ORIGINAL RESEARCH

Effects of Probiotics Mouthwash on Levels of Red Complex Bacteria in Chronic Periodontitis Patients: A Clinico-microbiological Study

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Abstract

Aim: The red complex includes *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, which are recognized as the most important pathogens and are the indicators of infection in chronic periodontal disease. This study was to assess the levels of red complex bacteria in chronic periodontitis patients following treatment with probiotic mouthwash.

Materials and methods: Twenty chronic periodontitis patients with ages ranging from 18 to 55 years were recruited for the study. The control group was given placebo mouthwash and the study group was given probiotic mouthwash. After clinical monitoring and scaling and root planing, the collected plaque samples at baseline and 14th day were transferred for microbiological analysis by transport media for Conventional Multiplex Polymerase Chain Reaction.

Results: On the 14th day, all the clinical parameters were significantly reduced in the study group with gingival index ($p = 0.003$ HS) and plaque index ($p = 0.001$ VHS). In the study group, there was significant bacterial cell reduction with *T. denticola* ($p = 0.041$ S) and *T. forsythia* ($p = 0.037$ S).

Conclusion: In patients with chronic periodontitis, treatment with probiotic mouthwash significantly reduces the levels of red complex bacteria.

Clinical significance: The use of probiotic mouthwash could be a useful adjunct to scaling and root planing in chronic periodontitis.

Keywords: Chronic periodontitis, Polymerase chain reaction, Probiotic, Red complex bacteria.

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Introduction

Based on the evidence, it's effectively recognized that both, the bacterial challenge and the host, are the main key factors to develop periodontal diseases.[1] The complexity and intimacy of the interactions which occur between host and bacteria cells during the infection process release proinflammatory mediators which result in periodontal tissue destruction. The presence of pathogenic bacteria, the absence of beneficial bacteria, and host susceptibility are the main causative factors of periodontal diseases.

Among the periodontal pathogens, the red complex which includes *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* (formerly *Bacteroides forsythus*), encompasses the most important pathogens in adult periodontal disease.[2] Clearly the major difference between health and disease was the increased prevalence and counts of the red complex species, *B. forsythus*, *P. gingivalis,* and *T. denticola*, in subjects with periodontal disease. Red complex species increased strikingly in prevalence and numbers with increasing pocket depth.[3] It's also described that the red complex comprising the above species appears later during biofilm development.[4]

So based on an understanding of the pathogenic role of these bacteria, the main aim of periodontal treatment is to make the hard tissue and soft tissue free from pathogenic bacteria by removing supragingival and subgingival plaque and calculus. If periodontal disease is in fact caused by a limited number of bacterial species, then nonspecific continuous plaque suppression is not the only possibility for prevention and therapy. Specific elimination or reduction of pathogenic bacteria from plaque becomes a valid alternative.[3] Nonsurgical therapy like scaling and root planing (SRP), is considered the gold standard for the initial treatment of inflammatory periodontal disease and extensively focuses on the reduction in the total subgingival microbiota.[5]

Although initially the number of pathogens can be greatly reduced by SRP, the periodontopathogens quickly recolonize the treated niches in the oral cavity.[6] In view of recolonization, systemic and local antibiotics, and antiseptics have been used as adjunctive to improve the outcome of periodontal therapy.[7,8] However, such use of antibiotics may result in the development of antibiotic resistance.

On the contrary, a group of beneficial bacteria called *lactobacilli* can fight several kinds of bad bacteria and may help restore a healthy balance in your mouth. Evidence is emerging that good bacteria can produce a variety of proteins that can inhibit the synthesis/release of inflammatory cytokines in the oral cavity. Such proteins also

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ensure bacterial survival and prevent the induction of damaging inflammation.[9] In this context, the application of beneficial bacteria has emerged as a promising concept in enhancing the microbial shift away from periodontopathogens.

