Effect of a Locally Delivered Probiotic-Prebiotic Mixture as an Adjunct to Scaling and Root Planing in the Management of Chronic Periodontitis

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Abstract

Objectives: This study aimed to determine the efficacy of a probiotic, *Saccharomyces boulardii* (*S. boulardii*), mixed with a prebiotic, fructooligosaccharide (FOS), in the treatment of periodontal disease when used as an adjunct to non-surgical periodontal therapy (NSPT).

Methods: The study was carried out on 30 subjects who were diagnosed as having chronic periodontitis and had at least two sites in two different quadrants with ≥ 5 mm probing pocket depth (PPD). Two sites in each subject were randomly selected: in control sites, scaling and root planing (SRP) was performed, while the study sites received SRP followed by placement of the probiotic mixture. The viability of the probiotic (*S. boulardii*) in the pocket (at baseline, 2 days, 4 days and 7 days after treatment) and *in vitro* (at baseline, 1 week and 2 weeks after treatment) was estimated at designated time periods. Site-specific measures of plaque, gingival inflammation and periodontitis were recorded at baseline and specific intervals.

Results: *S. boulardii in vivo* survived up to 4 days, while *in vitro* reduction in the mean counts of *S. boulardii* did not differ statistically from baseline to 14 days. Significant reduction in probing pocket depths and gain in clinical attachment level in the test sites was observed when compared to controls.

Conclusion: The results suggest that *S. boulardii* is effective in improving the clinical measures of periodontal disease. *S. boulardii* seems to thrive well in the subgingival environment and may function as an effective oral probiotic in subjects with periodontitis.

Key words: Periodontitis, Saccharomyces boulardii, probiotic

Introduction

Periodontitis is defined as an inflammatory disease of the supporting tissues of the teeth initiated by specific microorganisms or groups of specific microorganisms (Newman *et al.*, 2006). Periodontal diseases are caused by microorganisms that colonize the tooth surface at or below the gingival margin in the form of biofilm. The antimicrobial periodontal therapies at present can be grouped into three broad categories: therapies that physically eliminate microorganisms through mechanical debridement, treatment strategies that attempt to affect the metabolism of the microorganisms by using antiseptics or antibiotics, and agents that can potentially affect the environment of the organism (Socransky, 2002).

The initial phase of periodontal therapy is directed towards reduction or elimination of pathogens and establishment of a beneficial microbiota preventing pathogen recolonization. The mechanical subgingival debridement

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results in 2 to 3 $\log_{10}$ reductions in the total subgingival microbiota (Maiden *et al.,* 1991; Rhemrev *et al.,* 2006). However, recolonization to pre-treatment levels by both beneficial and periodontopathogenic bacteria is a realistic possibility (Magnusson *et al.,* 1984; Harper and Robinson, 1987).

One method of altering the subgingival environment is by using probiotics (Socransky, 2002). Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit on the host (Sanders, 2008) by passively occupying a niche that may otherwise be colonized by pathogens. This tends to limit a pathogen's ability to bind to tissue surfaces and to produce virulence factors (Nadkerny *et al.,* 2014). In the past few years, probiotics have been investigated for periodontal health. Studies have shown that certain gut bacteria can exert beneficial effects in the oral cavity by inhibiting pathogenic species (Anilkumar and Monisha, 2012). Teughels *et al.* (2007) showed that application of beneficial oral bacteria subgingivally after scaling and root planing (SRP) led to a more host compatible subgingival microbiota, which may also effect the promotion of a beneficial host response (Sareen *et al.,* 2012). Studies have revealed that probiotic *Lactobacillus* strains (*L. reuteri, L. salivarius, L. casei, L. acidophilus)* were useful in reducing gingival inflammation and the number of black-pigmented rods, including *Porphyromonas gingivalis*, in the saliva and subgingival plaque. *Streptococcus sanguinis* and *S. uberis* were found to inhibit the growth of periodontal pathogens, and a strong negative relation between *Aggregatibacter actinomycetemcomitans* and *S. sanguinis* was found (Deepak *et al.,* 2010). *Weissella cibaria* isolates in the form of probiotic rinses possess the ability to inhibit biofilm formation, both *in vitro* and *in vivo* (Kang *et al.,* 2005).

