The influence of a *Bifidobacterium animalis* probiotic on gingival health: a randomized controlled clinical trial

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One-sentence summary: The use of a probiotic yoghurt supplemented with *Bifidobacterium animalis* has a positive effect on plaque accumulation and gingival inflammatory parameters during a subsequent 5-day non-brushing period

Abstract

Background: There is a growing interest for probiotics in periodontal therapy, however, until to date, most research is focused on lactobacilli probiotics. The aim of this study was to evaluate the effect of the 4-week usage of a *Bifidobacterium animalis* subsp. *lactis* DN-173010 supplemented yoghurt versus a placebo yoghurt, followed by a 5-day non-brushing period.

Methods: Individuals were included in this single-blind, randomized controlled study if probing depth was (PD) ≤ 3 mm and attachment loss ≤ 2 mm. After professional prophylaxis, they were randomized into two groups receiving a placebo or *Bifidobacterium* containing yoghurt for 28 days, followed by a 5-day non-brushing period. The outcome measures were plaque and gingival indices, bleeding on probing, probing depth, gingival crevicular fluid (GCF) volume, total amount and concentration of interleukin-1β in GCF. These were measured at baseline, after 28 days of the study product usage and subsequently after the 5 days of plaque accumulation.

Results: 51 patients were analyzed. No intergroup differences could be detected before and after the intake of the study products. However, after plaque accumulation, significant better results for all parameters could be seen in the probiotic group compared to the control group ($p<0.001$): lower plaque and gingivitis scores, less bleeding on probing, less increase in GCF volume and lower total interleukin-1β amount/ concentration.

Conclusion: The use of a probiotic yoghurt supplemented with *Bifidobacterium animalis* can have a positive effect on plaque accumulation and gingival inflammatory parameters after refraining oral hygiene practices.

Keywords

Gingivitis, probiotics, Bifidobacterium, *Bifidobacterium animalis,* yoghurt

Introduction

Recently, there is a growing interest in the use of probiotic products for restoring a dysbiotic microbiota. Probiotics are defined as microorganisms which, when administered in adequate amounts, confer a health benefit for the host [1]. The beneficial impact of probiotics on certain gastrointestinal disorders is well-established [2]. More than a decade ago, probiotics were introduced for periodontal healthcare[3]. For the maintenance of periodontal health, the equilibrium between the microbial challenge and the host response is a determining factor [4–6]. In this context, probiotics might have a possible role, by suppressing and displacing harmful bacteria and indirectly by their immunomodulatory effects [7].

A number of in vitro and in vivo studies have been conducted focusing on the role of probiotics in the prevention and treatment of periodontal diseases [8–20]. It has been shown that probiotics were useful in reducing gingival inflammation [8–11], plaque accumulation [8,9,13], improving periodontal health [14–16], decreasing the number of black pigmented rods including *Porphyromonas gingivalis* in the saliva and/or subgingival plaque [14,17–20], and reducing pro-inflammatory cytokines in gingivitis patient [10]. Furthermore, it has also been reported that the application of probiotic bacteria as an adjunct to scaling and root planing can inhibit recolonization of pathogens in periodontal pockets and reduce plaque and gingival indices and bleeding [14–16,18,21].

Probiotic supplements come in a variety of forms: from powders, chewing gums and capsules to foods such as chocolates and dairy products that are supplemented with specific probiotic organisms. Most of the probiotic studies in the periodontal literature were performed using *Lactobacillus* species [22]. Besides *Lactobacillus* species, also *Bificobacteria* are often described as potent probiotics[23]. In the dental field, it was shown that probiotics containing *Bifidobacteria* can reduce the mutans streptococci counts [24–27]. However, to our knowledge, no studies have examined the effects of *Bifidobacterium* supplemented probiotics in (experimental) gingivitis or periodontitis patients. Hojo and co-workers (2007) evaluated the distribution of *Bifidobacterium* species in periodontitis patients, former periodontitis patients and in healthy individuals [28]. In their study, it has been suggested that bifidobacterial counts might be associated with periodontal health status. More recently, microbiological and immunoinflammatory effects of *Bificobacterium animalis* subsp. *Lactis* HN019 were shown by Oliveria and co-workers (2017) in experimental periodontitis in rats [29]. Therefore, the objective of this study was

to evaluate the effect of a 4-week usage of a *Bifidobacterium* supplemented yoghurt versus a placebo yoghurt, followed by a 5-day non-brushing period. The plaque accumulation was studied together with different parameters assessing the degree of gingival inflammation.

