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## **Effects of *Bifidobacterium* Probiotic on the Treatment of Chronic Periodontitis:**

### **A Randomized Clinical Trial**

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## **ABSTRACT**

**Aim:** This randomized placebo-controlled clinical trial evaluated the effect of *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) HN019-containing probiotic lozenges as adjuvant to scaling and root planing (SRP) in patients with generalized chronic periodontitis.

**Materials and Methods:** Forty-one chronic periodontitis patients were recruited and monitored clinically, immunologically, and microbiologically at baseline (before SRP) and 30 and 90 days after SRP. All patients were randomly assigned to a Test (SRP+Probiotic, n=20) or Control (SRP+Placebo, n=21) group. The probiotic lozenges were used twice a day for 30 days. The data were statistically analyzed.

**Results:** The Test group presented a decrease in probing pocket depth and a clinical attachment gain significantly higher than those of the Control group at 90 days. The Test group also demonstrated significantly fewer periodontal pathogens of red and orange complexes, as well as lower proinflammatory cytokine levels when compared to the Control group. Only the Test group showed an increase in the number of *B. lactis* HN019 DNA copies on subgingival biofilm at 30 and 90 days.

**Conclusion:** The use of *B. lactis* HN019 as an adjunct to SRP promotes additional clinical, microbiological, and immunological benefits in the treatment of chronic periodontitis (NCT03408548).

## CLINICAL RELEVANCE

*Scientific rationale for the study:* *Lactobacillus* probiotics have been investigated for the treatment of periodontitis. However, the effects of the genus *Bifidobacterium* on periodontitis are poorly known.

*Principal findings:* *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) HN019 probiotic significantly improved clinical periodontal parameters, reduced periodontal pathogens more effectively, and reduced the levels of proinflammatory cytokines in the gingival crevicular fluid.

*Practical implications:* The use of *B. lactis* HN019 as an adjunct to scaling and root planing may reduce the need for additional periodontal therapies after non-surgical treatment of chronic periodontitis.

## INTRODUCTION

Scaling and root planing (SRP) is the gold standard for the treatment of periodontitis (Berezow and Darveau, 2011). However, treated sites are subject to recolonization with a microbiota similar to that present before therapy (Mombelli, 2018). Given the limitations of SRP in the treatment of periodontitis (Berezow and Darveau, 2011), adjuvant therapies – antibiotic therapy, antimicrobial photodynamic therapy, and probiotic therapy – have been proposed (Feres et al., 2012, Petelin et al., 2015 Teughels et al., 2013).

The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) defined probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2002). The studies that evaluated the effects of probiotics on the treatment of periodontal disease demonstrated that they can reduce periodontopathogens, improve periodontal clinical parameters, decrease the levels of proinflammatory cytokines, and potentiate the effects of SRP (Ince et al., 2015, Shah et al., 2013, Tekce et al., 2015, Teughels et al., 2013, Vivekananda et al., 2010). In fact, two meta-analyses seem to support the adjunctive use of probiotics in the treatment of chronic periodontitis (Martin-Cabezas et al., 2016, Ikram et al., 2018). Nevertheless, further high-quality randomized clinical trials with microbiological outcomes are warranted to obtain strong conclusions in this regard (Ikram et al., 2018).

To date, studies on the effects of probiotics on periodontal diseases have used mainly microorganisms of the genus *Lactobacillus*. However, other potential probiotics should be investigated. Hojo et al. (2007) observed that *Bifidobacterium* species and their counts could be associated with periodontal health and with treatment success in patients with periodontitis. In an *in vitro* study, Jasberg et al. (2016) demonstrated that species of the genus *Bifidobacterium* can adhere strongly to the subgingival biofilm and significantly reduce the count of *Porphyromonas gingivalis*.

Only two pre-clinical studies evaluated the effect of bacteria of the genus *Bifidobacterium* on periodontal disease (Oliveira et al., 2017, Ricoldi et al., 2017). Oliveira et al. (2017) demonstrated that the topical use of *Bifidobacterium animalis subsp. lactis* HN019 promotes a protective effect against alveolar bone loss and connective tissue attachment loss attributable to experimental periodontitis in rats. Ricoldi et al. (2017) concluded that the oral administration of *B. lactis* HN019 potentiates the effects of SRP on experimental periodontitis in rats. Considering the clinical use of *Bifidobacterium* on periodontal diseases,

there is only one clinical trial that investigated the effects of 4-week use of yogurt supplemented with *Bifidobacterium animalis subsp. lactis* DN-173010 versus a placebo yogurt, followed by a 5-day non-brushing period, on gingivitis (Kuru et al., 2017). The authors concluded that the probiotic can have a positive effect on plaque accumulation and gingival inflammatory parameters after oral hygiene abstinence.

