

Clinical and microbiological effects of the adjunctive use of probiotics in the treatment of gingivitis: A randomized controlled clinical trial

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Abstract

Aim: To evaluate the efficacy of a probiotic combination in the treatment of gingivitis and to assess its impact on the subgingival microbiota.

Materials and Methods: A placebo-controlled clinical trial was conducted in gingivitis subjects during 6 weeks. Test treatment consisted of the administration of two oral tablets per day containing the probiotic strains *Lactobacillus plantarum*, *Lactobacillus brevis* and *Pediococcus acidilactici*; the control group received the same tablets but without live bacteria. The main outcome variable was the changes in gingival index (GI). Subgingival samples were collected and analysed by quantitative polymerase chain reaction (qPCR) for five putative periodontal pathogens. Outcome variables were compared between and within groups, and multiple regression analysis was performed.

Results: A total of 59 patients (29 tests, 30 placebos) were included in the analysis. Both treatment groups experienced a statistically significant improvement in mean GI ($p < .0001$), but no differences between treatment groups were found for any clinical index. A significantly higher reduction in the number of sites with higher GI scores (GI = 3 at baseline) was observed in the test group. In subgingival samples, a significant reduction in *T. forsythia* was significant only in the test group ($p < .008$).

Conclusions: The use of probiotic tablets containing *L. plantarum*, *L. brevis* and *P. acidilactici* did not lead to significant changes in mean GI; although a significant reduction occurred in the number of sites with severe inflammation. Furthermore, the adjunctive use of this probiotic promoted a significant microbiological impact.

KEYWORDS

gingival index, gingivitis, *Lactobacillus* spp., probiotics, subgingival microbiota

1 | INTRODUCTION

Gingivitis is characterized by redness, swelling and bleeding of the gingiva. It is caused by the accumulation of bacteria in the gingival crevice, which triggers an inflammatory reaction in the gingival tissues (Park et al., 2015). This condition is one of the most prevalent diseases affecting human beings (Albandar & Rams, 2002), and even though

not all patients with gingivitis will progress to periodontitis, management of gingivitis is critical in primary and secondary prevention of periodontitis (Chapple et al., 2015).

Regular mechanical removal of the dental biofilm through effective self-performed oral hygiene practices, together with professional intervention to eliminate already established biofilms and retentive factors (such as dental calculus, defective restorations or anatomic

factors), are the critical elements in gingivitis management (Lang, 2014). Unfortunately, a significant proportion of individuals fail to perform an effective supragingival biofilm control (Van der Weijden & Hioe, 2005) and subject-based factors, such as smoking, endocrine hormonal status, medication intake or systemic diseases, may modulate the inflammatory response to plaque and thus confer higher susceptibility to gingivitis (Tatakis & Trombelli, 2004). In these susceptible subjects, the adjunctive use of antimicrobial agents has been recommended (chemical plaque control; Van der Weijden & Hioe, 2005). However, the long-term use of antiseptics may be associated with unwanted side effects, which granted the search for alternative approaches (Wu & Savitt, 2002).

One of these alternative approaches has been the use of orally administered live microorganisms (probiotics) which, when administered in adequate amounts, may enhance the commensal flora and thus prevent the microbiological shift and colonization of true pathogens associated with gingival inflammation. Furthermore, probiotics may also benefit oral health by modulating the mucosal immunity in the oral cavity (Teughels, Teughels, Loozen, & Quiryren, 2011).

Several clinical studies have evaluated the effect of different strains of probiotics on gingival inflammation, reporting conflicting results, demonstrating that although a specific strain may exert a beneficial effect for general health, not all the probiotics may be useful in gingivitis management (Hallström et al., 2013; Iniesta et al., 2012; Krasse et al., 2006; Lee et al., 2015; Staab, Eick, Knöfler, & Jentsch, 2009).

Probiotic strains for oral use should be selected on the basis of their *in vitro* antimicrobial activity against oral pathogens, the ability to adhere to oral tissues, the tolerance to oral environmental stress factors and several safety aspects (such as their antibiotic resistance profile and lactic acid production). The strains *Lactobacillus plantarum* CECT 7481 (AB15), *Lactobacillus brevis* CECT 7480 (AB38) and *Pediococcus acidilactici* CECT 8633 (AB30) have shown *in vitro* activity against *Porphyromonas gingivalis*, *Treponema denticola* and *Fusobacterium nucleatum*, and the ability to colonize the oral cavity (Bosch et al., 2012). However, their clinical efficacy in terms of reducing gingival inflammation, when used as an orally administered probiotic, has not been demonstrated *in vivo*. It is therefore the purpose of this clinical trial to evaluate the efficacy of this probiotic combination when used orally to control gingivitis, and to evaluate its impact on the subgingival microbiota. The hypothesis is that the use of the present probiotic formulation could have an impact on the reduction in gingival inflammation.