Parker[10] was the first to use the term probiotics. The term probiotics is derived from the Greek language meaning "for life". The World Health Organization defines probiotics as "Live microorganisms if administered in adequate amounts confer a health benefit on the host".[11] Probiotics can be useful in eliminating pathogenic bacteria by various mechanisms. One is through competitive inhibition by consuming essential nutrients and inhibiting pathogens' adhesion capabilities through alteration of ph. Second probiotics can produce lactic acid, hydrogen peroxide, and bacteriocins which directly kill the periodontal pathogens. Further probiotics can have immunomodulatory effects by reducing the production of proinflammatory cytokines like IL-6, and IL-1β and increasing the production of anti-inflammatory cytokines like IL-10.[12–14]

The oral administration of probiotics was considered a useful adjunct to scaling and root planing in reducing plaque index and gingival index.[1,11] Sometimes, the clinical improvement after initial periodontal therapy indeed directly correlates with the degree to which pathogenic subgingival species are eradicated or reduced.[15,16]

Given the potent paradigm shift that this phenomenon of oral probiotics can give rise to the field of periodontal healthcare, probiotics might offer an opportunity as an alternative approach to achieve the reduction or elimination of pathogens like red complex in periodontitis.

However, data with respect to the effect of probiotics specifically on red complex pathogens are limited. Hence the present study was conducted to evaluate the clinical effect of probiotic mouthwash on the levels of red complex bacteria in chronic periodontitis cases.

Materials and Methods

This double-blind placebo-controlled parallel-arm clinical trial was approved by the Institutional Ethical Committee (Reference IEC/2020-21/02).

Patients seeking periodontal treatment care and who were referred to the Department of Periodontology were screened for the study based on the inclusion criteria and exclusion criteria.

Inclusion Criteria

- Systemically healthy patients with at least 18–55 years of age in both male and female patients.
- Previously untreated chronic stage II moderate periodontitis with probing pocket depth <5 mm.[17]

Exclusion Criteria

- Patients who have received oral prophylaxis/antibiotics/anti-inflammatory drugs for any purpose within 6 months prior to entering the study.
- Systemically compromised patients with a history of rheumatic fever, diabetes mellitus, thyroid disorder, cardiac issues, kidney or liver diseases, immunological diseases, neurological diseases, etc.
- Any medication influencing or affecting periodontal tissues (NSAIDs, cyclosporine, phenytoin, heparin, warfarin, commercially available mouthwash, probiotic products, etc.)
- Any type of smokers, pregnancy, and lactation.

Study Size

Twenty patients after consent were enrolled to take part in the study after fulfilling the inclusion and exclusion criteria. After taking into account the difference in mean bacterial cells in the control and study group, for a standard deviation of 1.12, the sample size was calculated at 80% study power (Beta 0.2) and an alpha error of 0.05. This sample size came to 8.86 in each group. Since 10 in each group were already recruited, the sample size was considered as adequately powered for the study.

All patients included in the study were explained in detail about the purpose of the study, its implications, and the potential benefits. Written informed consent was obtained from all participants. No changes were made in the study after the approval by the Institutional Ethical Committee.

Study Design (Flowchart 1)

A total of 20 participants and the examiner were enrolled in the study and were blinded regarding the product allocation (Flowchart 1). Ten patients received a placebo mouth rinse (control group) and 10 patients received a probiotic mouth rinse (study group). In the present study, the commercially available probiotic product Darolac (Aristo Pharmaceuticals, India, 23 A, Shah Industrial Estate, Off Veera Desai Road, Andheri West, Mumbai 400053, Maharashtra, India) containing 1 g powder of 1.25 billion freeze-dried combinations, of a mixture of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium longum*, and *Saccharomyces boulardii* was used in the test group. Each DAROLAC sachet powder was dissolved in 20 mL of distilled water by one of the study coordinators and used as a mouthwash which is used twice daily i.e. 10 mL in the morning after brushing and 10 mL at night before sleeping. The placebos mouthwash was also prepared by the same study coordinator using 20 mL of distilled water and used as a mouthwash which is used twice daily i.e. 10 mL in the morning after 30 minutes of brushing and 10 mL at night before sleeping in the control group. The mouthwash containers for the probiotic and placebo mouthwash were color-coded. The contents of the mouthwash container were blinded to the examiner. All the patients received clinical and microbiological monitoring at baseline, on the 14th day. The pre and post-intervention evaluations were conducted by a single trained examiner.

