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Use of the Probiotic *Limosilactobacillus reuteri* as an Adjunct to Subgingival Instrumentation in the Treatment of Periodontitis Patients With Diabetes: A Randomised Clinical Trial

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ABSTRACT

Aim: To evaluate the clinical and microbiological effects of a *Limosilactobacillus reuteri*-based probiotic as an adjunct to subgingival instrumentation in untreated periodontitis patients with diabetes.

Methods: A 6-month, randomised, triple-blinded clinical trial was conducted involving 40 patients, receiving steps 1 and 2 of periodontal therapy, including subgingival instrumentation, and randomised to receive tablets containing *L. reuteri* or placebo, for 3 months. Clinical and microbiological outcomes as well as glycosylated haemoglobin (HbA1c) concentrations were evaluated at baseline and at 3 and 6 months after therapy.

Results: At 6 months, both groups showed statistically significant reductions in the primary outcome (mean probing depth), with reductions of 0.9 mm in the probiotic group and 0.8 mm in the placebo group, with no significant differences between them. Both groups exhibited reductions in HbA1c levels after 6 months, more in the probiotic group (−0.6% vs. −0.1%), with statistically significant inter-group differences (−0.5%; 95% confidence interval [−1.0; 0.0]; $p < 0.001$). Microbiological outcomes were similar.

Conclusions: The adjunctive use of a *L. reuteri*-based probiotic did not provide additional clinical or microbiological benefits, compared to placebo, following subgingival instrumentation in patients with periodontitis and diabetes. However, statistically significant differences in HbA1c levels were observed, with larger reductions in the probiotic group, suggesting a potential systemic benefit.

Trial Registration: The protocol was approved by the Clinical Research Ethics Committee (CEIC) of Hospital Clínico de San Carlos (internal code 19/101-R_X) and registered a priori in [ClinicalTrials.gov](https://clinicaltrials.gov) (identifier NCT04069611)

1 | Introduction

Periodontal diseases are highly prevalent chronic inflammatory diseases associated with dysbiotic biofilms (Papapanou

et al. 2018a). Severe periodontitis, in particular, is the sixth most prevalent condition worldwide (Kassebaum et al. 2014; Papapanou et al. 2018b) and represents a major public health concern (Tonetti et al. 2017).

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The treatment of periodontitis is aimed at eliminating supragingival and subgingival biofilms, primarily by means of subgingival instrumentation, which effectively reduces and modifies the subgingival microbiota. However, this approach has several limitations, such as the presence of deep or tortuous pockets, challenging root anatomy, furcation involvement, vertical bone defects and the subgingival microbiological profile itself. For example, recolonisation of the subgingival environment typically occurs within 1–2 weeks after instrumentation (Teles et al. 2006). To overcome these limitations, the clinical practice guideline for the treatment of periodontitis in stages I–III has proposed the use of adjunctive therapies, including antiseptics, host-response modulators, systemic antibiotics and locally delivered antimicrobials (Sanz et al. 2020). Nevertheless, owing to concerns regarding the adverse effects associated with antibiotic use, particularly the rise of antibiotic-resistant bacteria (Herrera et al. 2008; Sanz et al. 2020), alternative compounds, such as probiotic formulations, have been proposed (Teughels et al. 2013).

Probiotics are defined as non-pathogenic live microorganisms that confer a health benefit to the host (Hill et al. 2014). They have been tested in the prevention and treatment of multiple systemic and oral conditions, including periodontal diseases (Iniesta et al. 2012; Montero et al. 2017). The rationale for using probiotics in dentistry includes (i) the production of antimicrobial substances that reduce cariogenic and periodontal pathogens (Shirbhate et al. 2023), and/or (ii) the modulation of the host's inflammatory response (Seminario-Amez et al. 2017).

Clinical studies evaluating the adjunctive use of probiotics to subgingival instrumentation have resulted in heterogeneous outcomes (Donos et al. 2020). Some studies show additional pocket depth reductions of >1 mm (Ince et al. 2015; Tekce et al. 2015), while others do not show any significant added benefit (Laleman et al. 2015; Teughels et al. 2013). The clinical practice guideline for the treatment of periodontitis in stages I–III (Sanz et al. 2020) does not recommend the adjunctive use of probiotics to subgingival instrumentation because of the lack of convincing scientific evidence. This suggests that further clinical research is needed.

Different reviews and consensus documents published in recent years have clearly indicated the bidirectional association between diabetes and periodontitis (Chapple, Genco, and Working Group 2 of Joint 2013; Lalla and Papapanou 2011; Preshaw et al. 2012; Sanz et al. 2018). If diabetic subjects can achieve appropriate glycaemic control, their response to periodontal therapy is equal to that of non-diabetic subjects (Christgau et al. 1998). However, not all diabetic patients are able to achieve optimum metabolic control, with epidemiological studies reporting $\geq 50\%$ for those presenting undesirable values of HbA1c (Bi et al. 2010; Casagrande et al. 2013; Grant, Buse, Meigs, and University HealthSystem Consortium Diabetes Benchmarking Project 2005). Furthermore, as a risk group, diabetic subjects are encouraged to undergo regular periodontal check-ups, as well as to keep a high standard of oral hygiene (Sanz et al. 2018). For these reasons, adjunctive therapies, such as local antimicrobials or probiotics, may add a beneficial effect when used along

with subgingival instrumentation in diabetic subjects and are worthy of being investigated. The present study was designed to evaluate the clinical and microbiological effect of a *Limosilactobacillus reuteri*-based probiotic formulation as an adjunct to subgingival instrumentation within step 2 of periodontal treatment.