2 | MATERIALS AND METHODS

2.1 | Study design

The study was designed as a randomized, double-blinded, placebo-controlled, parallel-group clinical trial in gingivitis patients, including subjects with minor attachment loss (≤ 2 mm). The protocol, informed consent and subject's information sheets were approved by the local research ethic committee (*Comité de Ensayos Clínicos del Hospital Universitario San Carlos, Madrid, Spain*) with the reference

Clinical Relevance

Scientific rationale for the study: Probiotics have been evaluated in the prevention and treatment of gingivitis with conflicting results, possibly due to the different probiotic strains administered. *L. plantarum*, *L. brevis* and *P. acidilactici* have shown antimicrobial activity against certain periodontal pathogens; however, their clinical efficacy has not been demonstrated *in vivo*.

Principal findings: The oral administration of the probiotic tablets did not lead to significant changes for mean GI but reduced the number of sites exhibiting severe gingival inflammation, as well as the counts of *T. forsythia* in subgingival samples.

Practical implications: The use of this probiotic formulation may be a useful tool to treat severe gingival inflammation.

number 14/465. Subjects attending the Faculty of Odontology, University Complutense (Madrid, Spain), from February to November 2015 were screened. Additionally, subjects studying or working in and around University Complutense were recruited by flyer advertisement. Those fulfilling the inclusion criteria were informed on the characteristics of the study; if they agreed to participate, they were recruited once they signed the informed consent forms.

2.1.1 | Inclusion criteria

- 18–55 years of age.
- Non-smokers (never smokers or former smokers for at least 6 months).
- Subjects with gingivitis defined as a mean gingival index [GI; Trombelli et al. (2004) modification of the Löe and Silness (1963)] > 1.3 .

2.1.2 | Exclusion criteria

- No interproximal attachment loss of ≥ 3 mm in ≥ 2 non-adjacent teeth (Tonetti & Claffey, 2005).
- Carious lesions and/or inadequate restorations.
- Subjects currently undergoing dental treatment.
- Subjects currently undergoing orthodontic therapy or wearing occlusal bite guards.
- Subjects suffering any systemic disease or condition which may affect the response of gingival tissues or the ability to perform adequate plaque control (pregnancy, diabetes, quantitative and/or qualitative polymorphonuclear neutrophils defects, other immune system disorders, etc.)
- Subjects taking medications that could interfere with the gingival tissues response (i.e. anti-inflammatory agents, diphenylhydantoin, calcium channel blockers, cyclosporin A, immunostimulants/immunomodulators).

- Subjects taking antibiotics, using antiseptics or probiotic products for oral health, within the previous 2 months.
- History of hypersensitivity or allergy to any component of the treatment products.

2.2 | Study visits and interventions

At day 0 (baseline), GI was recorded. Later on, microbiological samples were obtained from the site with the highest degree of gingival inflammation. Finally, PII and AngBS were registered. Then, patients received professional mechanical plaque removal (PMPR; “professional mechanical removal of supragingival plaque and calculus with subgingival debridement to the depth of the sulcus/pocket”; Sanz et al., 2015) and standardized oral hygiene instructions, consisting on tooth brushing with a fluoridated toothpaste (Fluor-Aid; Dentaid, Barcelona, Spain) twice per day. Use of mouthwashes was expressly prohibited.

Then, subjects were randomly assigned by blocks using a computer-generated list to one of the following two regimens:

- Test group: chewing, twice per day during 6 weeks (morning and night, after oral hygiene procedures), tablets containing the probiotic strains *L. plantarum* CECT 7481 (AB15), *L. brevis* CECT 7480 (AB38) and *P. acidilactici* CECT 8633 (AB30) at a dosage of 1.00×10^3 colony-forming units (CFUs) for each probiotic strain.
- Placebo group: same regimen, but using tablets containing the same excipients as the active product, but without live bacteria.

All tablets, as well as the cases containing them, were identical and were coded according to the computer-generated randomization list, which was only revealed at the end of the study. After 6 weeks, microbiological samples were taken, clinical variables were re-assessed, and evaluation of compliance and safety was conducted.