So far, no clinical study has investigated the effects of bacteria of the genus *Bifidobacterium* on non-surgical treatment of periodontitis. This randomized placebo-controlled clinical trial evaluated the effects of *B. lactis* HN019-containing probiotic lozenges as adjuvant to SRP in patients with generalized chronic periodontitis.

## **MATERIALS AND METHODS**

### *Patient population*

Forty-one patients were selected from the population referred to the Periodontal Clinic at the School of Dentistry of Ribeirao Preto – University of Sao Paulo (FORP-USP, Ribeirao Preto, SP, Brazil). All eligible patients (ClinicalTrials.gov ID: NCT03408548) were thoroughly informed of the nature and potential risks and benefits of their participation in the study and signed an informed consent form. The study protocol was reviewed and approved by the Research Ethics Committee of FORP-USP (protocol number: 06278012.1.0000.5419). The research was conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2008) and additional requirements.

The sample size was determined using Graphpad Statemate 2.0 (GraphPad Software, Inc., San Diego, CA, USA). An 80% power was adopted to recognize a significant difference of 1 mm (d) between groups with a 95% confidence interval ( $\alpha = 0.05$ ) and standard deviation (SD) of 1.13 mm (Vivekananda et al., 2010).

Considering changes in mean clinical attachment level (CAL) as the primary outcome variable and  $(Z\alpha + Z\beta)^2 = 7.84$  ( $Z\alpha = 1.96$  for 2-tailed 0.05 hypothesis test;  $Z\beta = 0.842$  for power = 0.8), sample size was calculated using the following formula:  $n = \{2[(SD)^2/(d)^2]\} \times (Z\alpha + Z\beta)^2$ . Therefore, a total of 36 patients were required. However, considering that some patients could be lost to follow-up, 41 patients were enrolled in this study.

#### *Inclusion and exclusion criteria*

All patients were diagnosed with generalized chronic periodontitis according to the classification of the American Academy of Periodontology (Armitage, 1999). The inclusion criteria were: (1) age over 30 years, (2) 30% or more of the sites with probing pocket depth (PPD)  $\geq 4$  mm and CAL  $\geq 4$  mm, (4) presence of bleeding on probing (BOP) and a minimum of five teeth with at least one site with CAL and PPD  $\geq 5$  mm. All patients had to be in good general health.

The exclusion criteria were: (1) cause-related periodontal therapy or antimicrobial therapy in the previous 6 months, (2) systemic conditions that could influence the progression of periodontitis or the treatment response (e.g. Down Syndrome, HIV, Diabetes Mellitus types 1 and 2), (3) smoking, (4) need of prophylactic antibiotic therapy for routine dental procedures, (5) long-term administration of anti-inflammatory medications and (6) usage of probiotics 6 months prior to the study.

#### *Experimental design, allocation concealment, and treatment protocol*

According to a random numeric table generated by computer software, the study coordinator (M.R.M.) allocated each patient to one of the following groups: Control (SRP+Placebo; 21 patients) or Test (SRP+Probiotic therapy, 20 patients). Before the study began, the selected individuals were identified by a numeric code that designated the experimental group to

which they belonged. The study coordinator (M.R.M.) revealed the meaning of each code only after conducting the statistical analysis of the experimental data.

The patients received lozenges containing 10 mg of probiotic (Test group) or placebo (Control group). In the Test group, the lozenges had  $10^9$  colony-forming units (CFUs) of *B. lactis* HN019 (HOWARU® Bifido LYO 40 DCU-S, DuPont™ Danisco® Sweeteners Oy, Kantvik, Finland). In general, products containing probiotic microorganisms must have a minimum number of viable cells, with proven efficacy established on human clinical studies, estimated between  $10^6$  and  $10^8$  CFUs per gram (CFUs/g) of the final product or  $10^8$  to  $10^{10}$  CFUs/day (considering 100 g or 100 ml of ingested food) (Champagne et al., 2011). A compounding pharmacy prepared identical probiotic and placebo lozenges. Identical plastic bottles containing the probiotic/placebos were sent to the study coordinator (M.R.M.), who wrote the number code of each patient on each bottle, according to the therapy they were assigned to. The coded bottles were given to the examiner (M.S.M.S.), who distributed them to the patients and did not have any access to information about the content of the lozenges. In addition, the patients were blinded to the content of the lozenges and treatment assignment during the study.