2 | Methods

2.1 | Study Design and Ethical Aspects

This study was designed as a randomised, parallel, triple-blinded, placebo-controlled clinical trial lasting 6 months.

2.2 | Study Population

Patients with diabetes (both types 1 and 2) and periodontitis were consecutively screened and eventually selected from among those attending the clinics of the Faculty of Dentistry, Complutense University of Madrid (Spain), according to pre-established inclusion and exclusion criteria. Selected patients received detailed information on the purpose, benefits and possible risks associated with the clinical trial, and if they agreed to participate, they were asked to sign an informed consent form.

2.2.1 | Inclusion Criteria

- Patients with diagnosed diabetes mellitus for at least 1 year;
- Presence of a minimum of 18 teeth;
- Untreated stages II or III periodontitis, with radiographic evidence of generalised alveolar bone loss of 30% and the presence of at least one pocket with a probing depth of 5 mm per quadrant with bleeding on probing.

2.2.2 | Exclusion Criteria

- Subgingival instrumentation within the previous 12 months;
- Use of systemic antibiotics in the 3 months prior to the study;
- Use of other probiotic products in the month prior to the study;
- Systemic diseases that could affect the course of periodontitis or its treatment, such as immunological disorders, excluding diabetes;
- Ongoing drug therapy influencing the evaluated clinical parameters (non-steroid inflammatory drugs, etc.);
- Compromised medical conditions requiring prophylactic antibiotic therapy (e.g., patients with valvular prostheses, patients with previous history of bacterial endocarditis, congenital heart diseases like shunts, or heart transplant recipients who developed valvular heart disease);

- Pregnancy;
- Stage IV periodontitis or acute periodontal conditions.

2.3 | Outcome Variables

Two calibrated (assessed using the intraclass correlation coefficient, yielding a value of 0.78 for probing depth measurements) and well-trained examiners (A.A. and R.B.) recorded blindly the clinical outcome measurements.

2.3.1 | Clinical Outcomes

The primary outcome of the study was the change in mean PD between baseline and 6 months.

The following clinical outcome variables were recorded at six sites per tooth, at all teeth excluding third molars, on Days 0, 90 and 180:

- Plaque index (PII), dichotomously assessed as present (1) or absent (0), following the criteria described by Ainamo and Bay (1975). A mean score was calculated for each patient at each visit.
- Bleeding on probing (BOP), dichotomously assessed as present (1) or absent (0). Similarly, the full-mouth BOP was calculated per patient at each visit as the average of all values recorded.
- Probing depth (PD), clinical attachment level (CAL) and gingival recession (REC), measured in millimetres, using a UNC-15 probe (Hu-Friedy, Chicago, IL, USA), and rounded to the nearest millimetre.

2.3.2 | Patient-Reported Outcomes and Adverse Event

Patient-reported outcomes (PROs) and the presence of adverse events (AEs) were recorded through a questionnaire consisting of nine open-ended questions based on possible changes in intestinal activity, abdominal pain, occurrence of any infection, use of antibiotics or probiotics (other than the study product), changes in taste of food or breath, their opinion on the taste and texture of the product and any experience of oral pain.

2.3.3 | Microbiological Samples

Microbiological samples were collected using sterile paper tips (No. 30, Maillefer, Ballaigues, Switzerland) at Days 0, 90 and 180. Four sites were selected, one per quadrant (those with the deepest pocket and BOP). Detailed information on the sampling is presented in [Supporting Information](#).

2.3.4 | Glycated Haemoglobin Levels

Blood samples were collected at Days 0, 90 and 180 via venipuncture, using a 0.8-mm gauge and 19-mm length butterfly needle,

and were placed in a PET tube containing K2EDTA anticoagulant (PET-K2EDTA). The samples were then sent to an external laboratory (Vivolabs, Madrid, Spain) for analysis, where HbA1c levels were measured using standard laboratory procedures by liquid chromatography.

2.4 | Study Visits and Interventions

2.4.1 | Screening Visit

Patient eligibility was determined according to the previously listed inclusion and exclusion criteria. Those who consented to participate signed the informed consent form and were subsequently scheduled for an appointment at the Postgraduate Clinic for Periodontology, Faculty of Dentistry, University Complutense of Madrid.

2.4.2 | Baseline Visit

A complete oral and periodontal clinical examination was performed, including panoramic and/or periapical radiographs. Additionally, microbiological and blood samples were collected as previously described, and relevant aspects of the medical and dental history were documented.

2.4.3 | Treatment Visits

Subjects underwent subgingival instrumentation in two consecutive days under local anaesthesia (lidocaine 8 mL, 2% with epinephrine 1:100,000), using Gracey curettes (Hu-Friedy, Chicago, IL, USA) and an ultrasonic scaler (EMS Piezon, Electro Medical Systems, Nyon, Switzerland) equipped with Perio Slim (PS) tips. Prior to each session, patients rinsed for 30s with 15 mL of 0.12% chlorhexidine (GUM Paroex, Sunstar, Etoy, Switzerland). All patients received standardised oral hygiene instructions, which included the use of a manual toothbrush (GUM Activital Toothbrush, Sunstar, Etoy, Switzerland) and interdental brushes (GUM Trav-ler and GUM Soft Picks, Sunstar, Etoy, Switzerland). These oral hygiene instructions were reinforced at the 3- and 6-month appointments. Immediately following the oral hygiene instructions, patients were randomly assigned to either the test or the control group, following a computer-generated randomisation list, using blocks of six. This list was prepared by the probiotic supplier (Sunstar, Etoy, Switzerland) before the start of the trial.