2.3 | Clinical outcome variables

Clinical parameters [(GI, plaque index (PII) and angulated bleeding score (AngBS)] were evaluated at four sites in all teeth (distobuccal, buccal, mesiobuccal and palatal/lingual). The primary outcome variable was the changes in GI [Trombelli et al. (2004) modification of the Löe and Silness (1963)]. PII [Furuichi, Lindhe, Ramberg, and Volpe (1992), modification of the Silness and Löe (1964) plaque index] was assessed with a disclosing solution to distinguish between PII scores 0 and 1. AngBS [Trombelli et al. (2004), modification of the angulated bleeding index of Van der Weijden, Timmerman, Nijboer, Reijerse, and Van der Velden (1994)] was evaluated after lightly drying the gingiva with compressed air; then, a periodontal probe (PCP 15; Hu-Friedy, Chicago, IL, USA) was held at an angle of approximately 60° to the longitudinal axis of the tooth and in contact with the sulcular gingival tissues, gently pushing the gingiva away from the tooth.

All indices were evaluated by two calibrated and trained examiners (EM for GI, MI for PII and AngBS; in all patients) who were blinded to the treatment assignment. An intra-examiner calibration was

performed before the start of the study, with five patients (providing 560 sites) fulfilling the same inclusion and exclusion criteria than the study participants. The percentage of agreement was 84.38% for GI (κ value 0.703) and 96.43% for AngBS (κ value 0.854).

2.4 | Microbiological procedures

One subgingival sample in each quadrant (the most accessible site with the highest degree of gingival inflammation) per patient was sampled, by means of two sterile paper points (# 30; Maillefer, Ballaigues, Switzerland) per site, consecutively placed and left subgingivally for 15 s. All paper points were pooled in one empty sterilized 1.5 ml Eppendorf tube. Samples were transported to the laboratory within 2 hr, where paper points were re-suspended in 1,000 μ l of water (Water PCR grade; Roche Diagnostic GmbH, Mannheim, Germany) and vortexed for 2 min at maximal setting. Then, paper points were removed, and the vials were centrifuged at 16500 g for 3 min, and the supernatant was discarded. The resultant pellets were processed with a commercial kit for DNA extraction (MoYsis Complete5, Molzym GmbH & Co.KG, Bremen, Germany) following manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) technology was used for detecting and quantifying the bacterial DNA. The qPCR amplification was performed with Taqman Probes using specific primers for five putative periodontal pathogens (*Aggregatibacter actinomycetemcomitans*, *P. gingivalis*, *Tannerella forsythia*, *Fusobacterium* spp. and *Campylobacter rectus*) targeting against 16S rRNA gene [obtained through Life Technologies Invitrogen (Carlsbad, CA, USA) and Applied Biosystems (Carlsbad, CA, USA)]. Primer sequences are presented in Table S1.

Each DNA sample was analysed in duplicate. Quantification cycle (C_q) values, previously known as cycle threshold (C_t) values, describing the PCR cycle number at which fluorescence rises above the baseline, were determined using the provided software package (LC 480 Software 1.5; Roche Diagnostic GmbH). Quantification of cells by qPCR was based on standard curves, following a protocol previously described (Figuro et al. 2014). The correlation between C_q values and CFU/ml was based on standard curves constructed with tenfold serial dilutions of each bacterial DNA. All assays were developed with a linear quantitative detection range established by the slope range of 3.3–3.6 cycles/log decade, $r^2 > .997$ and an efficiency range of 1.9–2.0. Measures to avoid carryover DNA were established. To prevent potential false-positive results, the limit of detection was calculated using the C_q value from the last point of the standard curve that is lower than C_q value from the NTCs obtained throughout experiments.

2.5 | Evaluation of compliance and safety

Subjects were asked to report the intake of tablets by filling daily questionnaires that were returned to the examiners at the 6 weeks visit, together with the blisters of unused tablets. Any adverse effect experienced by a subject participating in the study or detected by the examiner, irrespective of whether it might be related to the study intervention or not, was recorded, including information on the nature, severity, time of onset and duration of the adverse effect, as well as the measures

required for its treatment (when necessary), the possible association with the study intervention, as well as any other relevant information.

2.6 | Data analysis

Sample size was calculated using GI as the primary outcome variable, assuming a difference between groups of 0.36 with a standard deviation of 0.43 (Iniesta et al., 2012) for a statistical power of 85% and a α error of 0.05. A total of 26 subjects per group were needed, that after calculating for a potential dropout of 10%, resulted in 56 subjects (28 per treatment arm).

