliveira et al. 2022; Pudgar et al. 2021). Pathway analysis in periodontitis is still infrequent but has shown enrichment of anaerobic functions, while healthy sites were dominated by carbohydrate metabolism (Kumar et al. 2021; Wang et al. 2013; Shi et al. 2015). Our results indicated a general reduction in gene abundance rather than pathway-specific effect, although findings should be interpreted with caution due to saliva-based sampling, the incomplete KEGG database and the lack of metatranscriptomic data. Combined with the limitations of 16S sequencing, future studies should consider combining metagenomic and metatranscriptomic approaches to capture bacterial activity at the gene level (Belstrøm, Constancias, Markvart, et al. 2021; Belstrøm, Constancias, Drautz-Moses, et al. 2021).

Current probiotic evidence is affected by heterogeneity of dosages, frequencies and strains, along with a high prevalence of moderate to high bias (Ho et al. 2020; Hu et al. 2021; Ikram et al. 2018; Li et al. 2023; Martin-Cabezas et al. 2016; Mishra et al. 2021; Ram et al. 2024; Song and Liu 2020; Vives-Soler and Chimenos-Küstner 2020; Nguyen et al. 2020). Although probiotics appear to enhance clinical, microbiological and immunological outcomes when administered continuously, the formulation of definitive conclusions is complicated, and further well-designed, large-scale, standardised studies are needed.

Within the limitations of this exploratory pilot study, daily probiotic supplementation as an adjunct to NSPT reduces the abundance of periodontitis-associated species compared with placebo, supporting further investigations with longer follow-up and larger sample sizes.

Author Contributions

S.V.R.A., C.M.L.-O., D.B., C.D. and M.M. designed the study. Clinical examinations were performed by S.V.R.A. and PMPR and subgingival instrumentation was performed by C.M.L.-O. Subgingival plaque and saliva were collected by C.M.L.-O. at baseline and by S.V.R.A. at weeks 6 and 12. Cytokine activity was measured by M.G. and U.K.G. at Turku University, Finland, and proteases by A.S. and F.J.B. at the Academic Center for Dentistry Amsterdam (ACTA), Netherlands. Biostatistical analysis of clinical data was performed by C.M.L.-O. and levels of cytokines and proteases by C.M.L.-O. and L.M. C.M.L.-O. and D.B. wrote the first draft of the manuscript, which was critically revised by C.D., M.M., S.V.R.A., M.G., U.K.G., A.V.S., F.J.B. and L.M.

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Ethics Statement

The present study was conducted according to the Helsinki Declaration and approved by the regional ethical committee (H-21003295). The study was registered at ClinicalTrials.gov (NCT05518747) on 22 August 2022 and reported to the local data authorisation of the Faculty of Health and Medical Sciences, University of Copenhagen (514-0649/21-3000).

Consent

All participants signed informed consent before participation.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Raw sequences of 16S data are deposited at the European Nucleotide Archive (ENA, www.ebi.ac.uk, accessed on 1 June 2025) with the accession number PRJEB87651. Raw metagenome, cytokine, enzyme and albumin data, composition of the applied lozenges, trial protocol and analysis plan in Danish are available upon request to the corresponding author.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Histogram of compliance for the placebo group (A) and the probiotic group (B). **Figure S2:** Metagenomic pathway analysis comparing baseline with Week 6 and baseline with Week 12 within the placebo and probiotic groups and between the groups. A blue bar indicates a decrease in the given pathway from baseline to Week 6 or Week 12, while a red bar indicates an increase. Only pathways showing significant differences between groups are displayed. **Figure S3:** Microbiological sub-analyses excluding smokers and diabetics. The 20 most abundant genera at baseline, Week 6 and Week 12 are presented in (A). Significant differences are marked with as asterisk (*). Significantly different species identified by LEfSe are presented in (B) at Week 6 and Week 12. **Figure S4:** Subgingival PCA plots with clinical variables (A) and immunological variables (B), and salivary PCA plots with clinical variables (C) and immunological variables (D). **Figure S5:** Spearman correlations between the 20 most abundant subgingival species and clinical parameters (A) and immunological markers (B). Spearman correlations between the 20 most abundant salivary species and clinical parameters (C) and immunological markers (D). **Table S1:** Background information for the placebo and probiotic groups, presented by sex, age, distribution of diagnoses and distribution of self-reported smoking status, diabetes and previous periodontal treatment. **Table S2:** Clinical results reported for PI%, BoP%, PPD <5 mm, PPD ≥ 5 mm and CAL within the placebo group (A) and the probiotic group (B). **Table S3:** Salivary levels of IL-1β, IL-8, MIF, MCP-1, amylase activity, chitinase activity, total protease activity and albumin concentration presented within the placebo group (A), within the probiotic group (B) and between groups (C). **Table S4:** Sub-analyses excluding nicotine users and diabetics. Clinical results within the placebo group (A), clinical results within the probiotic group (B), clinical results between groups (C) and immunological results between the groups (D).