- The test group received two probiotic tablets administered daily for the first 3 months, one tablet in the morning and one in the evening after brushing their teeth. Test tablets contained two strains of *L. reuteri* (ATCC PTA 5289 [LR1] and DSM-17938 [LR2]). GUM Periobalance. Sunstar, Etoy, Switzerland) at a dose of 2×10^8 colony forming units (CFUs) per tablet.
- The placebo group received two tablets, which were identical in appearance to the probiotic tablets but did not contain live bacteria. Instructions for tablet intake were also identical.

Opaque containers were used to ensure the blinding of both the examiner and the patients. Patients also received a diary to record their pill consumption.

2.4.4 | Follow-Up Visits After 3 and 6 Months

Clinical measurements were repeated, and microbiological and blood samples were collected (at the same sites as at the initial visit). In addition, oral hygiene was reinforced and a questionnaire on the perception of the product and possible adverse effects was filled out at each visit.

2.5 | Microbiological Analyses

Microbiological samples were processed following two different analyses.

2.5.1 | Polymerase Chain Reaction (PCR) for the Detection of *L. reuteri*

The protocol of PCR and the primers used for detection of the two strains (ATCC PTA 5289 and DSM-17938) of *L. reuteri* contained in the commercially prepared probiotic tablets were provided by the Department of Microbiology, SLU, Sveriges Lantbruks

Universitet, Sweden. These recommendations were already followed in a previous study (Iniesta et al. 2012), and the correct amplification by PCR of these genes was tested by electrophoresis using positive controls. The specific primer sequences and the amplification protocol are described in detail in [Supporting Information](#).

2.5.2 | Quantitative Polymerase Chain Reaction (qPCR) for Selected Periodontal Pathogens and *Candida albicans*

The qPCR amplifications targeted six putative periodontal pathogens (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Campylobacter rectus*, *Treponema denticola* and *Fusobacterium* spp.) with primers and Taqman Probes specific to the 16S rRNA gene. The amplification protocol is described in detail in [Supporting Information](#).

2.6 | Data Analysis

2.6.1 | Sample Size Calculation

Sample size calculation was based on detecting an effect of 0.82 mm in the primary outcome variable (changes in PD from baseline to 6 months), assuming a standard deviation (SD) of 0.61 mm (Teughels et al. 2013), with a 5% α risk and 90%