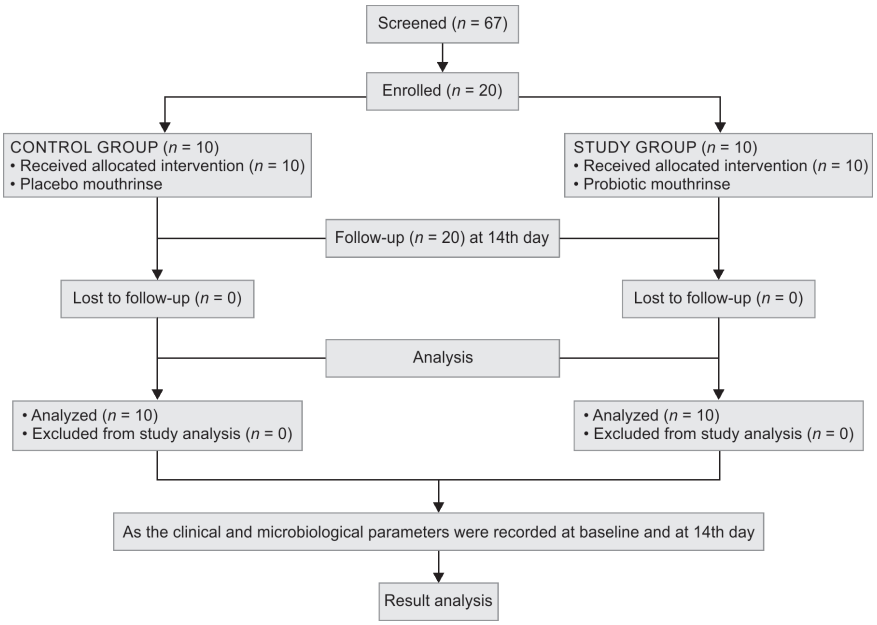
The gingival index and the plaque index of the volunteers were checked, at baseline and on the 14th day using plaque index (Silness and Loe 1964) and gingival index (Loe and Silness 1963) by the single examiner only.

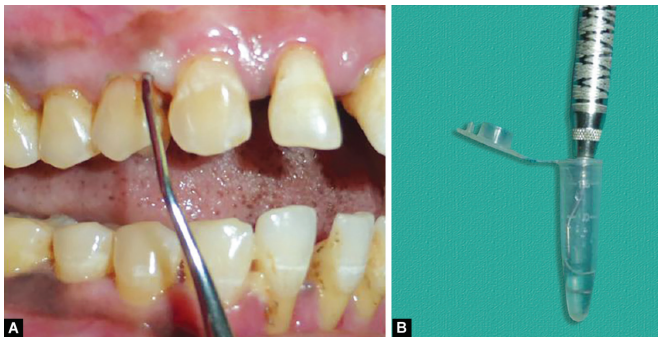
After thorough scaling and root planing, a subgingival plaque sample was collected with sterile Gracey curette from an average periodontal pocket depth of ≤5 mm.

Subgingival plaque samples were collected at baseline (Fig. 1) and each participant was given one of the test products with a given code according to the assigned group by the study coordinator. The participants were instructed to swish with the given mouthwash twice daily for 60 seconds and then expectorate and continue the same procedures for the next 14 days. Later, all the participants were recalled on the 14th day for subgingival plaque sample collection.

Each collected sample at baseline and on the 14th day was immediately transferred in transported media containing Tris-EDTA buffer (TE buffer) and was sent on the same day of sample collection for microbiological analysis by conventional multiplex polymerase chain reaction.

