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Evaluation of the efficacy of a probiotic drink containing *Lactobacillus casei* on the levels of periodontopathic bacteria in periodontitis: A clinico-microbiologic study

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ABSTRACT

Background and Objectives: This study was designed to evaluate whether the oral administration of lactobacilli could change the bacterial population in subgingival plaque.

Subjects and Methods: Forty-two healthy volunteers with chronic generalized mild to moderate periodontitis were given a probiotic drink containing *Lactobacillus casei* for 1 month. Subgingival plaque samples were collected at baseline, after which the patients were asked to consume the probiotic drink once daily for 1 month. At the 1 month interval, plaque samples were collected, and the drink discontinued. The patients were recalled at 2 months interval for collection of the final samples. The bacterial amounts in the plaque samples were analyzed by multiplex polymerase chain reaction procedure.

Results: Of the three periodontopathic bacteria selected, *Porphyromonas gingivalis* showed highly significant reductions in the bacterial levels at 1-month and 2 months intervals. In comparison, *Aggregatibacter actinomycetemcomitans*, when present higher than 10×10^3 at baseline, and *Prevotella intermedia* present higher than 2×10^3 at baseline, showed moderately significant reduction in their numbers.

Interpretation and Conclusion: Oral administration of the probiotic lactobacilli reduced the numerical sum of the three selected periodontopathic bacteria and could contribute to the beneficial effects on periodontal conditions.

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Probiotics have evolved for more than ten decades to present day. Metchnikoff introduced the concept of probiotics in his book on "prolongation of life."[1] In the early 20th century, disorders of the intestinal tract were frequently treated with viable nonpathogenic bacteria to change or replace the intestinal microbiota. Later the term "probiotics" was introduced by Lilly and Stillwell in 1965, who described it as microbial factors that stimulate the growth of other organisms.[2,3] and have a beneficial effect on the host.[4]

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Probiotics are broadly categorized into two genus: *Lactobacillus* and *Bifidobacterium*. In the earlier reports, consumption of products containing probiotic lactobacilli has shown to successfully reduce recurrence of apthous ulcers, decrease caries risk, and the number of *Streptococcus mutans* in the oral cavity.[5,6] Numerous modes of action of probiotics have been proposed, such as prevention of adhesion of pathogens to host tissues, stimulation, and modulation of the mucosal immune system.[7-13]

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It poses a great potential in the arena of periodontics in terms of plaque modification, halitosis management, altering anaerobic bacteria colonization, and improvement of pocket depth and clinical attachment levels (CAL).[2]

Studies have also revealed that probiotic *Lactobacillus* strains were useful in reducing gingival inflammation as well as the levels of pathogenic microorganisms in the saliva and subgingival plaque.[14-16] Concerning periodontal conditions, Teughels *et al.* have shown that application of beneficial bacteria, as an adjunct to scaling and root planing, can alter anaerobic bacteria colonization,[2] inhibit re-colonization of pathogens in periodontal pockets and reduce bleeding on probing.[17] Other clinical trials have demonstrated improved plaque index (PI) and probing depth and CALs[2] as well as a reduction in proinflammatory cytokines in periodontal patients using probiotics.[7,18]

The beneficial impact of probiotic bacteria has seen a paradigm shift away from treating dental diseases by targeting specific oral pathogens towards an ecological and microbial community-based approach to understand conditions, such as in periodontal disease.[19]

This study was performed to assess the effects of a Probiotic drink containing *Lactobacillus casei* strain *Shirota*, on the clinical and microbial parameters, namely:

- Gingival and plaque status: At baseline, and 1 month and 2 months interval after administration of the probiotic drink
- The levels of periodontopathic microorganisms (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia*), at baseline, and 1 month and 2 months interval after administration of the probiotic drink.

SUBJECTS AND METHODS

The inclusion criteria

Patients aged between 20 and 50 years, and suffering from mild to moderate periodontitis with good to fair oral hygiene were selected for the study [Figure 1].

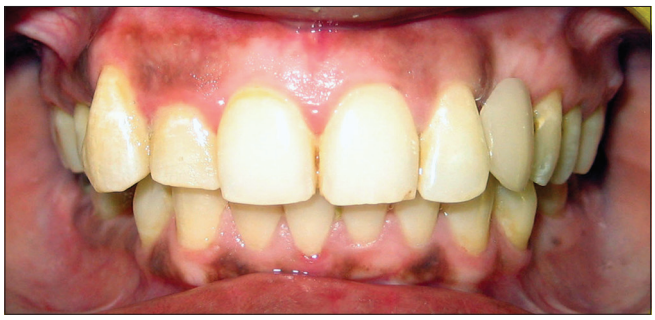


Figure 1: Patient selected for the study, suffering from chronic generalized mild to moderate Periodontitis

The exclusion criteria

- Patients using probiotic supplements
- Patients with allergy to lactose and fermented milk products
- Smokers
- Patients on antibiotic therapy or who were on antibiotic therapy in the past 6 months
- Patients with aggressive periodontitis
- Presence of excessive mobility of any tooth, which requires immediate treatment
- Presence of periapical and/or periodontal abscess
- Patient suffering from any systemic illness
- Patients who are deemed to be uncooperative.