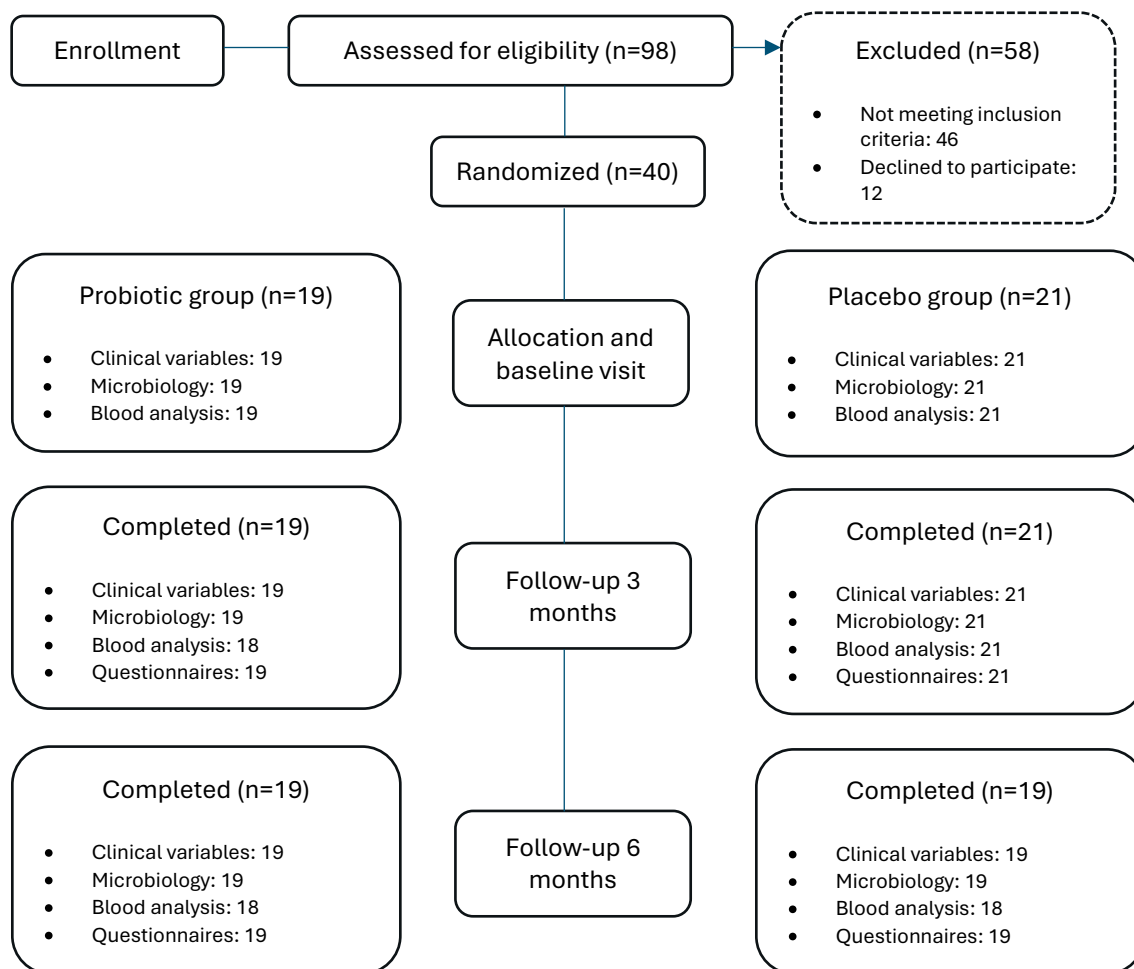


FIGURE 1 | Flow chart of patient inclusion and follow-up.

statistical power. This calculation resulted in a required sample size of 12 patients per group. To account for potential dropouts and based on power calculations performed in studies comparing the effect of systemic antimicrobials as adjuncts to subgingival instrumentation, 20 patients per treatment group were ultimately included.

2.6.2 | Statistical Analysis

Data were reported as means and SD, unless otherwise specified (e.g., n [%]). Normality tests were conducted, evaluating skewness and kurtosis and using the Kolmogorov–Smirnov test. Total anaerobic counts were log-transformed to fit a normal distribution.

Both intra- and inter-group differences for continuous clinical or microbiological periodontal outcomes were determined using ANOVA for repeated measures, and post hoc comparisons between visits (e.g., baseline vs. 6 months) were conducted using Bonferroni correction to control for multiple comparisons. The frequency distribution of probing depths was categorised into 1–3 mm, 4–5 mm and ≥ 6 mm. HbA1c (%) was modelled using multi-level linear regression, with the ‘xtmixed’ command in STATA. The model included the respective baseline measurement, treatment group, study visit (i.e., baseline, 3 and 6 months) and a treatment \times time interaction term as explanatory variables. Additional covariates included age, sex, smoking habit and diabetes mellitus type (1 or 2). PROMs, the presence of AEs and bacterial detection

frequencies were analysed as categorical variables and compared using the Chi-square test.

In addition, per-protocol (PP) analyses were conducted for all outcomes, and the estimates were reported if they differed from estimates obtained in the intention-to-treat (ITT) population. To perform descriptive and inferential analyses, two software packages, namely IBM SPSS Statistics 29.0.1.1 (244) (IBM Corporation, Armonk, NY, USA) and STATA 18.0 (StataCorp College Station, TX, USA) were used.

3 | Results

3.1 | Study Population

The study flowchart is shown in Figure 1. Ninety-eight patients were screened for eligibility, and 40 were finally included: 19 in the probiotic group (all of them completed the study) and 21 in the placebo group (19 completed the study and two dropped out for personal reasons).

The demographic characteristics of the patients are summarised in Table 1. Most patients were diagnosed with stage III periodontitis ($n = 35$; 92.5%) and had type 2 diabetes; only two patients (5%) were diagnosed with type 1 diabetes. The average age was 62.54 years (SD = 10.8) in the probiotic group and 62.01 years (SD = 12.6) in the placebo group. There were more males (65%) than females (35%); regarding smoking status, five patients in

TABLE 1 | Demographic characteristics of the recruited patient sample at baseline.

Variable		Probiotic ($n = 19$)	Placebo ($n = 21$)	Total ($n, \%$)
Age, mean (standard deviation)		62.54 (10.8)	62.01 (12.6)	62.26 (11.6)
Sex	Male	14	12	26 65.0%
	Female	5	9	14 35.0%
Periodontitis stage	II	2	1	3 7.5%
	III	17	20	37 92.5%
Diabetes type	1	0	2	2 5.0%
	2	19	19	38 95.0%
Hypertension	No	6	9	15 37.5%
	Yes	13	12	25 62.5%
Hypercholesterolemia	No	11	12	23 57.5%
	Yes	8	9	17 42.5%
Diagnosed psychological depression	No	18	17	35 87.5%
	Yes	1	4	5 12.5%
Self-reported high level of psychological stress	No	15	17	32 80.0%
	Yes	4	4	8 20.0%
Smoker	No	5	6	11 27.5%
	Yes	5	4	9 22.5%
	Former-smoker	9	11	20 50.0%

the probiotic group (26.3%) and four in the placebo group (19%) were smokers.

After 3 months, two patients (one per group) took systemic antibiotics for non-oral reasons, and three patients from the probiotic group used a probiotic different from the study product. Between the 3- and 6-month visits, three patients from the probiotic group took systemic antibiotics, with one patient taking them for oral reasons and two patients for non-oral reasons. These patients were excluded from the per-protocol analysis.