Flowchart 1: Flowchart of the study design





Figs 1A and B: Method of subgingival plaque collection

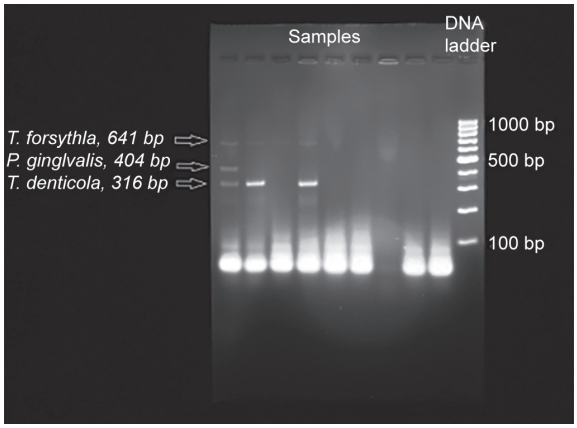


Fig. 2: Microbiological analysis by PCR technique

Microbiological Analysis

The DNA extraction procedure was done by using the modified proteinase-K method and samples were transferred to the tube containing TE buffer and were centrifuged at 5,000 rpm for 5 minutes supernatant was discarded and then 500 microliters of fresh TE buffer was added and centrifuge for 3–4 minutes. The above procedure was repeated 3–4 times with fresh TE buffer. The supernatant was discarded and 50 µL lysis buffer I was added, vortex it and kept for 5 minutes. Addition of 50 µL lysis buffer II and 10 µL proteinase—K (10 mg/mL), vortex vigorously done, and kept it in the water bath at 60°C for 2 hours than in boiling water bath for 10 minutes and transfer of the supernatant containing DNA to fresh tube done which stored the DNA at –20°C.

Ampliqon RED 2X master mix, PCR primers (Stock concentration 25 pmole), DNA template (Approximately 100 gm/mL), molecular grade water reagents, and the following set of PCR primers were used which are specific to respective organisms. *T. denticola*: 316 base pair TAA TAC CGA ATG TGC TCA TTT ACA T CA AAG AAG CAT TCC CTC TTC TTC TTA, *P. gingivalis* : 404 base pair AGG CAG CTT GCC ATA CTG CGACT GTT AGC AAC TAC CGA TGT, Tannerella forsythensis (*Bacteroides forsythus*): 641 base pair GCG TAT GTA ACC TGC CCG CA TGC TTC AGT GTC AGT TAT ACC T. Amplified products were subjected to electrophoresis through 2% agarose gel containing 1× TAE (Tris Acetate EDTA buffer), 20 µL of each amplified product were loaded into each well. Electrophoresis was performed at 25 V for 2 hours. The gel was visualized under UV light illuminator after staining with ethidium bromide (0.5 µg/mL). The gel image was captured and analyzed using Gel Documentation System (Major Science, USA). The gel was visualized under a UV transilluminator. Each organism will have specific band size based on the primer sequences selected. *P. gingivalis* has an amplified product of 404 base pair, *T. forsythia* gives the amplified product of 641 base pair and *T. denticola* gives the amplified product of 316 base pair. The DNA ladder was run simultaneously with each gel to obtain the bands of known sizes which will be used in locating the band positions of test samples. Total lab software (UK) was used to obtain the quantification of positive bands based on the intensity of the amplified products. The gel was uploaded into the software and quantification was obtained (Fig. 2).

Table 1: Age-wise distribution of cases

	Control group		*Study group*		*Total*	
Age in years	*No.*	*%*	*No.*	*%*	*No.*	*%*
26–35	5	50.0	3	30.0	8	40.0
36–45	4	40.0	5	50.0	9	45.0
46–55	0	0.0	1	10.0	1	5.0
>55	1	10.0	1	10.0	2	10.0
Total	10	100.0	10	100.0	20	100.0
Mean ± SD	36.70 ± 8.73		41.60 ± 9.08		39.15 ± 8.92	
t-test value and *p* value	$t = 1.230$; $p = 0.235$; NS					