Saccharomyces boulardii is commonly employed as a live non-pathogenic microbial food or food supplement that beneficially affects the host by improving its intestinal microbial balance (Mcfarland *et al.,* 1995; Attar *et al.,* 1999; Kotowska *et al.,* 2005; Can *et al.,* 2006; Sazawal *et al.,* 2006; Villarruel *et al.,* 2007). Culture of human dendritic cells in the presence of *S. boulardii* culture supernatant showed that secretion of key proinflammatory cytokines such as tumour necrosis factor-α and IL-6 were notably reduced, while the secretion of anti-inflammatory IL-10 increased (Thomas *et al.,* 2009), which is pertinent in the context of pathogenic mechanisms in periodontitis.

The objectives of the present study were to evaluate: 1) the role of a locally delivered probiotic on measures of periodontal health, and 2) the *in vitro* and *in vivo* vitality of the subgingivally delivered probiotic mixture.

Patients and methods

The purpose of this split-mouth, randomized controlled clinical trial was to evaluate the anti-inflammatory and anti-infective properties of *Saccharomyces boulardii* (Florafix™, Unique Biotech, Hyderabad, India) as a locally delivered probiotic when used as an adjunct to scaling and root planing in the management of chronic periodontitis. Approval from the Institutional Review Board was obtained and the study is listed on http://www.clinicaltrials.gov (NCT02645669).

Sample size calculation

Sample size was calculated according to the formula ($n \geq [z\alpha/2]2S2)/d2$, where n is the sample size, z is the normal distribution tabled value, d is the detection level considered important and S is the standard deviation from the pilot data. To detect a 0.1 $\log_{10}$ difference between two cultures, a minimum of 14 sites were required, assuming $z\alpha/2 = 2.5$, $S = 0.1453$ from pilot culture studies and $d = 0.1$.

Source of data

From a study pool of 62 subjects, 30 subjects were selected from the outpatient section of the department of Periodontology. Systemically healthy chronic periodontitis patients within the age group of 25 - 50 years having at least two periodontal pockets ≥ 5 mm with at least one pocket in each quadrant were included in the study. Smokers, medically compromised patients and subjects having received any form of surgical, non-surgical therapy or antibiotic therapy in the 6-month period prior to the study were not included.

Study protocol

After the prospective interdental areas were probed buccally and lingually/palatally, the site was considered for the study if the average probing pocket depth (PPD) was ≥ 5 mm. Probing pocket depth and clinical attachment level (CAL) were recorded at baseline (before SRP), 3 months and 6 months after treatment. Probing pocket depth and CAL were recorded using a University of North Carolina no.15 (UNC-15) color-coded periodontal probe. Gingivitis [using the modified gingival index (MGI); Lobene *et al.,* 1986] and plaque index (PI; Silness and Löe, 1964) were measured at baseline, 1 month, 3 months and 6 months after treatment. Probing pocket depth and CAL were recorded at baseline (before SRP), 1 month and 3 months and 6 months after treatment.

Study design

In all 30 subjects, for the purpose of standardization, two interdental suprabony pockets from the canine-premolar regions with 5-7 mm pocket depth in two different quadrants were chosen as the test and control sites. In control sites, only SRP was performed. In study sites, SRP was followed by placement of *S boulardii*-fructooligosaccharide (FOS) mixture *(Figure 1)*.