Study design

A total of 42 volunteers were selected for the study. The study period lasted 14 months, between March 2011 and May 2012. The patients were classified into a single group with chronic generalized mild to moderate periodontitis. Informed consent was obtained from individuals who wished to participate in the study.

At first visit the following clinical parameters were recorded for all the subjects:

- Probing pocket depth
- CALs
- Gingival index (GI) by Loe and Silness, 1963[20]
- PI by Silness and Loe, 1964.[21]

Administration of probiotics

After baseline parameters were recorded, the patients were advised to consume probiotic drink, Yakult®™, Yakult Danone India Pvt. Ltd. (Yakult is manufactured at an ISO 9001:2008, HACCP and OHSAS 18001:2007 certified manufacturing facility in Sonepat, Haryana, India), containing *L. casei* strain *Shirota,* once daily for 1 month [Figure 2]. The patients were asked to swish the probiotic drink in his/her mouth before swallowing, and

Figure 2: Yakult probiotic drink

also instructed to consume it half an hour after breakfast, so that there is no interference from the ingested food.

Clinical and microbiologic parameters were again checked after 1 month, following which the probiotic drink was discontinued. On the 2 months recall, the clinical parameters were recorded, and the plaque samples were collected for microbial analysis.

Collection of samples

- Subgingival plaque samples were collected on the buccal surfaces of all the teeth present at baseline, 1 month and 2 months intervals. The tooth was isolated with cotton rolls, and supragingival plaque was removed with cotton pellets. Then a sterile curette was inserted into the subgingival area and plaque removed. The sample was placed immediately in a sterile test tube and sealed to prevent any contamination of the sample
- The transport of samples to the lab was done via the use of tryptic-soy-serum-bacitracin-vancomycin transport media, and the samples were analyzed within 24 h.

Microbiologic examination

- Following extraction of DNA, both qualitative and quantitative analysis of the bacteria was carried out using the multiplex polymerase chain reaction (PCR) procedure.

DNA extraction procedure

The sample containing the plaque was transferred to a tube containing Tris-EDTA buffer (T.E. buffer) and centrifuged at 50,000 rpm for 2 min [Figure 3]. The supernatant which got collected at the top was discarded, and fresh T.E. buffer was added, which was then centrifuged for 3–4 min. The process was repeated 3–4 times, each with fresh T.E. buffer.

The supernatant was again discarded, after which 50 µl of lysis buffer II and 10 µl of proteinase-K was added [Figure 4]. This solution was placed in a water bath for 2 h, followed by placement in boiling water for 10 min. Thus, the final extracted DNA was then stored at –20°C, until it was to be amplified on the PCR.

Multiplex polymerase chain reaction procedure

The multiplex PCR was performed by using specific primers for the 16S rRNA gene of each bacterium [Figure 5]. PCR amplification reactions were carried out in a reaction mixture in a final volume of 100 µl consisting of 10 µl of DNA sample, and 90 µl of reaction mixture containing 30 pmol of each primer, 200 µM of a mixture of deoxy-nucleoside-triphosphates, 1.5 mM $MgCl_2$, 1X PCR buffer (10 mM Tris-HCl, pH 8.0), 50 mM KCl and 2.5 U Hot Start Taq™ DNA polymerase.[22,23]

The PCR protocol was as follows:

- 98°C for 15 min
- 40 cycles at 95°, for 30 s each

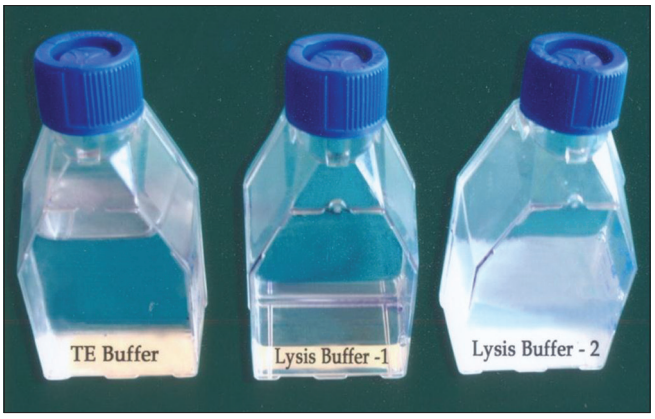


Figure 3: Buffer

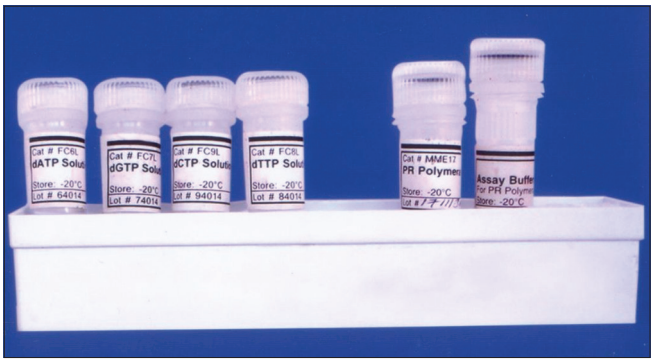


Figure 4: Dinucleotides, polymerase enzyme and assay buffer

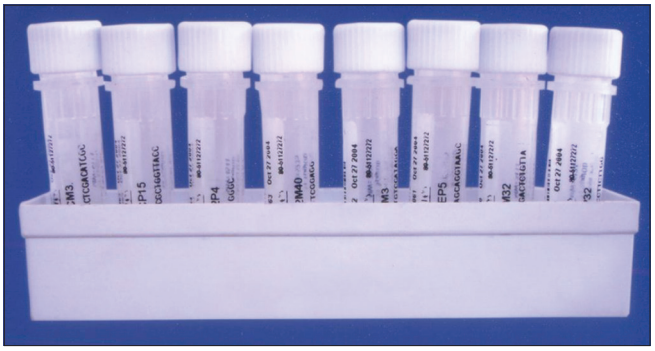


Figure 5: Primers

- 60°C for 1 min
- 72°C for 1 min, and
- A final step of 72°C for 10 min [Figure 6].

Amplicons were detected by electrophoresis of 20 µl of samples from each PCR tube in a 2% agarose gel in Tris-Acetate-EDTA buffer for 2 h at 80 V [Figures 7 and 8]. The amplification products were visualized and photographed under an ultraviolet light transilluminator after 30 min of ethidium bromide (1 µg/ml) staining [Figure 9]. The molecular sizes of the amplicons were determined by comparison to a commercial DNA molecular weight marker [Table 1]. The frequency of sites positive for each microbiota was reported.

Figure 6: Amplification of ribonucleic acid in the thermocycler

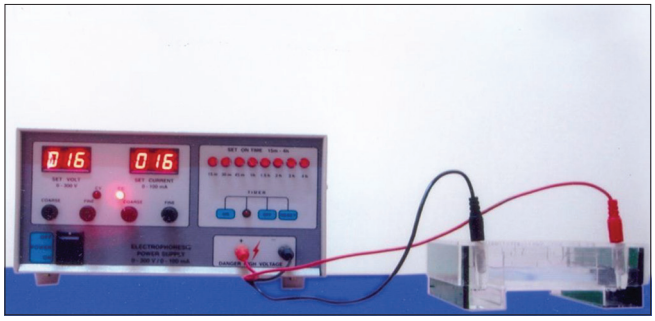


Figure 8: Gel electrophoresis

Table 1: Sequences and expected product size for polymerase chain reaction primers[22,23]

Primer pairs 5'-3'	Amplification length
Aggregatibacter actinomycetemcomitans GCT AAT ACC GCG TAG AGT CGG ATT TCA CAC CTC ACT TAA AGG T	443 base pairs
Porphyromonas gingivalis AGG CAG CTT GCC ATA CTG CG ACT GTT AGC AAC TAC CGA TGT	404 base pairs
Prevotella intermedia AAC GGC ATT ATG TGC TTG CAC CTC AAG TCC GCC AGT TCG CG	589 base pairs

Statistical analysis

Repeated measures analysis of variance has been used to find the significance of study parameters between three or more groups of patients and Student's *t*-test (two-tailed, dependent) has been used to find the significance of study parameters on the continuous scale within each group.[24] The statistical software namely SAS 9.2, SPSS 15.0, Stata 10.1, MedCalc 9.0.1, Systat 12.0, and R environment version 2.11.1 (Yakult Danone India Pvt Ltd, 52, Okhla Industrial Estate, Phase III, New Delhi, India) were used for the analysis of the data.

RESULTS

A total of 42 subjects, comprising of 27 (64.3%) males and 15 (35.7%) females participated in this study.