3.2 | Clinical Outcomes

Table 2 depicts the clinical outcomes in both treatment groups at each study visit. Significant reductions in both treatment groups were observed for mean PD at 3 and 6 months ($p < 0.001$). In the test group, the mean PD at baseline and at 6 months was 3.71 (SD=0.6) and 2.84 (SD=0.6) mm, respectively, while in the placebo group it was 3.59 (SD=0.5) mm and 2.72 (SD=0.4) mm, respectively. These correspond to a mean PD reduction of 0.9 mm (SD=0.4) in the probiotic group and 0.8 mm (SD=0.4) in the placebo group. No statistically significant inter-group differences were observed for the changes in mean PD.

The percentage of pockets with a PD of 1–3 mm increased between baseline and 6 months, from 54% to 80% ($p < 0.001$) in

the probiotic group and from 57% to 82% ($p < 0.001$) in the placebo group. Conversely, the percentage of pockets with a PD of 4–5 mm decreased markedly during the same period, from 36% to 15% ($p < 0.001$) in the probiotic group and from 33% to 14% ($p < 0.001$) in the placebo group. Similarly, pockets with a PD ≥ 6 mm also decreased significantly between baseline and 6 months: from 11% to 5% ($p = 0.018$) in the probiotic group and from 10% to 4% ($p = 0.002$) in the placebo group.

Similarly, statistically significant reductions were observed for BOP and PII between baseline and 3–6 months in both groups, without significant inter-group differences. Results of the PP analyses are presented in Table S1.

3.3 | Glycated Haemoglobin (HbA1c) Levels

Baseline HbA1c levels were similar in both treatment groups at baseline, being 7.1% (SD=1.1) in the probiotic group and 7.0% (SD=0.9) in the placebo group (Table 3). Both treatment groups demonstrated reductions in HbA1c at the 6-month visit compared to baseline, reaching levels of 6.5% (SD=1.2) in the probiotic and 6.9% (SD=0.8) in the placebo group, being statistically significant just in the probiotic group. Differences between the groups at 6 months were statistically significant (-0.5% ; 95% CI $[-1.0; 0.0]$; $p = 0.035$). However, the results of the PP analysis showed a significant reduction for the probiotic group when compared to baseline, but no significant

TABLE 2 | Periodontal clinical outcome variables at each study visit: Intention to treat analysis (ITT).

Variable	Group	Baseline (mean \pm SD) (<i>n</i> = 40)	3 months (mean \pm SD) (<i>n</i> = 40)	6 months (mean \pm SD) (<i>n</i> = 38)	Δ Baseline—6 months (mean \pm SD)
Mean PD (mm)	Probiotic	3.7 \pm 0.6	2.9 \pm 0.7 ^b	2.8 \pm 0.6 ^b	0.9 \pm 0.4
	Placebo	3.5 \pm 0.5	2.7 \pm 0.5 ^b	2.7 \pm 0.4 ^b	0.8 \pm 0.4
% PD < 4 mm	Probiotic	54% \pm 22%	79% \pm 17% ^b	80% \pm 16% ^b	-26% \pm 16%
	Placebo	57% \pm 18%	81% \pm 16% ^b	82% \pm 14% ^b	-25% \pm 15%
% PD 4–5 mm	Probiotic	36% \pm 17%	14% \pm 10% ^b	15% \pm 10% ^b	21% \pm 15%
	Placebo	33% \pm 15%	16% \pm 13% ^b	14% \pm 11% ^b	19% \pm 15%
% PD > 5 mm	Probiotic	11% \pm 12%	7% \pm 11%	5% \pm 8% ^b	6% \pm 5%
	Placebo	10% \pm 10%	4% \pm 4% ^b	4% \pm 4% ^b	6% \pm 9%
BOP	Probiotic	0.5 \pm 0.2	0.2 \pm 0.1 ^b	0.2 \pm 0.1 ^b	0.3 \pm 0.1
	Placebo	0.5 \pm 0.2	0.2 \pm 0.1 ^b	0.1 \pm 0.1 ^b	0.4 \pm 0.2
REC	Probiotic	1.3 \pm 0.6	1.6 \pm 0.7 ^{a,b}	1.5 \pm 0.7 ^b	-0.2 \pm 0.3
	Placebo	1.0 \pm 0.6	1.2 \pm 0.6 ^b	1.1 \pm 0.6	-0.9 \pm 0.2
CAL	Probiotic	5.0 \pm 0.8	4.5 \pm 1.0 ^{a,b}	4.4 \pm 1.0 ^b	0.6 \pm 0.5
	Placebo	4.5 \pm 0.8	3.8 \pm 0.7 ^{a,b}	3.8 \pm 0.8 ^b	0.7 \pm 0.4
PII	Probiotic	0.8 \pm 0.1	0.4 \pm 0.1 ^b	0.4 \pm 0.1 ^{a,b}	0.4 \pm 0.1
	Placebo	0.7 \pm 0.2	0.3 \pm 0.1 ^b	0.2 \pm 0.1 ^{a,b}	0.5 \pm 0.2

Abbreviations: BOP, bleeding on probing; CAL, clinical attachment level; PD, probing depth; PII, plaque index; REC, gingival recession; SD, standard deviation.

^aInter-group statistically significant differences ($p < 0.05$).

^bIntra-group statistically significant differences ($p < 0.05$) from baseline.

TABLE 3 | Glycated haemoglobin (HbA1c) levels at each study visit: Intention-to-treat analysis (ITT).