The subject was used as the experimental unit, and the primary outcome variable (mean GI), as well as the secondary variables, was generated at each visit, first for patient, then for treatment group. Means, standard deviations and 95% confidence intervals (CI) were calculated at each visit as well as the changes between baseline and follow-up visits.

To assess the normality of the distribution, Shapiro-Wilk test was performed. If data were parametrically distributed, differences in primary and secondary quantitative variables in and in between groups were determined by the unpaired *t* test and by means of ANCOVA for the comparison in between changes. If data were not parametrically distributed, the comparison of quantitative variables was carried out by nonparametric tests (Wilcoxon Rank-sum test for paired data). Inter-group comparison for the number of sites with GI = 3 at baseline and week 6 visits was performed by means of Chi-square test with Yates correction.

Certain demographic characteristics of the groups, such as gender, as well as prevalence of targeted periodontal pathogens, were compared with Fisher's exact test. Age distribution was compared with the Wilcoxon Rank-sum test.

In regard to the analysis of the microbiological data, correction for multiplicity using the Bonferroni-Holm approach was necessary when

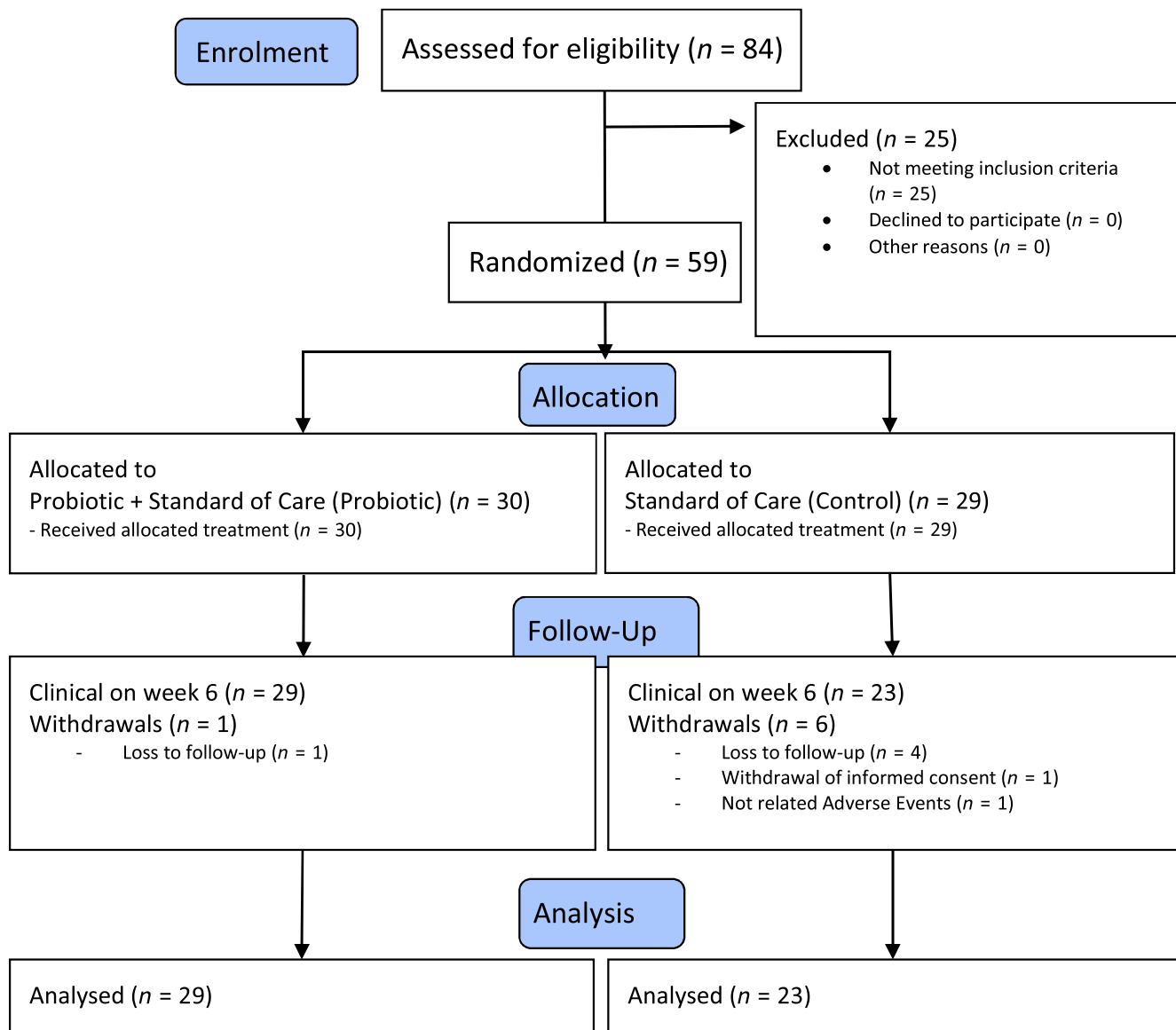


FIGURE 1 Flow chart of patient inclusion and follow-up

	Test group N = 30	Control group N = 29	Total N = 59	p Value
Age (mean ± SD)	30.9 ± 12.2	32.5 ± 13.6	31.7 ± 12.8	.780
Gender [n (%)]				
Female	19 (63.3%)	17 (58.6%)	36 (61.0%)	.710
Male	11 (36.7%)	12 (41.4%)	23 (39.0%)	

TABLE 1 Patient demographic characteristics

assessing the quantitative changes of each bacterial species and their correlation to the clinical outcome variables (considering the five species analysed). For each bacterial species analysed, when the reported concentration was "0" or "<LD" (below limit of detection) at both time points (baseline and week 6), that subject was not considered for calculating the median and range concentration values, nor for inferential statistics.

Pairwise correlation between variables was assessed using Spearman's rank test. Multiple linear regression analysis considered concentrations of all five bacterial species and study visit as potential predictor variables, and model optimization was achieved by means of backwards stepwise elimination.

All analyses were performed using Statistical Analysis Software (SAS; Cary, NC, USA under intention-to-treat criteria).

3 | RESULTS

3.1 | Study population

Fifty-nine patients, with a mean (standard deviation, SD) age of 31.7 (12.8) years, were participated in the study and randomized, with 30 subjects allocated to the test and 29 to the placebo group. Figure 1 depicts the flow chart of the study. One subject in the test and six in the control group were dropouts. Five were lost to follow-up, one withdrew the informed consent, and one presented an adverse event not related with the treatment. Table 1 depicts the subject characteristics at baseline. No significant differences between groups were detected either for age or gender.