Materials and methods

This examiner-blinded, randomized controlled study with two parallel groups was approved by the Marmara University Health Sciences Ethical Committee (MAR-2011-11/14) and registered at ClincialTrails.gov as NCT02546206. Potential participants were recruited from the Marmara University School of Dentistry, Oral Diagnosis and Radiology Department, where the patients were admitted first and screened for oral health problems to be referred to specialty clinics. This selection was conducted according to the following criteria: periodontally healthy patients [30] with at least 24 natural teeth (excluding third molars), probing depth (PD) ≤ 3 mm and without predisposing oral factors causing local irritation and plaque retention. The individuals were further evaluated periodontally and were solely included if attachment loss was ≤ 2 mm and gingival index (GI) ≤ 1 [31]. Exclusion criteria were 1) presence of systemic diseases, 2) pregnancy or breastfeeding, 3) history of drug abuse, 4) previous probiotic supplements in diet, 5) medications, in particular currently ingestion of non-steroidal or steroidal anti-inflammatory drugs, or antibiotics within 3 months before entering the study, 6) mouth breathing, 7) allergic reactions to lactose or fermented milk products, and 8) current smoker or smoker over the past year.

The study was performed between November 2011 and May 2012.

Sample size calculation and randomization

The sample size was calculated based on the study of Slawik et al. (2011)[32], considering 95% power and an α of 0.05, a mean difference of 15.51 and a standard deviation of 12.72 for bleeding on probing (BoP) score between the groups, the number of the patients needed was at least 16 for each group. A 10% drop-out rate was considered.

A computer-based randomization program[§] was used for assigning the patients randomly into 2 groups (by BEK). Every patient was sequentially numbered (1-51) and in the meantime coded as 1 (test) or 2 (control). The measurements and sampling was done by an examiner (TY) who was unaware of the patients' yoghurt type.

Treatment protocol

This study comprised firstly a period of 28 days of probiotic or placebo yoghurt consumption followed by a 5-day plaque accumulation period by refraining from any oral

[§] www.randomizer.org

hygiene measurement as seen in Figure 1. Seven days before the start of the study, participants were given verbal reinforcement of oral hygiene and a professional tooth cleaning was carried out using abrasives[**] and brushes[††]. All the patients were given the same toothpaste[‡‡]. On day 0 (D0), the patients were randomly assigned to one of the two groups as described above and the study started with the use of the probiotic and placebo yoghurts.

The yoghurts were handed out by BEK. Half of the participants were given 110g probiotic plain yoghurt/day[§§] containing ≥10^8 colony forming units (cfu)/g *Bifidobacterium animalis* subsp. *lactis* DN-173010. The participants in the control group received 110g of plain yoghurt without probiotic bacteria[***]. It was attempted to blind the type of the yoghurt as much as possible for the patients: the paper covering the body of the container was removed. It was recommended to use the study products in the morning between breakfast and lunchtime and to not eat, nor brush the teeth for at least 1 hour after yoghurt consumption.

Outcome variables of interest

At the start of the study on D0, firstly gingival crevicular fluid (GCF) was collected and afterwards clinical measurements were done: plaque index (PI), GI, PD and BoP. GCF sampling and clinical measurements were repeated both at the beginning and the end of the non-brushing period which are the days 28 (D28) and 33 (D33), respectively. Both the clinical measurements and the GCF sampling were done from 8 selected teeth: the maxillary incisors and canines and lower canines. At each experimental time-point (D0, D28, D33), the GCF samples and the clinical measurements were taken exactly from the same teeth and same periodontal sites.