Group	Baseline (n = 40)		3 months (n = 39)			6 months (n = 36)		
	Mean (SD)	p	Mean (SD)	Δ3M (95% CI)	p	Mean (SD)	Δ6M (95% CI)	p
Probiotic	7.1 (1.1)	0.712	7.1 (1.2)	-0.1 (-0.7, 0.3)	0.416	6.5 (1.2)	-0.5 (-1.0, 0.0)	0.035
Placebo	7.0 (0.9)		7.1 (1.0)			6.9 (0.8)		

Note: Data are unadjusted mean and SD. *p* values are for the calculated Δ values, which are adjusted for baseline values, age, sex, smoking habit and diabetes mellitus type. Δ 3M is the difference at 3 months between probiotic and placebo groups, adjusted for baseline values and other confounders. Δ 6M is the difference at 6 months between probiotic and placebo groups, adjusted for baseline values and other confounders.

Abbreviations: 3M, 3 months; 6M, 6 months; CI, confidence interval; SD, standard deviation.

^aInter-group statistically significant differences ($p < 0.005$).

^bIntra-group statistically significant difference when compared with the baseline visit ($p < 0.05$).

TABLE 4 | Detection of *Limosilactobacillus reuteri* (strains ATCC PTA 5289 [LR1] and DSM 17938 [LR2]) at each study visit and for each patient in subgingival samples.

Subgingival samples	Baseline	3 months	6 months
Probiotic group			
<i>n</i> (-)	19	14	18
<i>n</i> (+)	0	5 (2 × LR2 and 3 × LR1 and 2)	1 (LR2)
Placebo group			
<i>n</i> (-)	20	21	19
<i>n</i> (+)	1 (LR2)	0	0

Note: LR1 and 2: detection of strains ATCC PTA 5289 and DSM 17938. LR2: detection of strain DSM 17938.

inter-group differences were found at 6 months (-0.3%; 95% CI [-0.8;0.1]; $p = 0.290$, Table S2).

3.4 | Microbiological Outcomes

3.4.1 | PCR for the Detection of *L. reuteri*

L. reuteri strains (ATCC PTA 5289 [LR1] and DSM 17938 [LR2]) were positively detected in seven samples from seven different patients at baseline (six in the probiotic group and one in the placebo group) (Table 4). Five patients in the probiotic group tested positive for *L. reuteri* at 3 months: three were positive for both LR1 and LR2, while two were positive only for LR2. At 6 months, one patient in the probiotic group tested positive for LR2.

3.4.2 | qPCR for Selected Periodontal Pathogens

Similar frequencies of detection for the targeted bacteria were observed during all visits for both groups (Table 5a). Regarding counts, a statistically significant difference at baseline was noted between the groups for *C. albicans* ($p = 0.019$), with the placebo group displaying a mean of 3.69 (SD = 0.6) compared to 2.43 (SD = 0.3) in the probiotic group (Table 5b). After 6 months, the only statistically significant difference between the groups

was found for *A. actinomycetemcomitans* ($p = 0.044$), which was not detected in any patient in the probiotic group. Results of the PP analyses are presented in Table S3.

3.5 | Patient-Reported Outcome Measures and Adverse Events

Regarding AEs (Table 6), at 3 months, 11 patients (four [20.0%] from the probiotic group and 7 [33.0%] from the placebo group) reported changes in bowel activity, most of them reporting improvements. Only one patient in the probiotic group reported abdominal pain at 3 months. For PROMs, similar responses at 3 months were obtained, with a tendency towards significant differences observed for the question on product taste ($p = 0.053$), with six patients in the placebo group reporting dissatisfaction with the taste, compared to only one patient in the probiotic group. No serious adverse events were observed during the 6-month follow-up.

4 | Discussion

In the present randomised clinical trial, the use of probiotics as an adjunct to step 2 of periodontal treatment in patients with diabetes resulted in no additional benefits regarding periodontal clinical outcomes. However, the probiotic group showed significant reductions in HbA1c levels. No statistically significant differences between groups for the primary outcome variable (mean PD reduction) or in any other clinical or microbiological parameters were noted. The use of this probiotic formulation was safe, well tolerated and not associated with any ARs or complications.

The lack of additional clinical benefit to subgingival instrumentation has been previously reported when using adjunctive probiotic formulations (Theodoro et al. 2019; Thierbach et al. 2024). In a recent study evaluating the adjunct effect of a probiotic with *L. reuteri* in periodontitis patients with diabetes (Jardini et al. 2024), there were no significant differences in the clinical periodontal outcomes when compared to the placebo, but a positive effect was reported in the probiotic group on the lipoprotein subfraction, suggesting a benefit in the management of dyslipidaemia, a common comorbidity in patients with diabetes.

Other studies evaluating the adjunctive use of probiotics have resulted in significantly improved outcomes (Ince et al. 2015;

TABLE 5 | Microbiological outcomes: (a) frequency of detection, and (b) counts of target periodontal pathogens.