Appendix 1

CONSORT 2025 Checklist

Section/topic	No.	CONSORT 2025 checklist item description	Reported on page no.
Title and abstract			
Title and structured abstract	1a	Identification as a randomized trial	1
	1b	Structured summary of the trial design, methods, results, and conclusions	2
Open science			
Trial registration	2	Name of trial registry, identifying number (with URL) and date of registration	15
Protocol and statistical analysis plan	3	Where the trial protocol and statistical analysis plan can be accessed	15
Data sharing	4	Where and how the individual de-identified participant data (including data dictionary), statistical code and any other materials can be accessed	15
Funding and conflicts of interest	5a	Sources of funding and other support (e.g., supply of drugs), and role of funders in the design, conduct, analysis and reporting of the trial	15
	5b	Financial and other conflicts of interest of the manuscript authors	15
Introduction			
Background and rationale	6	Scientific background and rationale	4
Objectives	7	Specific objectives related to benefits and harms	4
Methods			
Patient and public involvement	8	Details of patient or public involvement in the design, conduct and reporting of the trial	5
Trial design	9	Description of trial design including type of trial (e.g., parallel group, crossover), allocation ratio, and framework (e.g., superiority, equivalence, non-inferiority, exploratory)	5
Changes to trial protocol	10	Important changes to the trial after it commenced including any outcomes or analyses that were not prespecified, with reason	—
Trial setting	11	Settings (e.g., community, hospital) and locations (e.g., countries, sites) where the trial was conducted	5
Eligibility criteria	12a	Eligibility criteria for participants	5,6
	12b	If applicable, eligibility criteria for sites and for individuals delivering the interventions (e.g., surgeons, physiotherapists)	
Intervention and comparator	13	Intervention and comparator with sufficient details to allow replication. If relevant, where additional materials describing the intervention and comparator (e.g., intervention manual) can be accessed	5–7
Outcomes	14	Prespecified primary and secondary outcomes, including the specific measurement variable (e.g., systolic blood pressure), analysis metric (e.g., change from baseline, final value, time to event), method of aggregation (e.g., median, proportion), and time point for each outcome	4
Harms	15	How harms were defined and assessed (e.g., systematically, non-systematically)	—
Sample size	16a	How sample size was determined, including all assumptions supporting the sample size calculation	5
	16b	Explanation of any interim analyses and stopping guidelines	—
Randomisation:			
Sequence generation	17a	Who generated the random allocation sequence and the method used	5
	17b	Type of randomisation and details of any restriction (e.g., stratification, blocking and block size)	5