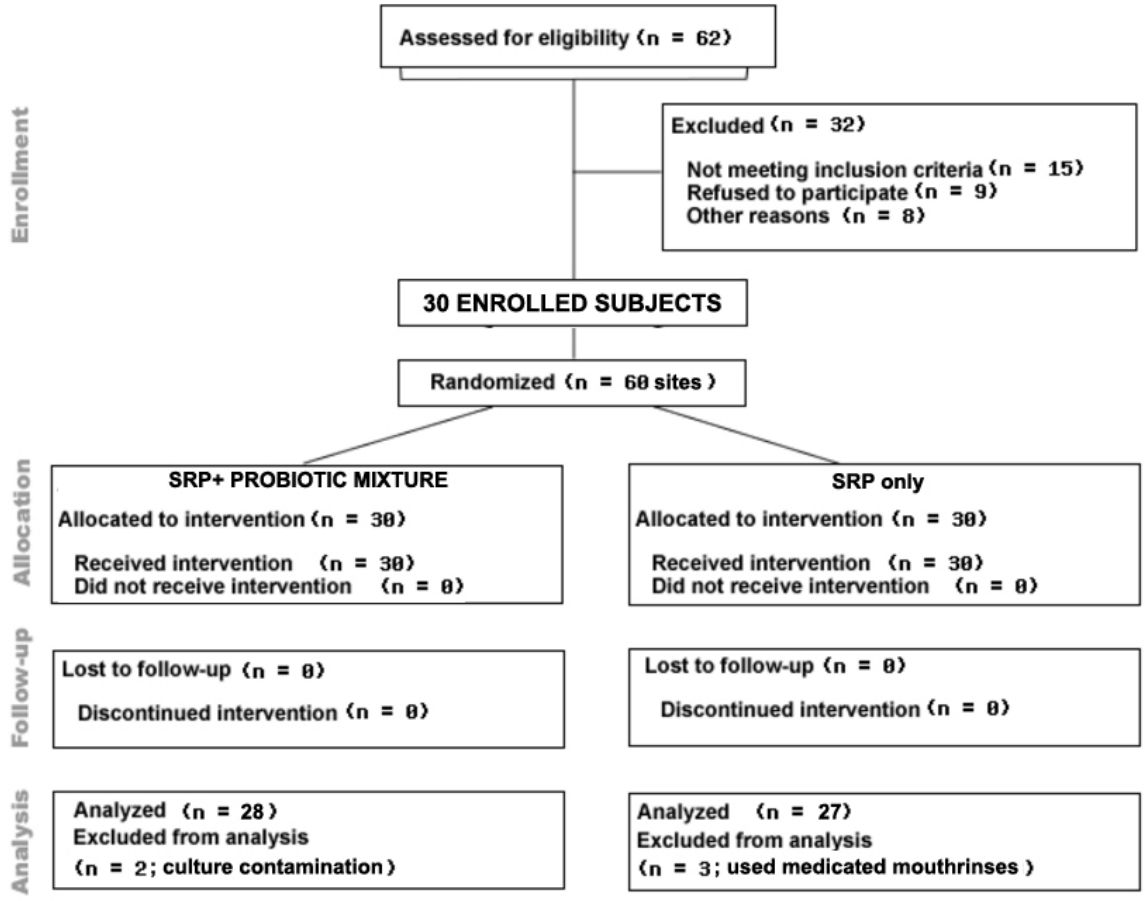

Figure 1. Flow chart of patient recruitment into test and control groups.

Formulation of the probiotic

The probiotic is in the form of a lyophilized powder (250 mg; Florafix®, Unique Biotech, Hyderabad, India) containing approximately 5 billion colony forming units (CFU) of the yeast *Saccharomyces boulardii*. The probiotic was mixed with a prebiotic (fructooligosaccharide, FOS; Mitushi Pharma, Ahmedabad, India), in the ratio of 4:1 to induce growth and activity in *S boulardii*. Briefly, 30 preparations of 1 g *S boulardii*-FOS mixture were made by stirring 200 mg of FOS and 800 mg of probiotic with a spatula. After mixing, the powder was gently sifted through a 6-size mesh sieve resulting in a homogenous and fine powder.