PI was determined using the Silness-Löe index at four surfaces of the teeth (mesio-buccal, mid-buccal, disto-buccal and mid-lingual)[33]. The plaque was assessed visually without staining and graded by four degrees. 0: no plaque, 1: little accumulation of plaque adhering to the free gingival margin and adjacent area of the tooth which can only be seen with the use of a probe, 2: moderate accumulation of plaque adhering to the free gingival

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margin and adjacent area of the tooth which can be seen with the naked eye, 3: pronounced accumulation of soft matter.

GI was recorded at four surfaces/tooth as for PI according to the Löe and Silness (1963) index with 0= normal gingiva, 1= mild inflammation, slight change in color, mild alteration of gingival surface structure, no bleeding on probing, 2 = moderate inflammation, redness, edema and swelling, bleeding on probing, 3 = severe inflammation, marked redness and edema, ulceration and tendency to spontaneous bleeding [31].

The PD, measured at six sites per tooth was defined as the distance between the base of the sulcus and the margin of the gingiva. The presence or absence of bleeding was measured at six sites per tooth after probing†††.

GCF was collected with an absorbent paper strip‡‡‡. The selected teeth were isolated by using cotton rolls. Following the elimination of supragingival plaque and saliva, paper strips were gently inserted in the gingival sulcus for 30 seconds and the volume of the GCF sample was immediately recorded§§§, expressed in Periotron units and followed by calculation of the volume values of each sample using a standard curve. The strips were then transferred to plastic tubes**** and stored at -70°C until analyzed. For this, the paper strips were allowed to thaw at room temperature for 30 min. Then pooled GCF samples were eluted from the 8 paper strips per patient by placing them in 150 µl of phosphate buffer saline and stored for up to 24 hours at 4^0C prior to use [34]. The levels of interleukin-1 beta (IL-1β) in GCF samples were analyzed by enzyme linked immunosorbent assay (ELISA), using a commercially available Solid Phase Sandwich ELISA Kit†††† according to manufacturer's instructions. The concentrations of IL-1β in each of the GCF sample were then calculated from the standard curve and presented as pg/ml per site.

Examiner calibration

A calibration exercise was performed for the examiner (TY) to determine the acceptable intra-examiner reproducibility. Five gingivitis patients (with both bleeding and non-bleeding sites upon probing) not included in the study were evaluated by the examiner

††† PCP 15UNC, Hu-Friedy, Chicago, IL, USA
‡‡‡ Periopaper Strip®, Pro Flow Incorporated, Amityville, NY, USA
§§§ Periotron® 8000, Proflow Incorporated. New York, NY, USA
**** Eppendorf , Millipore, Billerica, MA, USA
†††† Quantikine Human interleukin-1 beta HSLB00C, R&D Systems, Minneapolis, MN, USA

on two separate occasions 48 hours apart. The PI, GI, PD and BoP were measured. Calibration was accepted if the measurements at baseline and 48 hours were consistent in ≥90% of the measurements [35].

Compliance and adverse effects

The compliance was checked and confirmed verbally at D14 and D28 (BEK). For checking the adverse effects the patients were asked whether they got any of these symptoms: stomach gas, diarrhea, signs of infection (fever, chills), allergic reactions (rash, hives, itching, difficulty in breathing, swelling of mouth/ lips/ face/ tongue) and dizziness.

Statistical analysis

For all statistical evaluations, the patient was maintained as the unit of measurement. Quantitative data was taken as the mean value +/- standard deviation of 8 periodontal sites from 8 teeth in each individual for all parameters. Kolmogorow-Smirnow test was used to check the normality of the distribution. Two-way Friedman test was used for multiple intragroup comparisons for all parameters at 3 different time-point measurements (D0, D28, and D33). If this was found statistically significant, then intragroup comparisons in pairs (Wilcoxon test) between 2 time points (D0-D28; D28-D33; D0-D33) were done. Afterwards, these intra-group differences, as well as the mean values of the parameters at each time point were compared between the two groups (Mann-Whitney U test).