Section/topic	No.	CONSORT 2025 checklist item description	Reported on page no.
			Reported on page no.
Allocation concealment mechanism	18	Mechanism used to implement the random allocation sequence (e.g., central computer/telephone; sequentially numbered, opaque, sealed containers), describing any steps to conceal the sequence until interventions were assigned	5
Implementation	19	Whether the personnel who enrolled and those who assigned participants to the interventions had access to the random allocation sequence	5
Blinding	20a	Who was blinded after assignment to interventions (e.g., participants, care providers, outcome assessors, data analysts)	5
	20b	If blinded, how blinding was achieved and description of the similarity of interventions	6
Statistical methods	21a	Statistical methods used to compare groups for primary and secondary outcomes, including harms	8
	21b	Definition of who is included in each analysis (e.g., all randomized participants), and in which group	8
	21c	How missing data were handled in the analysis	8
	21d	Methods for any additional analyses (e.g., subgroup and sensitivity analyses), distinguishing prespecified from post hoc	—
Results			
Participant flow, including flow diagram	22a	For each group, the numbers of participants who were randomly assigned, received intended intervention, and were analysed for the primary outcome	9
	22b	For each group, losses and exclusions after randomisation, together with reasons	9
Recruitment	23a	Dates defining the periods of recruitment and follow-up for outcomes of benefits and harms	5
	23b	If relevant, why the trial ended or was stopped	—
Intervention and comparator delivery	24a	Intervention and comparator as they were actually administered (e.g., where appropriate, who delivered the intervention/comparator, how participants adhered, whether they were delivered as intended (fidelity))	6, 9
	24b	Concomitant care received during the trial for each group	—
Baseline data	25	A table showing baseline demographic and clinical characteristics for each group	9
Numbers analysed, outcomes and estimation	26	For each primary and secondary outcome, by group: • the number of participants included in the analysis • the number of participants with available data at the outcome time point • result for each group, and the estimated effect size and its precision (such as 95% confidence interval) • for binary outcomes, presentation of both absolute and relative effect size	9–11
Harms	27	All harms or unintended events in each group	—
Ancillary analyses	28	Any other analyses performed, including subgroup and sensitivity analyses, distinguishing pre-specified from post hoc	11
Discussion			
Interpretation	29	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	12–14
Limitations	30	Trial limitations, addressing sources of potential bias, imprecision, generalisability, and, if relevant, multiplicity of analyses	12–14

Appendix 2

Cytokines

Following manufacturer's protocol, samples were centrifuged for 5 min at 9300*g* and levels of interleukin (IL)-8, IL-1β, monocyte chemoattractant protein-1 (MCP-1) and macrophage migration inhibitory factor (MIF) were measured from salivary supernatants using the Luminex xMAP technique (Luminex Corporation, Austin, TX, USA) with optimised commercial kits (Bio-Plex Pro Human Cytokine Screening panel, Bio-Rad Laboratories). The limit of detection (LOD) was as follows: 0.36 pg/mL for IL-8, 0.24 pg/mL for IL-1β, 0.44 pg/mL for MCP-1 and 2.45 pg/mL for MIF. Mean intra-assay coefficient of variation (%) was 3.2% for IL-8, 3.6% for IL-1β, 3.2% for MCP-1 and 3.4% for MIF.

Appendix 3

Proteins

Saliva samples were centrifuged after collection at 10,000*g* at 4°C for 10 min and the supernatant was aliquoted. Per the manufacturer's instructions, the total protein concentration was analysed as described (Lundtorp et al. 2023; Lundtorp Olsen, Massarenti, et al. 2023; Morquecho-Campos et al. 2020) by Pierce BCA Protein Assay Kit (ThermoFisher, West Palm Beach, FL, USA, Cat#23227). Albumin was measured essentially as previously described (Bikker et al. 2019; Prodan et al. 2015) In brief, microplates were coated with rabbit anti-human albumin (cat# A0001 DAKO, Glostrup, Denmark). An albumin concentration series (BSA: 25–1500 ug/mL) was included as a reference on each plate and used to calculate the albumin concentration in samples. Saliva samples were 1:2000 diluted. Anti-human albumin (horseradish peroxidase [HRP]; Bior-byt via bioconnect, Cat# ORB243267) was used as conjugate and albumin was detected using o-phenylenediamine dihydrochloride (OPD) as substrate (Thermo Fisher #34006 West Palm Beach, FL, USA) according to the instructions of the manufacturer. Salivary amylase activity was measured by diluting the samples 1:100 in MILLI-Q and mixing them with alpha-amylase substrate consisting of 2-chloro-4-nitrophenyl-α-D-maltotrioside (Apollo Scientific, Denton, UK, BITJ00020) as described earlier (Morquecho-Campos et al. 2020). Total protease activity (TPA) was measured using PEK-54 substrate ([FITC]-NIeKKKK VLPIQLNAATDK-[KDbc]) as described elsewhere (Bikker et al. 2019), with a working solution of 32 μM diluted in TBS. Fifty microlitres of saliva and 50 μL of PEK-54 substrate were added to a black 96-well plate (non-binding), resulting in a final PEK-54 substrate concentration of 16 μM. Reaction mixtures were placed in a fluorescence microplate reader with a 485 nm excitation filter and a 530 nm emission filter (gain 800), and fluorescence was measured for approximately 1 h at 37°C using 5-min scanning intervals. Proteolytic activity was expressed as the increase in fluorescence per min (F/min). To measure the chitinase activity, 50 μL of 4-methylumbelliferyl β-D-*N*,*N*′,*N*′′-triacetyl chitotriose substrate with a final concentration of 12.7 μM was mixed with 50 μL saliva in a black 96-well plate (non-binding). In every plate, a chitinase control enzyme at a concentration of 0.001 mg/mL was used as reference. Reaction mixes were placed in a fluorescence microplate reader with a 360 nm excitation filter and a 450 nm emission filter and fluorescence was measured for approximately 1 h at 37°C at 5-min intervals.