Seven days prior to the non-surgical periodontal therapy, all patients received supragingival plaque control and oral hygiene instructions. Within 24 hours, a specialist in periodontics (M.M.I.), who was blinded to the experimental groups, performed supragingival and subgingival SRP on all teeth with periodontal involvement, using hand (Gracey Curettes, Hu-Friedy, Chicago, IL, USA) and ultrasonic instruments. The patients were instructed (immediately after SRP) to take one lozenge twice a day (after waking up and before bedtime) for 30 days. They were also instructed not to take any other probiotic product during the study. The patients received 14 lozenges (placebo or probiotic) per week. At the end of each week, they had to be seen at the FORP-USP Periodontal Clinic. During their

visit, they should bring the packs of lozenges taken during the week and then they received new lozenges for the subsequent week. During the visit, the patients answered a questionnaire about their perception of any side effect observed during the consumption of the dietary supplement. The patients were not subjected to any clinical procedure during these visits. One research assistant (P.H.F.S.) conducted these procedures and monitored the patient's compliance with medication dosage. This assistant was not the examiner or operator in this study.

All patients received microbiological, immunological, and clinical monitoring at baseline, at 30 days, and at 90 days. The evaluations (pre and post-intervention) were conducted by a single trained and calibrated examiner (M.S.M.S.), who was blinded to the experimental groups.

#### *Examiner calibration*

The Kappa coefficient greater than or equal to 0.85 was used for examiner calibration. Ten patients with at least five teeth with PPD and CAL  $\geq 5$  mm on proximal sites were selected. Each patient was examined twice by a universal North Carolina-15 periodontal probe (PCPUNC156, Hu-Friedy, Chicago, IL, USA), at a 48-hour interval between the first and second assessments.

#### *Clinical measurements*

Plaque index (PI) was employed to assess the patients oral hygiene status. BOP was recorded based on the presence or absence of bleeding up to 20 seconds after probing at the experimental sites. PI and BOP were scored as plaque and bleeding being absent or present (0 or 1, respectively). PPD was measured from the free gingival margin to the bottom of the periodontal pocket. CAL was measured from the cementoenamel junction to the base of the



periodontal pocket. Gingival recession (GR) was measured from the cemento-enamel junction to the free gingival margin. BOP, PPD, CAL, and GR were measured at six sites per tooth. All probing measurements were performed using a manual periodontal probe (PCPUNC156, Hu-Friedy, Chicago, IL, USA).

#### *Immunological monitoring*

Gingival crevicular fluid (GCF) was collected from four non-contiguous interproximal sites per patient using absorbent paper strips (Periopaper<sup>®</sup>, Oralflo Inc., Amityville, NY, USA), as previously described by Luchesi et al. (2013). Conventional enzyme immunoassays (ELISA) using commercially available kits (DCTM Protein Assay, Bio-Rad Laboratories, Inc. Berkeley, CA, USA) were performed to determine the amount of total protein in each sample. IL-1 $\beta$ , IL-10, and IL-8 levels in GCF samples were determined according to Moreira et al. (2015) using high sensitivity kits (LXSAHM-03 – Techne Corporation, R&D Systems, Minneapolis, MN, USA) and the multiplexing instrument (MAGPIX<sup>®</sup> analyzer, Luminex Corporation, Austin, TX, USA). These cytokines were selected considering the study by Ricoldi et al. (2017), which demonstrated the effects of *B. lactis* HN019 on these immunoinflammatory markers in periodontal tissues of rats. The concentrations of each cytokine were estimated from the standard curve using a five-parameter polynomial equation with specific software (Xponent<sup>®</sup> software, Luminex Corporation, Austin, TX, USA).

#### *Microbiological monitoring*

According to Moreira et al. (2015) subgingival plaque samples were obtained from four non-contiguous interproximal sites from each patient, grouped according to the following PPD categories: moderate pockets (PPD = 4 to 6 mm) and deep pockets (PPD  $\geq$  7 mm). The samples were individually analyzed for the presence of 40 subgingival bacterial species using

the checkerboard DNA-DNA hybridization technique (Socransky et al., 2004) at the Microbiology Laboratory of the Guarulhos University (UNG, Guarulhos, SP, Brazil).

*B. lactis* HN019 was detected and quantified by real-time polymerase chain reaction (qPCR) using specific primers, as proposed by Junick and Blaut. (2012). Genomic DNA was extracted and the qPCR technique was performed using the SYBRGreen system (Invitrogen Corp., Carlsbad, California, USA) in StepOne Plus™ Real-Time (Thermo Fisher Scientific).

#### *Outcome variables*

Changes in the mean CAL at 90 days post-SRP were defined as the primary outcome variable. All other parameters were considered secondary outcomes. Sub analyses were made considering PPD at baseline for clinical (PPD, CAL, and GR) and microbiological parameters. Periodontal pockets were classified at baseline as moderate (PPD = 4-6 mm) or deep (PPD  $\geq$  7 mm).