3.2 | Clinical outcomes

No statistically significant differences in mean GI between groups were detected at baseline ($p = .652$) and at week 6 ($p = .347$). GI decreased significantly from baseline to week 6 in test and control groups [-1.06 (0.3) and -1.08 (0.3), respectively ($p < .001$)]. No significant differences between treatment groups were found in the GI changes between baseline and week 6 [0.05; (-0.1, 0.2); $p = .481$] (Table 2).

Table 3 reports the intra-group change in the median number of surfaces per patient with each GI score. A significant intra-group reduction in the average number of sites with GI scores of 2 and 3 was observed in both treatment groups. Similarly, the analysis shows an intra-group increase in the average number of sites with GI scores of 0 and 1 in both treatment groups, although the increase in the median number of sites with GI = 1 in the placebo group did not reach statistical significance. No significant differences were found between groups. Table 4 depicts the frequency distribution of sites with different degree of severity of gingival inflammation at both time periods. At baseline, 401 sites (6.3%) demonstrated GI = 3, being the number of these sites significantly higher in the test group than in the control group [$n = 263$ (8.2%) versus $n = 138$ (4.4%), $p < .001$]. At week 6, however, there were no sites with GI = 3 in the test group, while five were still present in the control group (0.2%), and the difference between groups was statistically significant ($p = .042$). Furthermore, there were no subjects in the test group at 6 weeks with a mean GI > 1, while, in the control group, three subjects still presented that level of gingival inflammation (13%, $p = .080$).

TABLE 2 Gingival index (GI), plaque index (PII) and angulated bleeding score (AngBS) expressed as mean, standard deviation (SD) and 95% confidence intervals (95% CI), per visit, with inter-group and intra-group comparisons by unpaired *t* test. Intra-group comparisons for the change in between visits were determined by means of ANCOVA

	Visit	Test group				Control group				p Value (inter-group)		
		n	Mean	SD	CI 95%	n	Mean	SD	CI 95%			
GI	Baseline	30	1.65	0.3	1.5	1.8	29	1.62	0.2	1.5	1.7	.652
	Week 6	29	0.60	0.2	0.5	0.7	23	0.54	0.3	0.4	0.7	.347
	Baseline-week 6	29	-1.06*	0.3	-1.2	-1.0	23	-1.08*	0.3	-1.2	-1.0	.480
PII	Baseline	30	1.60	0.2	1.5	1.7	29	1.55	0.2	1.5	1.6	.384
	Week 6	29	1.22	0.2	1.1	1.3	23	1.08	0.3	0.9	1.2	.059
	Baseline-week 6	29	-0.39*	0.3	-0.5	-0.3	23	-0.44*	0.3	-0.6	-0.3	.180
AngBs	Baseline	30	0.57	0.2	0.5	0.6	29	0.57	0.2	0.5	0.7	.929
	Week 6	29	0.38	0.2	0.3	0.5	23	0.29	0.2	0.2	0.4	.044
	Baseline-week 6	29	-0.20*	0.2	-0.3	-0.1	23	-0.28*	0.2	-0.4	-0.2	.061

*Statistically significance difference for the intra-group comparison baseline-week 6, $p < .0001$.