For all measurements statistical significance was set as $p<0.05$, whereas in paired comparisons, p-values were corrected for multiple comparisons with the Bonferroni correction and the statistical significance was set as $p<0.017$.

Results

As shown in Figure 2, 63 patients were screened and 51 patients were found eligible to participate in the study. These 19 males and 32 females, aged 16 to 26, completed the study; other demographical data can be found in Table 1. When the compliance of the use of the study products was checked, all patients declared that they consumed the yoghurts without missing a day. The adverse effects were checked verbally with a list of possible side effects, but none of the patients reported a problem from this list.

Table 2 shows all intra- and intergroup comparisons for the examined parameters. The p-values for the intragroup differences in pairs are shown in table 3.

Concerning PI and GI, both in the probiotic as in the control group, no intragroup differences could be noted between D0 and D28. However, 5-days of not brushing led to a significantly higher plaque and gingival index compared to D0 and D28 regardless of the study group (for all these intragroup measurements, $p<0.001$). Concerning the intergroup comparisons, no statistically significant differences were found between the groups on D0 and D28. However, at the end of the non-brushing period, on D33, the means of PI and GI were significantly lower in the probiotic group than in placebo group. The increase in PI and GI scores between the measurements on D33 versus D28 and those on D33 versus D0 were statistically significantly different between the two groups; the mean increase between D33 and D28 or D0 were smaller in the probiotic group than in the control group. Concerning BoP the same trends can be seen as for PI and GI. The BoP was significantly higher both in the probiotic as in the control group on D33 than on D28 (respectively, $p<0.001$ and $p<0.001$) and on D33 versus D0 (respectively, <0.001 and $p<0.001$). The BoP was comparable between both groups on D0 and after the 28-days usage of the study products (D28). However, after plaque accumulation on D33, the BoP was significantly lower in the patients who used the probiotic yoghurts compared to the ones who consumed the placebo yoghurts. Looking at the intergroup comparison of the intragroup differences between the start of the non-brushing period on D28 and the end of this period on D33, the increase in bleeding sites was significantly lower in the probiotic group than in the control group (10.45% versus 21.23%).

For PD, the only statistically significant intragroup difference was that the mean PD in the control group was significantly deeper on D33 than on D0 ($p=0.005$). Accordingly, the only significant intergroup difference was the higher mean PD value in the control group

reached after the non-brushing period on D33. The intergroup comparison of the intragroup differences was not statistically significantly different.

Concerning the measurements carried out on GCF, no intragroup differences could be noted in the probiotic group, these were only detected in the control group. The volume of GCF/pooled site, the IL-1β concentration/pooled site and the IL-1β total amount/pooled site were significantly higher on D33 than on D0 or D28 (for all these measurements $p<0.001$). Also these measurements were also significantly higher in the control group on D28 compared with D0 (respectively, $p=0.009$, $p=0.001$ and $p=0.002$). Concerning the differences between the probiotic and control group, no statistically significant differences were found between the groups on D0 and D28 for any of the examined GCF parameters. However, when comparing both groups after the plaque accumulation period on D33, significantly more GCF volume, higher concentration and higher total amount of IL-1β could be detected in the patients of the control group. Furthermore, all intergroup comparisons of the intragroup differences between D33-D0, D33-D28 and D28-D0 were significantly different in favor of the probiotic group.

Discussion

This study in periodontally healthy individuals showed the effects of a 28 days probiotic yoghurt consumption containing at least 10^8 cfu/g *B. animalis* subsp. *lactis* versus placebo yoghurt and a subsequently 5-day non-brushing period. After the 5-day non-brushing period (D33) the clinical indices (PI, GI and BoP) were elevated in both groups compared to D0 and D28. For the GCF parameters, intragroup differences could only be found in the control group. Lower PI and GI scores, less BoP, lower GCF volume and a lower total amount and concentration of IL-1β were measured for the probiotic group compared to the control group. As a pro-inflammatory cytokine, IL-1β is released by macrophages following bacterial infection or tissue injury[36]. Higher IL-1β levels in GCF were detected in periodontitis patients compared to healthy and gingivitis patients and declined after mechanical periodontal therapy [37]. The finding that both concentration and total amount of GCF IL-1β were lower in the probiotic group than the control group could be interpreted as a result of the anti-inflammatory effect of the probiotic. Therefore, this study showed a positive effect on inflammatory parameters when plaque regrowth is induced after the consumption of this probiotic yoghurt.