NS, not significant

Table 2: Gender-wise distribution of cases

	Control group		*Study group*		*Total*	
Gender	*No.*	*%*	*No.*	*%*	*No.*	*%*
Males	2	20.0	3	30.0	5	25.0
Females	8	80.0	7	70.0	15	75.0
Total	10	100.0	10	100.0	20	100.0
χ^2-test value and *p* value	$\chi^2 = 0.267$; $p = 0.814$; NS					

NS, not significant

Table 3: Comparison of GI and PI between the control and study groups at baseline and day 14

		Control group	*Study group*		
Variables	*Time period*	*Mean ± SD*	*Mean ± SD*	*Unpaired t-test value*	*p value and significance*
Gingival index	Baseline	0.95 ± 0.19	0.95 ± 0.15	$t = 0.000$	$p = 1.00$, NS
	At 14th day	0.88 ± 0.17	0.67 ± 0.23	$t = 2.206$	$p = 0.041$, S
Plaque index	Baseline	1.01 ± 0.42	1.15 ± 0.37	$t = 0.775$	$p = 0.448$, NS
	At 14th day	0.89 ± 0.19	0.70 ± 0.14	$t = 2.399$	$p = 0.027$, S

NS, not significant; S, significant

Statistical Data Analysis

Statistical data were analyzed by IBM SPSS 20.0 version software. Collected data were spread on an excel sheet and prepared into a master chart. Through the master chart tables, graphs and diagrams were prepared. For qualitative data analysis Chi-square test was applied, for quantitative data analysis paired and unpaired *t*-test was applied for statistical significance. *p* values less than 0.05 were considered significant.

Results

On the basis of statistical analysis, there was no statistically significant difference in mean age and gender between both groups ($p > 0.05$) as shown in Tables 1 and 2 respectively. Age and sex controls were well matched.

The baseline characteristics of gingival index and plaque index were not significantly different in both groups (Table 3). This served as well-matched control to enable unbiased comparison between groups at day 14. On the 14th day, both the GI and PI were significantly lower in the study group when compared to the control group (Table 3).

Within the respective group, the GI and PI were then compared between baseline and day 14th (Table 4). Within the control group, the GI and PI did not alter significantly on day 14 when compared to baseline. However, within the study group, both GI and PI decreased significantly when compared to baseline (Table 4: GI $p = 0.003$; PI $p = 0.001$).

We then looked at the bacterial counts at baseline in the control group and study group which were not significantly different except for *P. gingivalis* wherein the baseline counts of *P. gingivalis* were already low (Table 5). On the 14th day, the number of bacterial cells of *T. denticola and T. forsythia* significantly reduced in the study group when compared to the control group (Table 5).

We then compared the bacterial counts between baseline and day 14th among the control and study groups (Table 6). Comparison of mean bacterial cells of *T. denticola*, and *T. forsythia* at baseline and at 14th day in the control group ($p > 0.05$) showed no statistically significant difference. However, in the study group, the difference in mean bacterial cells of *P. gingivalis*, *T. denticola*, and *T. forsythia* at baseline and 14th day were all statistically significant (Table 6) ($p < 0.05$).

Discussion

This double-blind placebo-controlled randomized clinical trial was conducted on a total of twenty chronic periodontitis patients which evaluated the effect of commercially available probiotics i.e DAROLAC on the levels of red complex bacteria after scaling and root planing, twice a day for 14 days, on microbiological and

Table 4: Comparison of GI and PI between baseline and 14th day within the control group and study group

		Baseline	*At 14th day*		
Groups	*Time period*	*Mean ± SD*	*Mean ± SD*	*Paired t-test value*	*p value and significance*
Control group	GI	0.95 ± 0.19	0.88 ± 0.17	$t = 1.95$	$p = 0.95$, NS
	PI	1.01 ± 0.42	0.89 ± 0.19	$t = 1.246$	$p = 0.244$, NS
Study group	GI	0.95 ± 0.15	0.67 ± 0.23	$t = 3.973$	$p = 0.003$, HS
	PI	1.15 ± 0.37	0.70 ± 0.14	$t = 4.714$	$p = 0.001$, VHS