Appendix 4

Biostatistics

Background information is presented by descriptive statistics. Clinical data and data on cytokines, enzymes and albumin were checked for normal distributions by Q–Q plots, and data on cytokines, enzymes and albumin were $\log_2$-transformed. Comparisons within groups were performed by ANOVA adjusted with Tukey's corrections for multiple testing and ANCOVA between groups with baseline measurements as covariates. Smoking is a known inhibitor of the treatment outcome of periodontitis and is considered a confounder. A p-value < 0.05 was considered significant for all analyses.

Bioinformatic processing of 16S samples was performed as previously described (Lundtorp et al. 2023; Lundtorp Olsen, Massarenti, et al. 2023; Lundtorp-Olsen, Enevold, Juel Jensen, et al. 2021; Lundtorp-Olsen, Enevold, Twetman, and Belstrøm 2021; Lundtorp Olsen, Markvart, et al. 2023), by matching the demultiplexed Illumina reads against the 16S rRNA Human Oral Microbiome RefSeq database (HOMD) v. 15.2 (Escapa et al. 2018). The subgingival microbiota was characterised and compared according to relative abundance adjusted for multiple testing by Benjamini–Hochberg's correction (Hochberg and Benjamini 1990). Linear discriminant analysis effect size (LEfSe) was used to identify differences between groups (Segata et al. 2011) with significant differences identified by the combined criteria of Kruskal–Wallis $p < 0.05$, Wilcoxon p-adjusted < 0.05 and LDAscore > 2. α-Diversity was compared between groups by ANCOVA with baseline adjustment.

Metagenomic data were processed as previously described with minor modifications (Rodenes-Gavidia et al. 2023). In brief, optical duplicates, human genome sequences and low-quality score reads were removed (BBMap/BBtools:Bushnell B 2015; Coelho et al. 2019). The number of reads was standardised to 15 million (Li, n.d.) and taxonomically assigned using Metaphlan v.4 (Blanco-Míguez et al. 2023), and sequences were mapped using Salmon (Patro et al. 2017) to eHOMD v.10.1 metagenome database (Escapa et al. 2018), which was enriched with other constructed metagenomes from our study as described (Wen et al. 2017). Metagenomes were assembled using MEGAHIT genome assembler (Li et al. 2015), and contigs > 500 bp were used to predict genes (Hyatt et al. 2010), which were annotated by the Kyoto Encyclopaedia of Genes and Genomes (KEGG) annotation (Kanehisa et al. 2016). Genes were selected if they had > 10 counts in > 10% of the samples and a KEGG annotation included in prokaryotic pathways.

Data were normalised by rarefaction (McMurdie and Holmes 2013) prior to α-diversity calculation, and its changes by groups were tested by the Wilcoxon test. β-Diversity was analysed by the Bray–Curtis dissimilarity matrix and PERMANOVA analysis (Vegan et al., 2019) after normalisation by relative abundances. DESeq2 (Love et al. 2014) was used to identify differentially abundant taxa and genes using its normalisation method. A taxon or gene was considered differentially abundant if the corrected p-value was < 0.05 and if present in at least 50% of the samples in one group. A gene set enrichment analysis (GSEA) was conducted on KEGG modules based on the differential abundance statistics from DESeq2 (Korotkevich et al. 2019). All analyses were performed using R and R Studio.