TABLE 3 Median number of sites per patient with each GI score (range). Inter-group comparison with Wilcoxon matched-pairs test

	GI score	Baseline	6 weeks	p Value
Control N = 23	0	1 (0–24)	57 (14–100)	<.0001
	1	40.5 (18–71)	46 (12–84)	.1940
	2	65 (35–88)	2 (0–21)	<.0001
	3	2 (0–27)	0 (0–4)	.0007
Probiotic N = 28	0	1 (0–33)	54 (26–85)	<.0001
	1	40 (8–59)	50 (19–75)	.0028
	2	56 (27–77)	4 (0–31)	<.0001
	3	2.5 (0–54)	0 (0–0)	<.0001

TABLE 4 Number of sites with gingival index (GI) = 3, with inter-group comparison by Chi-square test

	Test group	Control group	Chi-square test
Baseline			
GI = 3	263 (8.2%)	138 (4.4%)	<0.0001
GI < 3	2,937 (91.8%)	3,034 (95.6%)	
Week 6			
GI = 3	0 (0.0%)	5 (0.2%)	0.0418
GI < 3	3,088 (100%)	2,498 (98.8%)	
Change			
GI = 3 at baseline	263 (100%)	138 (96.5%)	0.0130
GI = 3 at week 6	0 (0.0%)	5 (3.5%)	

Table 2 shows the PII results. There were no significant differences between groups at baseline ($p = .385$) or at week 6 ($p = .059$). In both treatment groups, the plaque index decreased significantly [-0.39 (0.3) and -0.44 (0.3) in the test and control group, respectively (p value $<.001$)]. No significant differences were found between groups in the change of PII throughout the study period [diff. 0.10, CI95% ($-0.0, 0.2$), $p = .181$].

There were no significant differences in AngBS between groups at baseline ($p = .929$). At week 6, the mean AngBS was higher in the test group ($p = .044$). In both treatment groups, AngBS decreased significantly ($p < .001$) [0.20 (0.2) in the test group and 0.28 (0.2) in the control group]. No significant differences were found between groups in the baseline–week 6 [0.09 , CI 95% ($-0.0, 0.2$); $p = .061$; Table 2].

3.3 | Microbiological outcomes

The GI of the sites selected for subgingival plaque sampling was 2.06 (0.3) and 2.01 (0.5) for control and test group, respectively, at baseline visit, while it was 0.96 (0.4) and 0.97 (0.4) at the 6-week visit. No significant intergroup differences were found at any time point.

Table 5 depicts the frequency of detection of the selected bacterial species. Most subjects harboured the pathogens at both visits in both treatment groups, without differences between study visits.

In regard to the bacterial concentrations of the tested bacteria (Table 6), a significant reduction of *A. actinomycetemcomitans* occurred

TABLE 5 Number and percentage of subjects testing positive for target bacterial species

	Test group N = 29	Control group N = 23	Total N = 52
<i>P. gingivalis</i>			
Baseline	27 (93.1%)	20 (87.0%)	47 (90.4%)
Week 6	28 (96.6%)	22 (95.7%)	50 (96.2%)
<i>A. actinomycetemcomitans</i>			
Baseline	13 (44.8%)	15 (65.2%)	28 (53.8%)*
Week 6	7 (24.1%)	8 (34.8%)	15 (28.8%)*
<i>F. nucleatum</i>			
Baseline	29 (100%)	23 (100%)	52 (100%)
Week 6	29 (100%)	23 (100%)	52 (100%)
<i>T. forsythia</i>			
Baseline	28 (96.6%)	20 (87.0%)	48 (92.3%)
Week 6	27 (93.1%)	20 (87.0%)	47 (90.4%)
<i>C. rectus</i>			
Baseline	28 (96.6%)	22 (95.7%)	50 (96.2%)
Week 6	29 (100%)	20 (87.0%)	49 (94.2%)

*Statistically significant difference between visits, $p = .016$.

in both the test and control groups [-0.97 (1.3) and -1.06 (1.3); $p = .044$ and $p = .017$, respectively], while a significant reduction of *T. forsythia* only happened in the test group [-1.06 (1.6); $p = .008$].