Gingival inflammatory changes as well as GCF parameters are indicative for local host response. Bleeding is the most sensitive clinical indicator for gingival health and provides a reliable assessment for gingival inflammatory changes[38]. Gingival inflammation is also associated with increased levels of a variety of inflammatory mediators[39]. An increase in IL-1β release rates has been found in the GCF after at least 3 days of plaque accumulation[40]. There is no doubt that after 5 days of refraining from mechanical plaque control, microbial dental plaque accumulates. It is important to discard the possible immediate effect of probiotic on the plaque accumulation during the non-brushing period to evaluate the differences between the test and the control groups. Therefore, no probiotic yoghurts were given during the non-brushing period.

To our knowledge, this is the first study examining the influence of a single strain *Bifidobacterium* probiotic on gingival health in healthy dentate people. Positive effects of single strain *Bifidobacterium* probiotics on mutans streptococci in young adults were already described [24,26,41]. Also a decreased PI and GI could be seen after a four week usage of a multistrain probiotic (*Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* BB-12) in healthy adults[23]. It was also suggested that periodontal health might be associated with high bifidobacterial counts [28]. Moreover, Bifidobacteria, isolated

from probiotic yoghurt, can -at least in vitro- inhibit the growth of several periodontopathogens, such as *Porphyromonas gingivalis, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans* [42], when the Bifidobacteria were inoculated before the periodontal pathogens. Van Essche and co-workers (2013) confirmed these results using *Bifidobacterium dentium* strains which were isolated from healthy individuals[43]. From all the isolated inhibitory bacteria in the latter study, the bifidobacteria were the strongest inhibitors of *P. gingivalis*. Additionally, in an experimental periodontitis model in rats, it was shown that the topical use of *B. animalis* subsp. *lactis* HN019 promotes a protective effect against alveolar bone and connective tissue attachment loss.

The present study uses a similar set up as Staab (2009) and Slawik and co-workers (2011)[12,32]. Staab and co-workers investigated the effect of the consumption of a milk drink containing *Lactobacillus casei Shirota* for 8 weeks, followed by a 4-day experimental gingivitis period [12]. Clinically no statistically significant differences were found between patients using the probiotic milk drink for 8 weeks and control patients although the polymorphonuclear elastase activity was significantly lower in the test group after this 8-week period. Additionally, at the end of the 4-day experimental gingivitis period the test group had a significantly lower myeloperoxidase activity than the control group. Slawik and co-workers (2011) used the same probiotic milk drink in periodontally healthy patients[32]. The patients were instructed to use the product for 2 weeks prior to starting the 2-week experimental gingivitis period [32]. After the experimental gingivitis period BoP and GCF volume were significantly lower in the test group when compared to the control group. This experiment also revealed a positive effect on clinical parameters in the probiotic group.

The reason why this *B. lactis* containing yoghurt showed this effect is highly speculative. Besides the above mentioned antimicrobial properties of bifodobacteria towards periodontopathogens, it was shown that bifidobacteria can survive in saliva and bind to *F. nucleatum*-covered hydroxyapatite in vitro[44]. Although a microbiological analysis was not performed, these properties could have influenced the biofilm composition by inhibiting the periodontopathogens during the non-brushing period. Consequently, this may have had an impact on the inflammatory response and in turn, since inflammation increases plaque growth, resulted in a reduced PI [45].