(a)	Group	Baseline (n = 40)		3 months (n = 40)		6 months (n = 38)	
		Freq (%)	p	Freq (%)	p	Freq (%)	p
P.g	Probiotic	94.7	0.366	100	0.386	94.7	0.271
	Placebo	95.2		90.5		85.7	
A.a	Probiotic	5.3	0.239	15.8	0.727	5.3	0.236
	Placebo	23.8		23.8		14.3	
T.f	Probiotic	94.7	0.366	94.7	0.942	100	0.335
	Placebo	95.2		95.2		95.2	
F.spp.	Probiotic	94.7	0.366	94.7	0.942	100	0.335
	Placebo	95.2		95.2		95.2	
C.r	Probiotic	89.5	0.510	94.7	0.628	100	0.386
	Placebo	90.5		90.5		90.5	
T.d	Probiotic	94.7	0.366	94.7	0.628	94.7	0.628
	Placebo	95.2		90.5		90.5	
C.a	Probiotic	15.8	0.308	10.5	0.688	10.5	0.118
	Placebo	28.6		19		38.1	

(b)	Group	Baseline (n = 40)		3 months (n = 38)		6 months (n = 38)	
		Mean + SD	p	Mean + SD	p	Mean + SD	p
P.g	Probiotic	5.43 ± 1.57	0.938	5.52 ± 1.62	0.859	5.53 ± 1.49	0.648
	Placebo	5.09 ± 1.73		5.41 ± 1.21		5.39 ± 1.41	
A.a	Probiotic	.	0.359	2.8 ± 1.23	0.414	.	0.044*
	Placebo	3.47 ± 1.28		3.54 ± 1.11		4.32 ± 0.34	
T.f	Probiotic	6.45 ± 1.16	0.772	6.25 ± 1.25	0.827	6.10 ± 1.31	0.260
	Placebo	6.57 ± 1.19		6.23 ± 0.91		6.54 ± 0.63	
F.spp.	Probiotic	6.14 ± 0.65	0.388	6.19 ± 0.70	0.811	6.30 ± 0.67	0.680
	Placebo	6.28 ± 0.69		6.12 ± 0.48		6.26 ± 0.38	
C.r	Probiotic	4.56 ± 0.77	0.435	4.45 ± 0.84	0.195	4.44 ± 0.73	0.350
	Placebo	4.42 ± 0.81		3.99 ± 0.76		4.21 ± 0.80	
T.d	Probiotic	4.79 ± 1.17	0.872	4.71 ± 1.01	0.846	4.64 ± 1.24	0.940
	Placebo	4.69 ± 1.28		4.62 ± 0.81		4.88 ± 0.88	
C.a	Probiotic	2.43 ± 0.30	0.019*	2.91 ± 0.86	0.499	2.43 ± 0.11	0.269
	Placebo	3.69 ± 0.67		2.50 ± 0.53		3.43 ± 3.43	

Note: Intention to treat analysis (ITT). Counts are expressed as logarithm of means of colony forming units (CFUs) per mL.

Abbreviations: A. a., *Aggregatibacter actinomycetemcomitans*; C. a., *Candida albicans*; C. r., *Campylobacter rectus*; F. spp., *Fusobacterium* spp.; Freq, frequency of detection; P. g., *Porphyromonas gingivalis*; SD, standard deviation; T. d., *Treponema denticola*; T. f., *Tannerella forsythia*.

^aIntra-group statistically significant difference when compared with the baseline visit ($p < 0.05$).

*Inter-group statistically significant differences ($p < 0.005$).

Tekce et al. 2015; Teughels et al. 2013). Teughels et al. (2013) reported statistically significant reductions in PD favouring the probiotic group, although this effect was limited to deep pockets (≥ 7 mm). Similarly, a significantly added effect was reported with the use of adjunctive probiotics in a population with deep PDs at baseline (mean PD of 5.2 mm) (Vivekananda et al. 2010). This may explain the lack of an additional effect reported in the

present study, since our population had a relatively shallow baseline PD, with only 10%–11% of the initial sites with PD ≥ 6 mm. Owing to the heterogeneity in the reported results and the absence of clear benefits, the recently published clinical practice guideline for treating periodontitis stages I–III concluded that adjunctive probiotics to subgingival instrumentation is ‘not suggested’ (Sanz et al. 2020).

TABLE 6 | Patient-reported outcomes and adverse events: Inter-group comparisons.

	Probiotic		Placebo		<i>p</i>
	Yes	No	Yes	No	
3-month visit					
Have you noticed a change in bowel activity/motility?	4 (20.0%)	15 (78.9%)	7 (33.0%)	14 (70.0%)	0.385
Have you noticed abdominal pain?	1 (5.0%)	18 (94.0%)	0 (0.0%)	21 (100%)	0.287
Have you had any infection?	0 (0.0%)	19 (100%)	2 (10.0%)	19 (90.0%)	0.168
Have you taken any antibiotic/other probiotic?	4 (21.1%)	15 (78.9%)	5 (23.8%)	16 (76.2%)	0.835
Have you noticed any change in the taste of food?	0 (0.0%)	19 (100%)	0 (0.0%)	21 (100%)	.
Have you noticed any change in breath?	2 (10.0%)	17 (90.0%)	6 (30.0%)	15 (70.0%)	0.154
Do you like the taste of the product?	18 (94.7%)	1 (5.3%)	15 (71.4%)	6 (28.6%)	0.053
Do you like the texture?	16 (84.2%)	3 (15.8%)	17 (81.0%)	4 (19.0%)	0.787
Have you had any oral discomfort?	2 (10.5%)	17 (89.5%)	4 (19.0%)	17 (81.0%)	0.451
6-month visit					
Have you noticed a change in bowel activity/motility?	2 (10.0%)	17 (90.0%)	1 (5.0%)	19 (95.0%)	0.517
Have you had any infection?	1 (5.0%)	18 (95.0%)	0 (0.0%)	20 (100%)	0.299
Have you taken any antibiotic/other probiotic?	5 (26.5%)	14 (73.7%)	1 (5.0%)	19 (95.0%)	0.065
Have you noticed any change in the taste of food?	0 (0.0%)	19 (100%)	0 (0.0%)	20 (100%)	.
Have you noticed any change in breath?	0 (0.0%)	19 (100%)	0 (0.0%)	21 (100%)	.
Have you had any oral discomfort?	1 (5.0%)	18 (95.0%)	1 (5.0%)	19 (95.0%)	0.970