The “need for additional periodontal treatment” was determined according to Cionca et al. (2009). “Risk for periodontal disease progression” was defined at the patient level according to Lang and Tonetti. (2003).

#### *Statistical analysis*

Each outcome was computed per participant and then averaged across patients in both groups. The significance level was set at 5%.

Within-group and between-group differences in PPD, CAL, GR, PI, BOP, and residual periodontal pockets were assessed by repeated-measures analysis of variance (ANOVA) followed by Bonferroni *post-hoc* test and by Student’s t test, respectively. The differences between groups considering the outcomes “need for additional periodontal treatment” and “risk for periodontal disease progression” were assessed by the Chi-square test.

Microbiological data were presented as mean counts ( $\times 10^5$ ) of individual bacterial species in both groups. Bacterial species were also grouped into complexes, according to Socransky et al. (1998). Friedman's test was used to detect significant differences within each group. The analyses were performed after adjustments for multiple comparisons (Socransky et al., 1991). The between-group differences in the changes observed at 90 days regarding the mean counts of each bacterial species compared to baseline values were assessed by the Mann-Whitney test. The within-group and between-group differences in the number of copies of *B. lactis* HN019 DNA were also assessed by the Mann-Whitney test. Total protein values were converted to pg/mL. Final cytokine levels were obtained by dividing the initial values provided by the MAGPIX<sup>®</sup> system by the total protein content in GCF samples (pg/mL). Within-group and between-group differences in the mean levels of IL-1 $\beta$ , IL-8, and IL-10 were assessed by ANOVA followed by Bonferroni *post-hoc* test and Student's t test, respectively.

## RESULTS

This study started in December 2015 and ended in August 2016. Figure 1 shows the study design. All patients successfully completed the study. The demographic characteristics didn't show any difference between groups (for additional details, see online supplementary material). No adverse effects were observed after the use of probiotics.

### *Clinical monitoring*

Table 1 shows the PI, BOP, PPD, CAL, and GR values for the Control and Test groups. In moderate and deep pockets, the Test group had larger clinical attachment gain and lower PPD than the Control group at 90 days ( $P < 0.05$ ).

Regarding the number of residual pockets (Table 1), the Control group had a higher number of moderate (at 30 and 90 days) and deep (at 90 days) pockets than the Test group ( $P<0.05$ ).

As to the risk of progression of periodontal disease (Table 2), while 55% of the patients in the Test group were at a low risk, only 30% of the patients in the Control group were classified as such. The Test group had a lower rate of patients in need for additional periodontal treatment on more than three sites when compared to the Control group at 90 days ( $P<0.05$ ).

#### *Microbiological monitoring*

Figure 2 shows the mean total count ( $\times 10^5$ ) of 40 subgingival species in deep and moderate periodontal pockets for the Control and Test groups, as well as changes in the mean counts of each bacterial species at 90 days relative to baseline values. The Test group exhibited a larger count of *Actinomyces naeslundii* and *Streptococcus mitis* and a more pronounced reduction in the count of *P. gingivalis*, *Treponema denticola*, *Fusobacterium nucleatum vincentii*, *Campylobacter showae* and *Eubacterium nodatum* than the Control group ( $P<0.05$ ) for deep periodontal pockets.

Figure 3 shows the mean cumulative proportions of microbial complexes observed in both groups in different experimental periods. Test group showed mean proportions of orange (at 30 days) and red (at 90 days) complexes significantly lower than those of the Control group ( $P<0.05$ ). A significantly larger proportion of blue complex bacteria was also observed in the Test group when compared to the Control group at 90 days ( $P<0.05$ ).

The qPCR analysis (Figure 4) demonstrated that only the Test group had an increase in the number of copies/ $\mu\text{L}$  of the DNA of *B. lactis* HN019 in subgingival biofilm samples at 30 and 90 days ( $P<0.05$ ).

### *Immunological monitoring*

IL-8, IL-1 $\beta$ , and IL-10 levels are shown in Figure 5 (A-C). Only the Test group had higher levels of IL-10 than those at baseline at 30 days ( $P<0.05$ ). Figure 5 (E-G) shows the mean ratios between the levels of each cytokine and those at baseline. The Control group had a higher ratio of IL-1 $\beta$  (at 30 and 90 days) and of IL-8 (at 30 days) when compared to the Test group ( $P<0.05$ ).