The findings must be interpreted with the consideration of the following points. First, although a well-established non-brushing model was used [12], despite similar amounts of plaque accumulation, patients may respond differently to experimentally induced gingival inflammation [46–49]. Secondly, although this study was primarily designed to evaluate the clinical parameters, an analysis of the microbiota could have given more information about the observed effect. Since data are still sparse to explain the molecular and biological mechanisms of probiotics on oral health, a microbiological analysis could have given us for example more information about the (temporary) colonization by the probiotic microorganisms. Furthermore, possibly the participants could still deduct the type of yoghurt, when the specific green color and the text on the upper seal of the probiotic yoghurt were taken into account. This could have affected the compliance and retention of the trial participants [50]. However, all patients declared to have never missed one yoghurt and the examiner who did the clinical measurements was blinded to the treatment allocation.

Conclusion

This study demonstrated reduced clinical and immunological signs of inflammation in a non-brushing model after 28 days of $\geq 10^8$ cfu/g *B. animalis* subsp. *lactis* yoghurt consumption. This effect was seen both on the clinical level (PI, GI, BoP and PD) as on GCF markers (GCF volume, interleukin-1β concentration and interleukin-1β total amount). This is the first study describing Bifidobacteria as a potential probiotic to combat gingival inflammation. The effect of *B. animalis* on "real" gingivitis patients should be investigated together with their effects on microbiological parameters, the ideal concentration, method of administration and duration of the positive effect after the product usage.

Acknowledgments and Conflict of Interest

Authors declare no conflicts of interest. This study was not funded, the used probiotic and placebo yoghurts were supplied by the researchers.

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Figures and Tables

Figure 1: course of the study
Figure 2: Flow chart of the study

Table 1: Patient demographics

Variable	Treatment group		
	Probiotic	Control	*p-value*
Number of patients	26	25	
Number of males	9	10	
Number of females	17	15	
Age, range (mean ± SD)	17-25 (22.8 ± 3.52)	16-26 (21.64 ± 4.15)	
Plaque index	0.28 ± 0.14	0.37 ± 0.15	0.080
Gingival index	0.19 ± 0.08	0.22 ± 0.07	0.196
Bleeding on probing (%)	1.42 ± 0.92	1.36 ± 0.65	0.843
Probing Depth	1.49 ± 0.20	1.53 ± 0.13	0.434
GCF volume/ pooled sites (µl)	0.19 ± 0.04	0.14 ± 0.04	0.101
IL-1β concentration/ pooled sites (pg/ml)	125.21 ± 136.45	89.84 ± 105.49	0.152
IL-1β total amount/ pooled sites (pg)	0.05 ± 0.05	0.03 ± 0.04	0.080