Treatment phase including randomization and blinding

Randomization included computerized generation of the allocation sequence in random permuted blocks. Allocation was performed by assigning the block of sites to study and control sites according to the specified sequence. All the therapies were performed by a designated operator (TS) for the sake of uniformity, whereas the relevant readings were recorded by another operator (YSHSC) who was blinded to the nature of the site. The blind was not broken until this clinical trial was completely finished. After recording relevant parameters, thorough scaling and root planing was done by using appropriate site-specific curettes.

Scaling and root planing was performed at baseline by using an ultrasonic scaling unit and a universal curette until the root surface was considered smooth and clean by the operator (TS). Following SRP, small increments of the preparation were taken and mixed with distilled water until a paste-like consistency was achieved. Sequential increments of the mixture were placed until the pocket was entirely filled to the gingival margin. The remaining powder was weighed to estimate the amount delivered into a site in mg. A similar amount of the mixture was weighed separately for *in vitro* viability of the probiotic mixture. Apart from oral hygiene instructions, the periodontally affected sites in quadrants not involved in the study received routine periodontal maintenance care, and additional nonsurgical or surgical therapy was instituted after determining the response to initial therapy after three months.

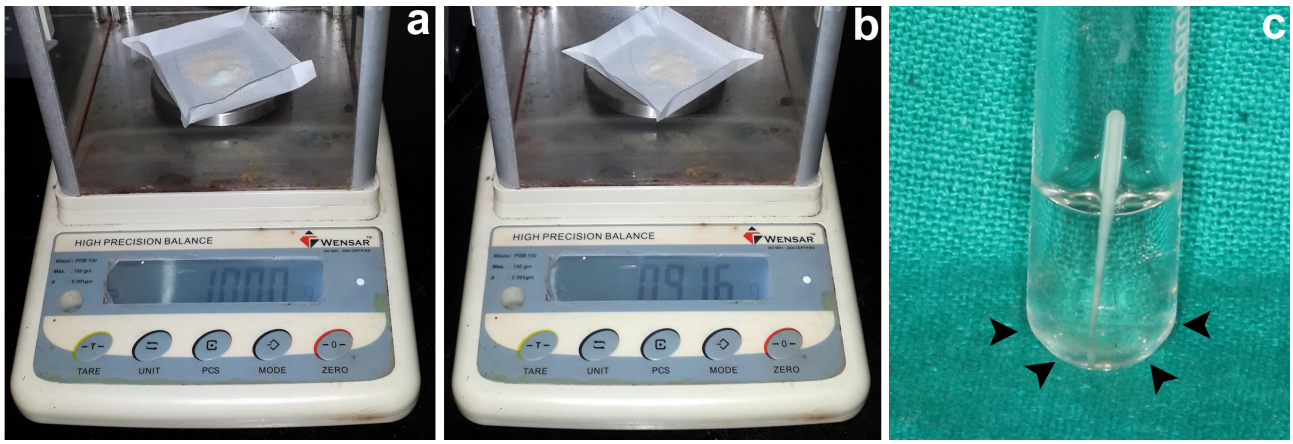

Figure 2. The preparation was weighed before (a) and after (b) subgingival delivery to estimate the amount (in mg) delivered into a site. During subgingival sampling, residue of the preparation could be seen up to 4 days in the gingival sulcus (c, arrows).