NS, not significant; HS, highly significant; VHS, very highly significant

Table 5: Comparison of the number of bacterial cells of *P. gingivalis*, *T. denticola*, and *T. forsythia* at baseline and 14th day between the control group and study group

		Control group	*Study group*	*Paired t-test*	
Groups	*Time period*	*Mean ± SD*	*Mean ± SD*	*value*	*p value and significance*
At baseline	*P. gingivalis*	381	$7.27 \times 10^8 \pm 9.19 \times 10^3$	—	—
	T. denticola	$1.16 \times 10^7 \pm 2.86 \times 10^6$	$1.15 \times 10^8 \pm 3.04 \times 10^3$	$t = 0.542$	$p = 0.812$, NS
	T. forsythia	$3.59 \times 10^8 \pm 5.07 \times 10^8$	$3.59 \times 10^9 \pm 5.77 \times 10^4$	$t = 0.317$	$p = 0.897$, NS
At 14th day	*P. gingivalis*	0.0 ± 0.0	$4.10 \times 10^3 \pm 1.15 \times 10^2$	—	—
	T. denticola	$1.23 \times 10^5 \pm 1.13 \times 10^3$	$2.87 \times 10^3 \pm 1.81 \times 10^2$	$t = 7.41$	$p = 0.041$, S
	T. forsythia	$1.28 \times 10^5 \pm 1.32 \times 10^4$	$1.28 \times 10^3 \pm 1.23 \times 10^2$	$t = 6.93$	$p = 0.037$, S

NS, not significant; S, significant

Table 6: Comparison of the number of bacterial cells of *P. gingivalis*, *T. denticola*, and *T. forsythia* between baseline and 14th day within the control group and study group

		Baseline	*At 14th day*		
Groups	*Time period*	*Mean ± SD*	*Mean ± SD*	*Paired t-test value*	*p value and significance*
Control group	*P. gingivalis*	381	0.0 ± 0.0	—	—
	T. denticola	$1.16 \times 10^7 \pm 2.86 \times 10^6$	$1.23 \times 10^5 \pm 1.13 \times 10^3$	$t = 1.001$	$p = 0.363$, NS
	T. forsythia	$3.59 \times 10^8 \pm 5.07 \times 10^8$	$1.28 \times 10^5 \pm 1.32 \times 10^4$	$t = 1.000$	$p = 0.500$, NS
Study group	*P. gingivalis*	$7.27 \times 10^8 \pm 9.19 \times 10^3$	$4.10 \times 10^3 \pm 1.15 \times 10^2$	$t = 11.26$	$p = 0.043$, S
	T. denticola	$1.15 \times 10^8 \pm 3.04 \times 10^3$	$2.87 \times 10^3 \pm 1.81 \times 10^2$	$t = 6.93$	$p = 0.036$, S
	T. forsythia	$3.59 \times 10^9 \pm 5.77 \times 10^4$	$1.28 \times 10^3 \pm 1.23 \times 10^2$	$t = 7.91$	$p = 0.031$, S

NS, not significant; S, significant

clinical parameters in chronic periodontitis patients. DAROLAC is an approved probiotic product for commercial use containing the established oral probiotic organisms (*Lactobacillus*, *Bifidobacterium,* and *Saccharomyces*). In the present study, the choice for probiotic use was based on the presence of beneficial bacteria present in the product and its beneficial effect on the oral tissues like gingiva. Among the various selection criteria for probiotics, adhesion of the good bacterium to the tooth surface was considered of primary importance that favored the expression of probiotic activity. In the past, many studies have been done to assess the adhesion by measuring the bacteria attachment to the oral epithelium and saliva-coated hydroxyapatite and these studies have shown that among probiotics strains *L. rhamnosus GG* exhibited the highest values of adhesion properties, compared to the early tooth colonizer like *Streptococcus sanguinis*.[18] It is observed that some probiotic bacteria showed immunostimulatory activity on the oral epithelial tissue by inducing remarkable expression of human b-defensin (hbD-2) an antimicrobial peptide resulting in a reduction in periodontal inflammation. The expression of hbD-2 is due to a high level of exposure to commensal microorganisms.[19]

Since all the three members of the red complex are anaerobes and are nutritionally demanding and fastidious, it is difficult to isolate and identify by culture method. Hence, the conventional multiplex polymerase chain reaction (PCR) technique was used for microbiological analysis. The detection of bacteria was done using a conventional multiplex polymerase chain reaction by using a set of PCR primers, specific to respective organisms.