Table 2: Overview of all examined parameters

Variable		Treatment group						Intergroup *p*-value		
		Probiotic			Control					
		Mean ± SD	Delta vs DO ± SD	Delta vs D28 ± SD	Mean ± SD	Delta vs DO ± SD	Delta vs D28 ± SD	For mean	For delta vs DO	For delta vs D28
Plaque index										
Overall	D0	0.28 ± 0.14			0.37 ± 0.15			NS		
	D28	0.27 ± 0.13	0.01 ± 0.10		0.38 ± 0.15	0.01 ± 0.18		NS	NS	
	D33	0.80 ± 0.30*†	0.52 ± 0.35	0.53 ± 0.34	1.80 ± 0.42*†	1.43 ± 0.44	1.42 ± 0.45	<0.001	<0.001	<0.001
Intragroup *p value*		<0.001			<0.001					
Gingival index										
Overall	D0	0.19 ± 0.08			0.22 ± 0.07			NS		
	D28	0.19 ± 0.07	0.00 ± 0.05		0.22 ± 0.05	0.00 ± 0.08		NS	NS	
	D33	0.80 ± 0.33*†	0.61 ± 0.34	0.61 ± 0.35	1.52 ± 0.44*†	1.30 ± 0.44	1.30 ± 0.45	<0.001	<0.001	<0.001
Intragroup *p value*		<0.001			<0.001					
Bleeding on probing (%)										
Overall	D0	1.42 ± 0.92			1.36 ± 0.65			NS		
	D28	1.42 ± 0.66	0.01 ± 1.22		1.58 ± 0.91	0.21 ± 1.05		NS	NS	
	D33	11.87 ± 4.12*†	10.46 ± 4.21	10.45 ± 4.22	22.81 ± 6.12*†	21.44 ± 6.20	21.23 ± 6.32	<0.001	<0.001	<0.001
Intragroup *p value*		<0.001			<0.001					
Probing depth (mm)										
Overall	D0	1.49 ± 0.20			1.53 ± 0.13			NS		
	D28	1.47 ± 0.17	0.02 ± 0.21		1.52 ± 0.13	0.00 ± 0.25		NS	NS	
	D33	1.44 ± 0.15	0.05 ± 0.24	0.03 ± 0.20	1.58 ± 0.12*	0.06 ± 0.10	0.06 ± 0.14	0.002	NS	NS
Intragroup *p value*		0.881			0.003					
GCF volume/pooled sites (µl)										
Overall	D0	0.19 ± 0.04			0.14 ± 0.04			NS		
	D28	0.21 ± 0.09	0.02 ± 0.11		0.19 ± 0.09*	0.05 ± 0.09		NS	0.03	
	D33	0.19 ± 0.07	0.01 ± 0.08	0.01 ± 0.11	0.33 ± 0.12*†	0.18 ± 0.12	0.13 ± 0.14	<0.001	<0.001	<0.001
Intragroup *p value*		0.448			<0.001					
IL-1β concentration/ pooled sites (pg/ml)										
Overall	D0	125.21 ± 136.45			89.84 ± 105.49			NS		

	D28	132.36 ± 184.35	7.14 ± 136.34		154.99 ± 179.56*	65.15 ± 131.75		NS	0.01	
	D33	144.72 ± 97.21	19.50 ± 96.67	12.36 ± 130.74	1267.05 ± 848.31*†	1177.21 ± 832.41	1112.06 ± 777.78	<0.001	<0.001	<0.001
Intragroup *p value*		0.151			<0.001					
IL-1β total amount/ pooled sites (pg)										
Overall	D0	0.05 ± 0.05			0.03 ± 0.04			NS		
	D28	0.05 ± 0.07	0.00 ± 0.05		0.06 ± 0.06*	0.02 ± 0.04		NS	0.01	
	D33	0.09 ± 0.07	0.04 ± 0.10	0.04 ± 0.12	0.63 ± 0.44*†	0.60 ± 0.44	0.57 ± 0.42	<0.001	<0.001	<0.001
Intragroup *p value*		0.028			<0.001					

SD: standard deviation, D0: day 0, D28: day 28, D33: day 33, * Intragroup significant difference from D0, † Intragroup significant difference from D28

Intergroup comparisons were performed by Mann-Whitney u test for the mean and delta values. Intra-group comparisons were performed by two way Friedman test for the three time points by blocking on subject, If there was a statistically significant difference, Wilcoxon test was performed for multiple comparisons in pairs. p values were corrected for multiple comparisons and with the Bonferroni correction, the statistical significance was set as $p<0.017$.

Table 3: intragroup comparisons in pairs

Variable	*p*-values					
	Probiotic			Control		
	D28-D0	D33-D0	D33-D28	D28-D0	D33-D0	D33-D28
Plaque index	NS	<0.001	<0.001	NS	<0.001	<0.001
Gingival index	NS	<0.001	<0.001	NS	<0.001	<0.001
Bleeding on probing (%)	NS	<0.001	<0.001	NS	<0.001	<0.001
Probing depth (mm)	NS	NS	NS	NS	NS	0.01
GCF volume/pooled sites (µl)	NS	NS	NS	0.01	<0.001	<0.001
IL-1β concentration/ pooled sites (pg/ml)	NS	NS	NS	<0.001	<0.001	<0.001
IL-1β total amount/ pooled sites (pg)	NS	NS	NS	<0.001	<0.001	<0.001

NS: not significantly different