3.4 | Correlation between clinical and microbiological outcomes

The number of individual GI scores = 3 was correlated with the amounts of all bacterial species, except *P. gingivalis*. Only *A. actinomycetemcomitans* and *T. forsythia* showed correlation of mean GI after correcting for multiple testing (Table 7). Neither PII nor AngBS correlations survived correction for multiple testing.

In the multiple regression analysis, after adjusting for visit, only the concentration of *T. forsythia* remained a significant linear predictor of the number of individual GI scores = 3. Similarly, only concentration of *A. actinomycetemcomitans* remained a significant linear predictor of mean GI in multiple regression analysis after adjusting for visit (Tables S2 and S3).

3.5 | Compliance and adverse events

From the whole sample, 94.2% of patients took at least 75% of treatment, 89.7% in the test group and 100.0% in the control group. Thus, there were no significant differences in treatment compliance between groups. Of 59 treated patients, five reported at least one adverse event (8.5%), four in the test and one in the placebo group (13.3% versus 3.4%, $p = .3533$), being the most frequent event abdominal pain due to increased intestinal motility. No patient reported serious adverse events, and only one patient (belonging to the control group) discontinued treatment due to an adverse event.

	Test group				Control group			
	n	Mean (SD)	CI 95%		n	Mean (SD)	CI 95%	
<i>P. gingivalis</i>								
Baseline	28	2.61 (1.5)	2.0	3.2	23	3.02 (2.2)	2.1	4.0
Week 6	28	2.24 (0.9)	1.9	2.6	23	2.44 (1.2)	1.9	2.9
Change		-0.37 (1.2)	-0.9	0.1		-0.58 (2.0)	-3.9	2.6
<i>A. actinomycetemcomitans</i>								
Baseline	14	2.25 (1.3)	1.5	3.0	16	2.24 (1.0)	1.7	2.8
Week 6	14	1.29 (1.6)	0.4	2.2	16	1.18 (1.3)	0.5	1.9
Change		-0.97 ^a (1.3)	-1.7	-0.2		-1.06 ^a (1.3)	-1.7	-0.4
<i>F. nucleatum</i>								
Baseline	29	4.89 (0.7)	4.6	5.2	23	4.81 (0.8)	4.4	5.2
Week 6	29	4.79 (0.6)	4.6	5.0	23	4.53 (0.8)	4.2	4.9
Change		-0.10 (0.7)	-0.4	0.2		-0.28 (0.8)	-0.6	0.1
<i>T. forsythia</i>								
Baseline	28	4.40 (1.4)	3.9	4.9	21	4.04 (1.9)	3.2	4.9
Week 6	28	3.34 (1.5)	2.8	3.9	21	3.53 (1.6)	2.8	4.3
Change		-1.06 ^{ab} (1.6)	-1.7	-0.4		-0.51 (2.0)	-1.4	0.4
<i>C. rectus</i>								
Baseline	29	3.31 (1.1)	2.9	3.7	23	3.23 (1.2)	2.7	3.8
Week 6	29	3.32 (0.8)	3.0	3.6	23	2.61 (1.3)	2.0	3.2
Change		0.01 (0.8)	-0.3	0.3		-0.63 (1.4)	-1.2	0.0

^aIntra-group difference with baseline for the less stringent correction, $p < .05$.

^bIntra-group difference with baseline for the more stringent correction, $p < .05$.

4 | DISCUSSION

The results from this randomized clinical trial have shown that the adjunctive use of probiotic tablets containing strains *L. plantarum*, *L. brevis* and *P. acidilactici* was able to reduce gingivitis when adjunctively used to PMPR. When considering changes in mean GI, in both groups, a significant improvement was observed, while differences between groups were not significantly different. When evaluating sites with higher GI scores (GI = 3 at baseline), treatment with probiotic tablets resulted in a significantly higher reduction of these specific sites. Furthermore, all subjects in the probiotic group demonstrated gingival health (as identified with a mean GI < 1) at the re-evaluation visit,

while three patients in the control group still shown gingival inflammation (mean GI > 1).