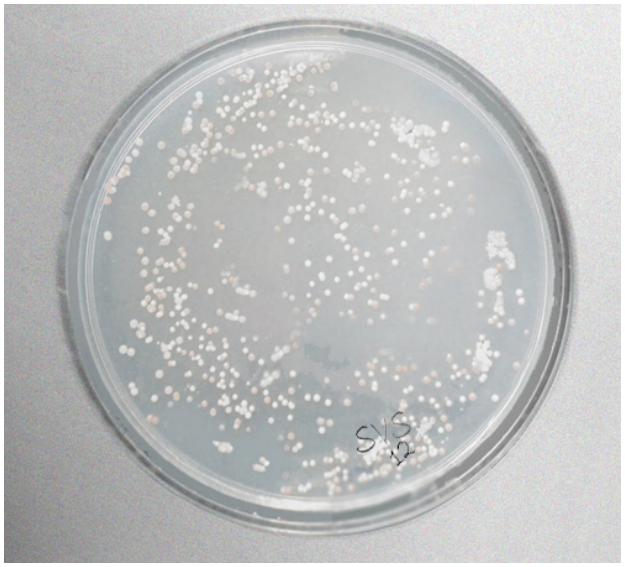

Figure 3. S. boulardii colonies cultured on Emmons' modification of Sabouraud agar medium.

Estimation of S. boulardii viability

In vitro viability of the probiotic mixture

The *S. boulardii-FOS* mixture was stored for 3 weeks at 25°C for periodic analysis (1, 7 and 14 days) of micro-organism viability on a selective medium (Emmons' modification of Sabouraud agar medium; Himedia Laboratories, Mumbai, India). Briefly, the mixture was plated on Sabouraud agar plates at 30°C and CFU were enumerated. Colonies were expressed as total log count per mg of sample ($\log_{10}$ CFU/mg). The growth proportion index (GPI; Patel *et al.,* 2014) of the organism at the end of each time period was calculated as follows: GPI = final cell population ($\log_{10}$ CFU/mg)/initial cell population ($\log_{10}$ CFU/mg).

Viability of the probiotic mixture in the periodontal pocket

On days 1, 2, 4 and 7, the test sites were isolated with cotton rolls and supragingival plaque was carefully removed with a sterile scaler to prevent the contamination of the samples with saliva or supragingival plaque. A sterile paper point was inserted slowly with a sterile dental tweezers into the pocket until tissue resistance was felt. The paper point was left for 30 sec and then it was carefully removed without touching the adjacent unrelated tissues and placed into a special sterile test tube containing 1 ml of distilled water *(Figure 2)*. Viable cell count was determined by serial dilution method on Emmons' modification of Sabouraud agar medium and colonies were expressed as total log count per ml of sample ($\log_{10}$ CFU/ml). Only colonies that were opaque, light brown, smooth and 2-3 mm in diameter were counted (Patel *et al.,* 2014; *Figure 3)*. The growth proportion index of the organism at the end of each time period was calculated.

Statistical analysis

Intragroup comparison was performed using repeated measures analysis of variance (ANOVA) followed by multiple comparisons using Bonferroni correction. One-way ANOVA followed by a post hoc test was used for intragroup comparison, and intergroup comparison was performed using a *t*-test. A p-value of ≤ 0.05 was considered statistically significant and a p-value of ≤ 0.001 was considered highly significant.

Results

Viability of S. boulardii in the gingival sulcus

A mean quantity of 88.57 ± 10.27 mg of the preparation was delivered into the sulcus. The reduction in the count of *S. boulardii* from baseline to 4 days was highly statistically significant ($p < 0.001$). The mean differences in levels of *S. boulardii* in sulci were 0.88095, -0.46238, and 1.34333, between baseline to 2 days, 2 days to 4 days and from baseline to 4 days, respectively. These mean differences at different time intervals were highly statistically significant ($p < 0.001$). The GPI at the end of the 4th day was significantly less than the GPI on the 2nd day ($p < 0.001$; *Table 1)*.

Table 1. Survival of *Saccharomyces boulardii* in the gingival sulcus at different time intervals using ANOVA.

	Days	Mean ± SD	f value	*p* value	GPI 2	GPI 4
S. boulardii Sulcus (n = 28) (log_{10} CFU/ml)	Baseline	4.24 ± 0.25	90.592	0.0001**	0.795	0.688**
	2	3.36 ± 0.30				
	4	2.90 ± 0.41				

**Highly significant

Table 2. Survival of *Saccaromyces boulardii in vitro* using ANOVA.