## **DISCUSSION**

This double-blind, randomized controlled trial assessed the effects of *B. lactis* HN019 on the non-surgical treatment of individuals with advanced generalized chronic periodontitis by analyzing clinical, immunological, and microbiological parameters. The results show that probiotic therapy as an adjunct to SRP promoted additional benefits in assessments at 30 and 90 postoperative days.

The growing prevalence of antimicrobial resistance has prompted the development of new antimicrobial therapeutic approaches for the treatment of biofilm-related oral diseases. In the present study, the results obtained for PPD and CAL in deep pockets in the Test group at 90 postoperative days were similar to or higher to those obtained in some studies that used antibiotics as adjuvants to SRP for the treatment of chronic periodontitis (Silva et al., 2011, Feres et al., 2012, Saleh et al., 2016).

In the present study, whereas 58% of pockets diagnosed at baseline with PPD  $\geq 7$  mm decreased to PPD  $\leq 3$  mm at 90 days in the Test group, only 22% of deep pockets in the Control group were reduced. Teughels et al. (2013) also verified that the use of probiotics as adjuvants to SRP reduced the risk of progression of periodontitis and the number of patients,

teeth, and sites with indication of surgical periodontal treatment. These findings demonstrate the clinical significance of periodontal treatment.

It should be emphasized that the outcomes obtained with probiotics can not be generalized (Teughels et al., 2011), as they depend on the strain, dosage, frequency, and mode of administration. In the present study, probiotic therapy with bacteria of the genus *Bifidobacterium* promoted a reduction of PPD (3.5 mm) in deep pockets larger than those obtained by Teughels et al. (2013) and Laleman et al. (2015) (2.88 and 2.37 mm, respectively) at 90 days, with the administration of probiotics of the genera *Lactobacillus* and *Streptococcus*, respectively.

Modulation of the host's microbiota is one of the basic mechanisms that may explain the beneficial effects of probiotics on periodontal health. The Test group presented higher rates of commensal bacteria on subgingival biofilm when compared with the Control group at 90 days. These commensal bacteria are associated with periodontal health (Aas et al., 2005, Lucas et al., 2000, Hajishengallis et al., 2012, Abusleme et al., 2013, Aruni et al., 2015) and they can help control inflammation and infection of periodontal tissues (Devine et al., 2015, Kumar and Mason, 2015). Compared with the Control group, the Test group presented lower proportions of some Gram-negative anaerobic bacteria species involved in the pathogenesis of periodontal diseases. In a preclinical study, Oliveira et al. (2017) demonstrated that *B. lactis* HN019 reduces the amount of periodontopathogens on biofilm. The possible coaggregation of *Bifidobacterium* with *Fusobacterium nucleatum* can reduce the amount of binding sites for periodontopathogens on biofilm (Haukioja et al., 2006). Furthermore, probiotic bacteria can produce several components that act as antimicrobial agents (Gillor et al., 2008, Gordon, 2009). Some *in vitro* studies have shown that *Bifidobacterium* can inhibit the growth of periodontopathogens such as *P. gingivalis* (Zhu et al., 2010, Jasberg et al., 2016).

In the present study, whereas no differences were observed between the Control and Test groups at 30 postoperative days regarding the proportions of red complex bacteria, the Control Group had higher percentual of these bacteria at 90 days. It could suggest that probiotic might have acted delaying the recolonization of periodontal pockets by these bacteria in the Test Group. Tekce et al. (2015) observed that patients treated with SRP and probiotics presented improvements in the rates of strict anaerobic microorganisms on subgingival biofilm, and that these rates became similar to those of the patients treated exclusively with SRP only at 360 postoperative days. Differently from these results with probiotics, the effects of antibiotics on delayed recolonization of periodontal pockets seem to occur in the short term. Mdala et al. (2013) and Bizzarro et al. (2016) have shown that no differences were seen in the number of red complex pathogens between patients treated with SRP and those treated with SRP combined with antibiotic therapy after 3 months.

The Test group had a greater number of copies/ $\mu$ L of the DNA of *B. lactis* HN019 in subgingival biofilm samples than the Control group at 30 and 90 days, which suggests exogenous translocation of the probiotic to the subgingival biofilm. *B. lactis* HN019 was detected in the Test group 60 days after discontinuation of probiotic therapy. This fact suggests that *B. lactis* HN019 might have temporarily integrated into the subgingival biofilm. Meurman et al. (1994), Iniesta et al. (2012), and Tekce et al., (2015) also noted the persistence of bacteria of the genus *Lactobacillus* in the oral cavity at 2, 8, and 9 weeks, respectively, after discontinuation of probiotic therapy.