In terms of the impact of the tested treatments on the concentrations of glycosylated haemoglobin, both showed reductions 6 months after treatment, with a magnitude ranging from 0.1% to 0.6%, although this reduction reached statistical significance only in the probiotic group. This tendency aligns with the existing literature, as periodontal therapy has demonstrated a positive and clinically relevant effect on glycaemic control, resulting in reductions in HbA1c levels of 0.27%–0.48%, 3 months post treatment (D'Aiuto et al. 2018; Engebretson and Kocher 2013; Montero et al. 2020; Sanz et al. 2018), a magnitude of reduction that has been associated with a 35% lower risk of cardiovascular complications and a 10% reduction in overall mortality, respectively (Stratton et al. 2000; Khaw et al. 2001).

Probiotics have emerged as a promising complementary therapy for type 2 diabetes mellitus due to their ability to modulate the gut microbiota, improve intestinal barrier function and reduce systemic inflammation, all of which contribute to increased insulin sensitivity and better glucose metabolism. Several studies support these benefits, showing that probiotic supplementation significantly reduces fasting glucose, HbA1c and insulin resistance (Tao et al. 2020; Li et al. 2023). Interestingly, in our study, the significant reduction in HbA1c in the probiotic group emerged only at the 6-month follow-up, while no significant changes were observed after 3 months. This delayed response may reflect the cumulative and progressive effects of probiotics on host metabolic and immune functions. Indeed, several clinical trials have reported that the glycaemic benefits of probiotics often become statistically significant only after 8–12 weeks or longer (Miao et al. 2021; Sabico et al. 2019; Yao et al. 2017).

These findings support the notion that the HbA1c reduction observed at 6 months in our study, despite the lack of significant changes at 3 months, may still be attributed to the probiotic intervention, emphasising the importance of extended follow-up periods when assessing systemic outcomes.

In terms of the microbiological outcomes, and similar to the findings reported in the present study, the adjunctive use of probiotics has resulted in only limited benefits or minor changes in bacterial counts (Montero et al. 2017; Romani Vestman et al. 2015; Schlagenhauf et al. 2016). Regarding the colonisation pattern of the probiotic strains, our results showed that both strains could colonise the subgingival niche, with LR2 colonising more effectively (detected in seven patients, whereas LR1 was found only in three patients). In contrast, the study by Iniesta et al. (2012) found higher subgingival colonisation for LR1, supporting the idea that host factors may play a significant role in successful colonisation. This study showed that the ability of *L. reuteri* strains to colonise the oral cavity was very limited once the administration stopped, since only one patient tested positive for LR2 at 6 months. Nevertheless, it is controversial whether probiotic colonisation is essential for achieving beneficial effects, given that most probiotics used for gastrointestinal disorders do not typically colonise the gastrointestinal tract but still provide positive outcomes (Myllyluoma et al. 2007).

This study has some limitations, which should be considered when interpreting the results. First, the plaque index of the patients at the final visit was relatively high ($\approx 40\%$), which may have contributed to the absence of significant clinical or

microbiological differences between the groups. Additionally, the sample size was calculated to identify differences in PD changes but not for other outcomes such as glycated haemoglobin or the microbiological results. Furthermore, both types 1 and 2 diabetes mellitus were included, which may involve different metabolic responses, but diabetes type was included as a covariate in the multilevel regression model to control for potential confounding. In addition, as highlighted in a recent consensus report (Herrera et al. 2023). Despite differences in pathophysiology and treatment, both types of diabetes share similar pathogenic mechanisms in their relationship with periodontitis (primarily through systemic inflammation), supporting the inclusion of both groups in the same analysis.

In conclusion, and within the limitations of the present randomised clinical trial, the adjunctive use of probiotics to subgingival instrumentation in patients with stage II–III periodontitis and diabetes did not result in any additional clinical or microbiological improvements compared to placebo. However, a significant reduction in glycated haemoglobin levels was observed in the probiotic group, suggesting a potential systemic benefit. Probiotics were safe, well tolerated and not associated with ARs or complications in patients with diabetes and periodontitis.

Author Contributions

R.B. coordinated the trial, performed clinical assessments and drafted the manuscript; E.M. wrote the study protocol, coordinated the trial and drafted the manuscript; J.D.G. and M.J.M. performed the microbiological assessments; A.A. performed clinical assessments; M.I. coordinated the trial; M.S. and D.H. wrote the study protocol and critically revised the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** jcpe70035-sup-0001-supinfo.docx. **Data S2:** jcpe70035-sup-0002-Tables.docx.