	Days	Mean ± SD	f value	*p* value	GPI 7	GPI 14
S. boulardii (log_{10} CFU/mg) (n = 28)	Baseline	6.80 ± 0.39	0.210	0.811†	1.013	1.015†
	7	6.88 ± 0.52				
	14	6.89 ± 0.43				

†Not statistically significantly different

Table 3. Intergroup comparison of plaque and gingival index at different time intervals.

Time	Groups	Mean ± SD	t value	*p* value
Plaque index				
Baseline	Test (n = 28)	1.58 ± 0.34	-1.941	0.059
	Control (n = 27)	1.79 ± 0.36		
1 week	Test	0.52 ± 0.10	-1.478	0.153
	Control	0.65 ± 0.39		
3 months	Test	0.54 ± 0.15	-5.139	0.0001**
	Control	0.85 ± 0.23		
6 months	Test	0.64 ± 0.28	-3.331	0.002*
	Control	0.92 ± 0.27		
Gingival index				
Baseline	Test	2.09 ± 0.30	0.0001	1.000
	Control	2.19 ± 0.50		
1 week	Test	1.19 ± 0.40	-2.346	0.024*
	Control	1.52 ± 0.51		
3 months	Test	0.85 ± 0.35	-2.331	0.025*
	Control	1.09 ± 0.30		
6 months	Test	0.90 ± 0.30	-4.779	0.0001**
	Control	1.80 ± 0.92		

*Significant; **highly significant

In vitro viability of S. boulardii

The difference in the counts of *S. boulardii*, from baseline to 14 days was not statistically significant. The mean differences in levels of *S. boulardii in vitro* were, -0.07333, -0.01048, 0.08381 between baseline to 2 days, 2 days to 4 days, and from baseline to 4 days, respectively. These mean differences at different time intervals were not statistically significant. No significant differences were seen in the GPI values at the 7th and 14th day *(Table 2)*.

Intragroup comparisons

The decrease in plaque scores and gingival index scores from baseline to the end of 1 week, 3 months and 6 months was highly statistically significant ($p < 0.001$) in both treatment groups. The intragroup reduction in pocket depth and gain in clinical attachment level from baseline to 3 months and 6 months was highly statistically significant in both treatment groups ($p < 0.001$).

Intergroup comparisons

There were no significant differences in the clinical parameters between the test and control groups at baseline. Intergroup comparison of the two study groups (test and control) was performed at different time-based intervals for PI, MGI, PPD, and CAL.

Reduction in PI at the test site was observed at 1 week, but this did not differ statistically from the control, whereas this reduction was highly significant at 3 months and 6 months ($p \leq 0.001$) and significant at 6 months ($p \leq 0.05$) when compared to the control site *(Table 3)*.

At baseline, both groups showed similar scores of MGI. The test group showed a significant reduction in the MGI both at 1 week and 3 months ($p \leq 0.05$) compared to the control group. At 6 months, the reduction in MGI was highly significant in test group compared to the control group ($p \leq 0.001$; *Table 3)*.

Significant reduction in PPD and gain in CAL at the test site was observed at 3 months ($p \leq 0.05$), whereas these measures were highly significant at 6 months ($p \leq 0.001$) when compared to the control site *(Table 4)*.

Discussion

Saccharomyces boulardii is a non-pathogenic yeast used as a preventive and therapeutic agent for the treatment of a variety of gastrointestinal diseases. The pharmacokinetic data seem to indicate that *S. boulardii* reaches a steady-state concentration quickly and maintains a high stable level (Canani *et al.,* 2011). Studies on animal models and evidence from human volunteers indicate that administration of *S. boulardii* is safe for oral ingestion, and review of published cases indicates that *S. boulardii* results in a very low risk in immunocompetent subjects. Only 9 cases relating to the adverse effects of oral administration of *S. boulardii* have been reported (Riquelme *et al.,* 2003). The beneficial effects of *S. boulardii* in gastrointestinal inflammatory conditions are mediated through modulation of host proinflammatory responses by interfering with the host's signaling molecules controlling inflammation at different levels, such as the NF-κB and MAP kinase pathways. Additionally, *S. boulardii* whole yeast administration substantially reduces mucosal levels of the proinflammatory mediators IL-1β, IL-6, TNF-α, and iNOS (Pothoulakis, 2009).