The Control group presented higher levels of IL-1 $\beta$  (30 and 90 days) and of IL-8 (30 days) when compared with the Test group. This finding demonstrates the possible effect of probiotic therapy on modulation of the periodontal inflammatory process. In a recent clinical study, administration of probiotic lactic acid bacteria to patients with chronic periodontitis decreased IL-1 $\beta$  levels in GCF (Szkaradkiewicz et al., 2017). Kuru et al. (2017) demonstrated

that patients with experimental gingivitis previously treated with *B.lactis* DN-173010 presented lower IL-1 $\beta$  levels in GCF when compared to untreated patients. With respect to IL-10, only the Test group had higher levels of this cytokine when compared with baseline at 30 days. Ricoldi et al. (2017) also demonstrated that *B. lactis* HN019 can increase IL-10 levels at periodontal sites in rats.

The present study is the first one to demonstrate the potential effect of a probiotic bacterium of the genus *Bifidobacterium* on the non-surgical treatment of chronic periodontitis. The short period of assessment was a limitation of this study. A long-term follow-up of these patients would be important to evaluate whether the additional effects obtained with probiotic therapy as an adjunct to SRP are sustained over time. It is also important that future investigations evaluate the persistence of *B. lactis* HN019 in the oral cavity after discontinuation of probiotic therapy, new modes of administration, and other therapeutic regimens.

## CONCLUSION

The use of *B. lactis* HN019 as an adjunct to SRP offers additional clinical, microbiological, and immunological benefits in the treatment of periodontal pockets in patients with generalized chronic periodontitis.



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Table 1 – Means and SD of clinical parameters (PPD, CAL, GR, PI, and BOP), number of moderate and deep pockets and follow-up of pockets detected at baseline in groups Test and Control.

VARIABLE	TIME POINT	EXPERIMENTAL GROUPS				INTERGROUP COMPARISONS		
		Test N=20		Control N=21		Mean difference	Student's <i>t</i> test <i>p</i> value	
		Mean ± SD	Delta± SD Δ 0-30/ 0-90	Mean ± SD	Delta± SD Δ 0-30/ 0-90		Mean	Delta
PPD (mm)								
Overall	Baseline	3.01±0.27	-	3.10±0.43	-	0.09	NS	-
	30 days	2.53±0.25*	-0.48±0.30	2.78±0.37*	-0.32±0.27	0.25	≤0.05	0.1579
	90 days	2.49±0.27*	-0.52±0.32	2.85±0.34*	-0.25±0.22	0.36	≤0.05	0.0048
Moderate pockets	Baseline	4.47±0.20	-	4.44±0.27	-	0.02	NS	-
	30 days	3.29±0.47*	-1.18±0.37	3.33±0.42*	-1.11±0.41	0.04	NS	NS
	90 days	3.19±0.52*	-1.28±0.41	3.50±0.45*	-0.94±0.42	0.31	≤0.05	0.01
Deep pockets	Baseline	7.27±0.29	-	7.26±0.35	-	0.14	NS	-
	30 days	4.07±1.14*	-3.20±1.00	4.47±1.23*	-2.79±1.11	0.39	NS	NS
	90 days	3.75±1.32*	-3.52±1.19	4.64±1.00*	-2.62±0.88	0.90	0.019	0.0103
CAL (mm)								
Overall	Baseline	3.26±0.39	-	3.42±0.54	-	0.16	NS	-
	30 days	2.77±0.44*	-0.49±0.37	3.13±0.50*	-0.29±0.26	0.36	≤0.05	0.0855
	90 days	2.77±0.38*	-0.49±0.37	3.24±0.51*	-0.18±0.23	0.47	≤0.05	0.0025
Moderate pockets	Baseline	4.63±0.42	-	4.70±0.45	-	0.07	NS	-
	30 days	3.51±0.65*	-1.12±0.44	3.69±0.56*	-1.01±0.44	0.18	NS	NS
	90 days	3.48±0.59*	-1.15±0.45	3.94±0.63*	-0.76±0.41	0.45	0.022	0.0082
Deep pockets	Baseline	7.48±0.53	-	7.62±0.72	-	0.13	NS	-
	30 days	4.36±1.45*	-3.12±1.27	5.08±1.49*	-2.54±1.33	0.72	NS	NS
	90 days	4.03±1.44*	-3.45±1.21	5.55±1.37*	-2.07±0.97	1.51	0.001	0.0004
GR (mm)								
Overall	Baseline	0.25±0.35	-	0.32±0.33	-	0.07	NS	NS
	30 days	0.23±0.32	-0.02±0.16	0.35±0.33	0.03±0.13	0.12	NS	NS
	90 days	0.27±0.87	0.02±0.12	0.38±0.97	0.06±0.19	0.11	NS	NS
Moderate pockets	Baseline	0.15±0.27	-	0.26±0.25	-	0.10	NS	-
	30 days	0.21±0.33	0.06±0.16	0.36±0.34	0.10±0.24	0.14	NS	NS
	90 days	0.28±0.34	0.13±0.27	0.43±0.48	0.17±0.31	0.15	NS	NS
Deep pockets	Baseline	0.26±0.45	-	0.50±0.77	-	0.24	NS	-
	30 days	0.28 ± 0.47	0.02±0.37	0.61±0.91	0.11±0.47	0.33	NS	NS
	90 days	0.28 ± 0.66	0.02±0.62	0.90±1.09	0.40±0.47	0.61	0.038	0.0360
PI after OHI								
Mean Number ± SD (%)								
	Baseline	23.85±15.33 (25.90)	-	26.71±16.60 (27.40)	-	2.71 (1.50)	NS	-
	30 days	14.20±12.73* (15.40)	-9.65±10.52	20.24±17.53* (20.80)	-6.47±11.59	6.51 (5.40)	≤0.05	NS
	90 days	21.65±13.13 (23.50)	-2.20±12.30	27.14±18.64 (27.90)	0.47±12.07	5.49 (4.40)	NS	NS