The use of mean GI as the main outcome measurement for assessing the efficacy of the adjunctive use of new agents for gingivitis management, such as probiotics, may not be appropriate. As shown in the reported results from the present investigation, the dilution effect of the most frequently reported event (GI ≤ 1) may mask the positive effect of the agent on sites with clear signs of inflammation (GI ≥ 2). In fact, when analysing these sites (GI = 3), the adjunctive effect of the probiotic was statistically significant. The lack of statistically significant differences in the primary outcome variable (mean GI) could also be explained by the selection of mild-moderate gingivitis cases (instead of

TABLE 6 Subgingival samples: mean, standard deviation (SD) and IC 95% of log-transformed counts/ml by visit for target bacterial species

Bacterial species	Number of subjects with GI scores = 3		Mean GI	
	Spearman coefficient	p Value	Spearman coefficient	p Value
<i>P. gingivalis</i>	0.284	.0038	0.053	.5993
<i>A. actinomycetemcomitans</i>	0.303	.0185*	0.392	.0020*
<i>F. nucleatum</i>	0.330	.0006*	0.176	.0745
<i>T. forsythia</i>	0.415	<.0001*	0.301	.0026*
<i>C. rectus</i>	0.301	.0019*	0.153	.1218

*Statistically significant correlation.

TABLE 7 Correlation of individual species to number of individual gingival index (GI) scores = 3 and mean GI

more severe conditions), the possible effect of PMPR, the Hawthorne effect or the limited follow-up, which may have prevented to evaluate the effect of the probiotic on bacterial recolonization patterns. In fact, similar results demonstrating lack of statistically significant differences for mean GI have been reported in other studies evaluating probiotic tablets although using different strains, such as *Lactobacillus salivarius* WB21 (Shimauchi et al., 2008) or *Lactobacillus reuteri* (DSM-17938 and ATCC PTA 5289; Iniesta et al., 2012). Lack of significant differences was also reported in experimental gingivitis studies (Hallström et al., 2013; Slawik et al., 2011; Staab et al., 2009). Only one study demonstrated significant differences in mean GI scores (Krasse et al., 2006), but the sample was not well balanced at baseline, with PII scores showing significant differences; in addition, intergroup differences were not reported at follow-up.

The present study has also evaluated, in this mild to moderate gingivitis population, the microbiological effect over five selected periodontal pathogens. Most subjects harboured target species at both visits, while the amounts were significantly reduced in both groups. The use of probiotic-containing tablets was associated with a significant reduction in the levels of *T. forsythia*. Similar findings have been reported by Mayanagi et al. (2009), after administering *Lactobacillus salivarius* WB21 for 8 weeks. *T. forsythia* is a member of the red complex bacteria and one of the most prevalent bacterial species in subgingival plaque samples from periodontitis subjects (Socransky & Haffajee, 2005; Tomita et al., 2013; Wara-aswapati et al., 2009). Furthermore, it has been associated with periodontitis severity and poorer response to therapy (Lanza et al., 2016; Ready et al., 2008). Its relevance in gingivitis is unknown, but the significant reductions in the amounts with the use of probiotics reported in this study, and its possible impact in preventing future periodontitis deserves further investigation. The significant correlations between the concentrations of the tested bacteria and the number of tooth surfaces with GI = 3, specifically the concentrations of *T. forsythia* (correlation coefficient = .415, <.0001), underline the possible target effect of the adjunctive probiotics on those sites harbouring more pathogenic species, which have more severe gingival inflammation. In future studies, more targeted outcome measurements, such as the number of sites with overt inflammation or the concentrations of *T. forsythia*, should be further investigated to evaluate the effect of the adjunctive use of probiotic strains.

Few adverse effects were reported by the participating subjects. Four subjects in the test group and one in the control group complained of changes in intestinal motility, eventually leading to abdominal pain. A recent systematic review of RCTs reported that probiotics did not affect gastrointestinal motility (Asrani, Yoon, Megill, Windsor, & Petrov, 2016). However, sorbitol, which was a component of both test and placebo tablets, has been associated with transitory diarrhoea in certain individuals (Oku & Nakamura, 2002). This could explain the fact that all patients but one (in the control group) were able to continue the treatment provided.

Within the referred limitations of the present investigation, it can be concluded that the use of probiotic tablets containing *L. plantarum*, *L. brevis* and *P. acidilactis* did not lead to significant changes in mean GI; however, they were able to reduce the number of sites with severe

inflammation in gingivitis patients after PMPR, when compared with the use of similar tablets without the probiotic strains. The adjunctive use of the probiotic also demonstrated a significant microbiological impact by reducing the counts of *T. forsythia*.

CONFLICT OF INTEREST

The authors have stated explicitly that there is no conflict of interests in connection with this research.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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