This study evaluated the efficacy of a prebiotic (FOS) – probiotic (*S. boulardii)* mixture in the treatment of periodontitis. The probiotic and prebiotic were mixed in the ratio of 4:1 to induce growth and activity in *S boulardii* at the time of administration. The viability of the probiotic was analysed in two independent experimental methods, i.e., *in vitro* viability of the probiotic mixture for periodic analysis at 1, 7 and 14 days, and *in vivo* viability of the probiotic mixture in the periodontal pocket on days 1, 2, 4 and 7.

S.boulardii is classified as a facultative anaerobe, meaning it can grow under aerobic or anaerobic conditions. Although it utilizes glucose as a substrate, it can thrive on monosaccharides, polysaccharides, oligosaccharides, ethanol, acetate, glycerol, pyruvate and lactate (de Souza *et al.,* 2012). Addition of FOS substantially increases the survivability of *S. boulardii.* In various gastrointestinal tract studies, addition of FOS significantly improved the growth of *S. boulardii* (Gibson and Roberfroid, 1995; George *et al.,* 1999; Mitterdorfer *et al.,* 2001; Pandiyan *et al.,* 2012). However, in the present study, *S. boulardii* was not detected by day 7 when administered as a local drug delivered subgingivally into the periodontal pocket. These findings are in agreement with Elmer *et al.* (1999) who noticed that *S. boulardii* is eliminated within 24 -72 hours if not re-inoculated. Blehaut *et al.* (1989) and Schneider *et al.* (2005) in their studies stated that *S. boulardii* disappeared from the faeces within five days of discontinuation of oral intake.

Table 4. Intergroup comparison of probing depths and clinical attachment level at different time intervals.

Time	Groups	Mean ± SD	t value	*p* value
Probing depth (in mm)				
Baseline	Test (n = 28) Control (n = 27)	5.66 ± 0.73 5.52 ± 0.74	0.626	0.535
3 months	Test Control	3.19 ± 0.67 3.76 ± 0.88	-2.34	0.025*
6 months	Test Control	2.19 ± 0.51 3.61 ± 0.97	-5.953	0.0001**
Clinical attachment level (in mm)				
Baseline	Test Control	3.57 ± 0.74 3.52 ± 0.74	0.206	0.838
3 months	Test Control	1.42 ± 0.59 1.90 ± 0.88	-2.037	0.049*
6 months	Test Control	0.61 ± 0.58 1.80 ± 0.92	-4.96	0.0001**

*Significant; **highly significant

S. boulardii preparations are stable and physiologically active. In this study, the mean counts of *S. boulardii in vitro* were 6.80 ± 0.39, 6.88 ± 0.52, and 6.89 ± 0.43 CFU at baseline, 7 days and 14 days, respectively. The difference in the counts did not differ statistically from baseline to 14 days. The values are similar to a study done by Pandiyan *et al.* (2012) in which the counts of *S. boulardii* in the treatment group did not differ statistically from baseline to 15 days (6.80 ± 0.02, 6.37 ± 0.02). Probiotic products may lose their culturability or may become dormant during commercial processing (Shinde, 2011); however, the results seem to suggest that the mixture is stable and remains culturable even after 14 days.