Table 1 (continued)

<b>BOP after OHI</b>								
Mean Number $\pm$ SD (%)								
	<i>Baseline</i>	30.80 $\pm$ 22.07 (22.10)	-	35.00 $\pm$ 25.84 (24.10)	-	4.20 (2.00)	NS	-
	30 days	17.05 $\pm$ 14.60* (12.20)	-13.75 $\pm$ 14.16	24.05 $\pm$ 19.83* (16.50)	-10.50 $\pm$ 16.17	7.00 (4.30)	$\leq 0.05$	NS
	90 days	18.80 $\pm$ 16.14* (13.50)	-12.00 $\pm$ 15.49	30.71 $\pm$ 27.86 (21.10)	-4.29 $\pm$ 18.61	11.91 (7.60)	$\leq 0.05$	NS
<b>Moderate pockets</b>								
Mean Number $\pm$ SD (%)								
	<i>Baseline</i>	28.45 $\pm$ 9.72 (20.40)	-	33.90 $\pm$ 13.12 (23.30)	-	5.45 (2.90)	NS	-
	30 days	13.90 $\pm$ 7.64* (10.00)	-14.55 $\pm$ 8.63	22.86 $\pm$ 14.1* (15.70)	-11.04 $\pm$ 10.35	8.96 (5.70)	$\leq 0.05$	NS
	90 days	12.50 $\pm$ 6.95* (9.00)	-15.95 $\pm$ 10.58	23.67 $\pm$ 11.6* (16.30)	-10.23 $\pm$ 8.82	11.17 (7.30)	$\leq 0.05$	0.0674
<b>Deep pockets</b>								
Mean Number $\pm$ SD (%)								
	<i>Baseline</i>	5.60 $\pm$ 3.69 (4.00)	-	6.00 $\pm$ 5.25 (4.10)	-	0.40 (0.10)	NS	-
	30 days	1.25 $\pm$ 2.26* (0.90)	-4.35 $\pm$ 3.66	1.90 $\pm$ 3.41* (1.30)	-4.10 $\pm$ 4.27	0.65 (0.40)	NS	0.8391
	90 days	1.25 $\pm$ 2.42* (0.90)	-4.35 $\pm$ 3.95	2.90 $\pm$ 4.14* (2.00)	-3.10 $\pm$ 2.40	1.65 (1.10)	$\leq 0.05$	0.2242
<b>Follow-up of moderate pockets detected at baseline</b>								
Mean Number $\pm$ SD (%)								
PPD $\leq$ 3mm	90 days	19.60 $\pm$ 9.33 (68.90)	-	18.38 $\pm$ 8.80 (54.20)	-	1.22 (14.70)	$\leq 0.05$	-
PPD $\geq$ 4 $\leq$ 6 mm	90 days	8.40 $\pm$ 5.39 (29.10)	-	14.81 $\pm$ 10.54 (43.70)	-	6.41 (14.60)	$\leq 0.05$	-
PD $\geq$ 7 mm	90 days	0.45 $\pm$ 1.23 (1.60)	-	0.71 $\pm$ 1.30 (2.10)	-	0.26 (0.40)	NS	-
<b>Follow-up of deep pockets detected at baseline</b>								
Mean Number $\pm$ SD (%)								
PPD $\leq$ 3mm	90 days	3.25 $\pm$ 2.50 (58.00)	-	1.33 $\pm$ 1.20 (22.20)	-	1.92 (35.80)	$\leq 0.05$	-
PPD $\geq$ 4 $\leq$ 6 mm	90 days	1.65 $\pm$ 1.53 (29.50)	-	2.8 $\pm$ 2.56 (46.80)	-	1.15 (17.30)	$\leq 0.05$	-
PPD $\geq$ 7 mm	90 days	0.7 $\pm$ 1.42 (12.50)	-	1.86 $\pm$ 2.85 (31.00)	-	1.16 (18.50)	$\leq 0.05$	-