Results showed that patients using probiotic mouthwash along with routine mechanical oral hygiene procedures benefited. In addition, patients using the probiotic mouthwash showed a significant reduction in plaque index and gingival index and also these patients had a significantly more pronounced reduction in *P. gingivalis*, *T. denticola,* and *T. forsythia* bacteria in the study group. A statistically significant reduction in the mean plaque score was found on the 14th day for the study group when compared to the baseline and as well as compared to the control group.

A Brazilian study by Marcos et al. studied the effects of Bifidobacterium probiotics on the treatment of chronic periodontitis. The test group showed a decrease in probing depth and a clinical attachment gain significantly higher than those of the

control group at 90 days and they also demonstrated significantly fewer periodontal pathogens of red and orange complexes when compared to the control group.[20]

Ince et al. used lozenges of *Lactobacillus reuteri* as an adjunct to nonsurgical periodontal therapy in chronic periodontitis wherein the test group showed significant improvement in the plaque index, gingival index, and probing depth when compared to the placebo group.[11]

The results in our study are similar to the study done by Jindal[21] and Shimauchi[22] reporting a statistically significant decrease in plaque index and gingival index when compared with baseline values when probiotics as an adjunct to SRP were used. On the contrary, Staab showed a statistically significant increase in gingivitis index when compared with baseline values due to an increase in myeloperoxidase activity.[23]

Teughels et al. study showed a reduction in periodontal pocket depth, *P. gingivalis*, and *P. intermidia* and again in clinical attachment level.[1] The present study also showed a statistically significant reduction of mean bacterial cells of *P. gingivalis*, *T. denticola*, and *T. forsythia* on the 14th day along with a reduction in GI and PI. On the 14th day mean bacterial cells of *P. gingivalis*, *T. denticola*, and *T. forsythia* were observed significantly low as compared to baseline in the study group. In this study, we could not compare *P. gingivalis* in the control vs the study group as the baseline number of *P. gingivalis* cells in the study group was already low. However, within the study group, there was a significant decline ($p = 0.043$) in the number of *P. gingivalis* cells on the 14th day from the baseline. This suggests that probiotic mouthwash is helpful in reducing the bacterial load and thereby it could serve as a useful adjunctive therapy.

It would be interesting to see the long-term follow-up of such patients in terms of recolonization of bacteria in terms of recurrence of disease or in terms of the need for repeated nonsurgical therapy. Long-term follow-up studies will be required to establish this. Considering that this therapy is easily acceptable to the patients, and the fact that it is cost-effective, this therapy could turn out to be a promising option for the routine treatment of mild to moderate chronic periodontitis.

Conclusion

In patients with chronic periodontitis, the use of probiotic mouthwash significantly reduces the levels of red complex bacteria along with significant improvement in clinical parameters.

Scientific Rationale for the Study

Lactobacillus probiotics and *Bifidobacterium* as probiotics have been separately investigated for the treatment of periodontitis. However, there is limited data on the effect of a mixture of probiotics on red complex pathogens which are the key periodontal pathogens.

Principal Findings

Probiotic mouthwash consisting of a mixture of *Lactobacillus*, *Bifidobacterium,* and *Saccharomyces boulardii (Darolac)* significantly reduced levels of red complex bacteria. It also significantly improved clinical periodontal parameters.

Clinical Significance

The use of probiotic mouthwash can be a useful adjunct to scaling and root planing, and may obviate the need for systemic antibiotic therapy.

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