In the present study, the probiotic and prebiotic mixture was placed subgingivally into the periodontal pocket using distilled water as a vehicle. In both the test and control groups, highly significant intragroup improvements in PD and CAL levels were seen. Both groups received SRP, and the positive effect of SRP on PD and CAL is well documented in periodontal literature (Socransky, 2002). However, test sites showed significant intergroup reduction in PPD and gain in CAL compared to control sites at 3 months and 6 months as well. Teughels *et al.* (2007) stated that the application of selected beneficial bacteria as an adjunct to SRP would inhibit the periodontopathogenic recolonization of periodontal pockets. In a beagle dog model, analysis of the data showed that beneficial bacteria when applied in periodontal pockets adjunctively after root planing lead to delay and reduction in subgingival recolonization of periodontopathogens (Teughels *et al.,* 2007). The study confirmed the hypothesis and provided a proof of concept for a guided pocket recolonization (GPR) approach in the treatment of periodontitis. Challa *et al.* (2013) evaluated the clinical and microbiological efficacy of local delivery of probiotics in the treatment of chronic periodontitis and found a positive change in the subgingival microbiota. But in terms of clinical parameters, no significant difference was found between the probiotic and the placebo groups.

The mean plaque index and modified gingival index scores in our study showed significant reduction from baseline to 6 months. The reduction in PPD and gain in CAL were significant from baseline to 6 months. Krasse *et al.* (2006) showed a significantly reduced gingival index and amount of bacterial plaque in patients treated with *Lactobacillus reuteri* compared to a placebo group and they concluded that this oral probiotic was effective in reducing gingivitis and bacterial plaque deposition in patients with moderate to severe gingivitis. Another study used epidemiological data to assess the relationship between periodontal health and the consumption of dairy products such as cheese, milk and yogurt. The authors found that individuals who regularly consumed yogurt or beverages containing probiotics had lower probing depths and less loss of clinical attachment than individuals who consumed few of these dairy products (Shimazaki and Shirota, 2008). In another corresponding study, lozenges containing *L. reuteri* significantly reduced PI, GI, and GBI when compared to a placebo (Vivekananda *et al.,* 2010). Another study showed that there was a significant difference in the gingival index between probiotic and chlorhexidine groups, with the probiotic group reduction being better when compared to the chlorhexidine group (Harini and Anegundi, 2010). This improvement in the periodontal parameters was attributed to reduction in periodontopathic oral bacteria (Tsubura *et al.,* 2012), and formation of lactic, acetic and other short-chain organic acids that may be antagonistic to the potentially pathogenic competitors (Mutanda *et al.,* 2014).

This study has some limitations. No specific vehicle apart from distilled water was utilized in this study. This must have contributed to the short term survival of *S. boulardii* in periodontal pockets. As the objective of this study was to compare the efficacy of SRP versus probiotic mixture, a positive control arm with prebiotic or probiotic without SRP was not incorporated. Probiotics can inhibit the growth of periodontal pathogens, and FOS in itself as a prebiotic can aid in the growth of beneficial periodontal bacteria (Gibson and Roberfroid, 1995); however, disruption of the biofilm is essential prior to the administration of a probiotic (Teughels *et al.*, 2007; Vivekananda *et al.,* 2010; Nadkerny *et al.,* 2014) and any delivery of a prebiotic/probiotic without SRP may not be beneficial. Moreover, as this study utilizes *S. boulardii* – FOS as a probiotic-prebiotic mixture, no attempt was made to filter the effects of FOS in itself. No fixed probiotic-prebiotic ratio exists, and ratios of 2:1 and 4:1 have been recommended if FOS is the intended prebiotic (Ann *et al.,* 2007). A mixture at a 4:1 ratio was utilized in this study as it results in an increased concentration of the prebiotic.

In the present study, it was observed that SRP + *S. boulardii* was more effective in improving measures of periodontal disease than SRP alone. *S. boulardii* seems to thrive well in the subgingival environment and may function as an effective oral probiotic in subjects with periodontitis.

Acknowledgments

The authors wish to thank Dr. Anirudh Mukkamala and Dr. Srikanth Reddy for the microbiological analysis in this study. The authors report no financial or other conflicts of interest relevant to the subject of this article.

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