PPD = probing pocket depth; CAL = clinical attachment level; GR=gingival recession; PI = plaque index; BOP = bleeding on probing; SD = standard deviation; OHI = oral hygiene instructions; Moderate pockets = PPD  $\geq$  4  $\leq$  6 mm; Deep Pockets = PPD  $\geq$  7 mm; NS = no significant difference; \*Significant difference when compared to baseline (repeated measures ANOVA, Bonferroni *post hoc* test,  $P < 0.05$ ).

Table 2 – Number and percentage of subjects presenting low ( $\leq 4$  sites with PPD  $\geq 5$  mm), moderate (5-8 sites with PPD  $\geq 5$  mm) or high ( $\geq 9$  sites with PPD  $\geq 5$  mm) risk for disease progression, as well as presenting 0, 1-2 or  $\geq 3$  sites in need for additional therapy.

VARIABLE	TIME POINT	EXPERIMENTAL GROUPS		INTERGROUP COMPARISONS
		Test N=20 Number (%)	Control N=21 Number (%)	Chi-square for trend <i>p</i> value
Risk for disease progression				
Low	90 days	11 (55.00)	6 (28.60)	0.0863
Moderate	90 days	3 (15.00)	4 (19.00)	
High	90 days	6 (30.00)	11 (52.40)	
Need for additional therapy				
0 sites	90 days	4 (20.00)	1 (12.20)	0.0306
1-2 sites	90 days	9 (45.00)	6 (36.60)	
≥ 3 sites	90 days	7 (35.00)	14 (51.20)	

## FIGURE LEGENDS

**Figure 1.** Flowchart of the study design.

**Figure 2.** Profiles of the mean counts ( $\times 10^5$ ) of 40 taxa in subgingival biofilm samples at baseline and at 30 and 90 days post-treatment. The panel at the far right presents the changes in the mean counts of each species between baseline and 90 days post-treatment. The species were ordered according to the microbial complexes described by Socransky et al. (1998). The significance of differences among time points was determined using Friedman test ( $^*P < 0.05$  at 30 days and  $^{\#}P < 0.05$  at 90 days) and between groups at 90 days using Mann–Whitney test ( $^{\&}P < 0.05$ ). All the analyses were adjusted for multiple comparisons (Socransky et al. 1991). (A) deep pockets, (B) moderate pocktes.

**Figure 3.** Cumulative mean proportions of microbial complexes in subgingival biofilm samples taken from subjects at baseline and at 30 and 90 days post treatment. The colors represent different microbial complexes (SOCRANSKY et al. 1998). The light green color represents the proportion of *Aa*. The gray color represents species that did not fall into any complex, and *Actinomyces* species are represented in blue. The significance of differences among time points was determined using Friedman and Dunn multiple-comparison tests (different letters represent significant differences between time points,  $P < 0.05$ ). The significance of differences between the 2 groups at 30 days and 90 days was determined by using Mann–Whitney test ( $^*P < 0.05$  at 30 days and  $^{**}P < 0.05$  at 90 days).



**Figure 4.** Number of copies/ $\mu\text{L}$  of *B. lactis* HN019 DNA and standard deviations (numbers above the columns) in samples of subgingival biofilm of Test and Control groups at baseline, 30 days and 90 days, as well as intra and inter group comparisons. Different majuscule letters indicate significant differences in each group over experimental time (Friedman test, Dunn,  $P<0.05$ ). Different minuscule letters indicate inter group differences within the same time point (Mann-Whitney test,  $P<0.05$ ).

**Figure 5.** Levels (pg/mL) of IL-8 (A), IL-1 $\beta$  (B), and IL-10 (C) for each subject of groups Test and Control at baseline and at 30 and 90 days post-treatment. The mean changes in cytokines levels in relation to baseline values for Control and Test groups can be observed in E-G. \*Significant difference in Test group when compared with baseline values (Repeated Measures ANOVA, Bonferroni,  $P<0.05$ ). †Significant difference in Control group when compared with baseline values (Repeated Measures ANOVA, Bonferroni,  $P<0.05$ ). ‡Significant difference among groups in the same time point (Student t test,  $P<0.05$ ).