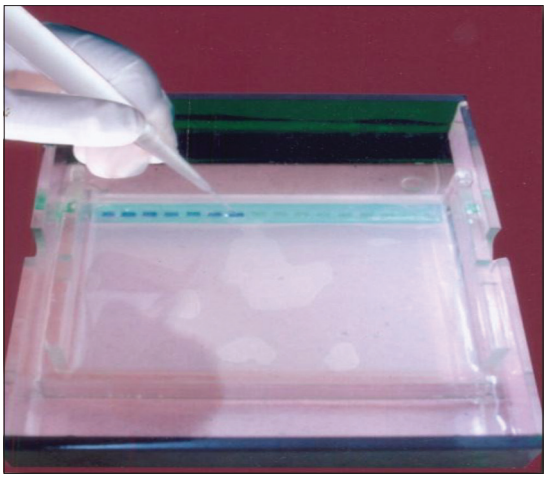


Figure 7: Transfer of amplification product on agarose gel

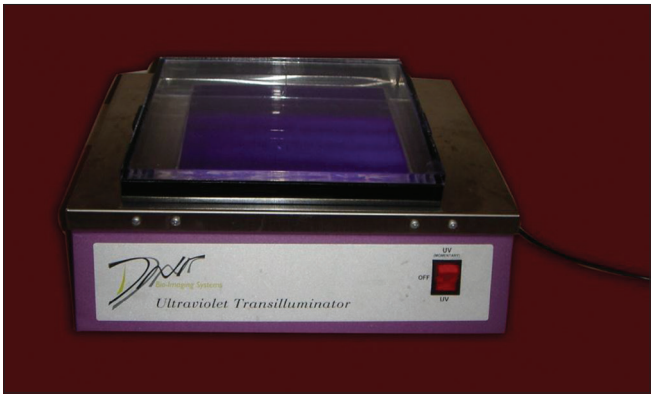


Figure 9: Ultraviolet transilluminator

On assessment of the clinical parameters, the reduction in PI and GI was at baseline, 1 month and 2 months recalls, and was found to be statistically nonsignificant [Tables 2 and 3].

In the microbiologic analysis of the plaque samples, using multiplex PCR, the levels of three periodontopathic microorganisms were detected, namely *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia*.

On comparison of *A. actinomycetemcomitans* levels at baseline, 1 month and 2 months showed the levels among 0–1 × 10^3, 1–2 × 10^3, and 2–10 × 10^3 was found to be statistically nonsignificant. Patients with levels of the microorganism between > 10 × 10^3 were found to be moderately statistically significant [Table 4 and Graph 1].

The microbial levels of *P. gingivalis* levels when compared at baseline, 1 month and 2 months showed highly statistical significance for levels from 0 to 100 × 10^3, 1000 × 10^3 to 10,000 × 10^3, and > 10,000 × 10^3. However microbial levels ranging from 1000 × 10^3 to 10,000 × 10^3 showed moderately significant results [Table 5 and Graph 2].

Table 2: Evaluation of gingival index at baseline, 1 month and 2 months

GI	Range	Mean±SD	Difference from baseline	*P* value from baseline
Baseline	1.0-2.0	1.41±0.48	-	-
1 month	0.90-2.0	1.38±0.47	0.0275	0.062
2 months	1.0-2.0	1.39±0.48	0.015	0.244

Student t test was used to find the pair wise significance and repeated measures. ANOVA was used to find the overall significance with F=2.631; P=0.078. GI=Gingival index, SD=Standard deviation, ANOVA=Analysis of variance

Table 3: Evaluation of plaque index at baseline, 1 month and 2 months

PI	Range	Mean±SD	Difference from baseline	*P* value from baseline
Baseline	1.0-2.50	1.41±0.49	-	-
1 month	1.0-2.40	1.39±0.49	0.0255	0.212
2 months	1.0-2.50	1.41±0.50	0.0025	0.323

Student *t*-test was used to find the pair wise significance and repeated measures. ANOVA was used to find the overall significance with F=1.447; P=0.241. SD=Standard deviation, ANOVA=Analysis of variance, PI=Plaque index

Table 4: Polymerase chain reaction analysis of microbial samples for *Aggregatibacter actinomycetemcomitans*

A. actinomycetemcomitans	Baseline (%)	1 month (%)	2 months (%)	*P*
0-1×10^3	35 (83.3)	39 (97.5)	38 (95.0)	0.290
1-2×10^3	3 (7.1)	1 (2.5)	1 (2.5)	0.174
2-10×10^3	0	0	1 (2.5)	0.159
>10×10^3	4 (9.5)	0	0	0.023*
Total	42 (100)	40 (100)	40 (100)	-
Lost to follow-up		2	2	-

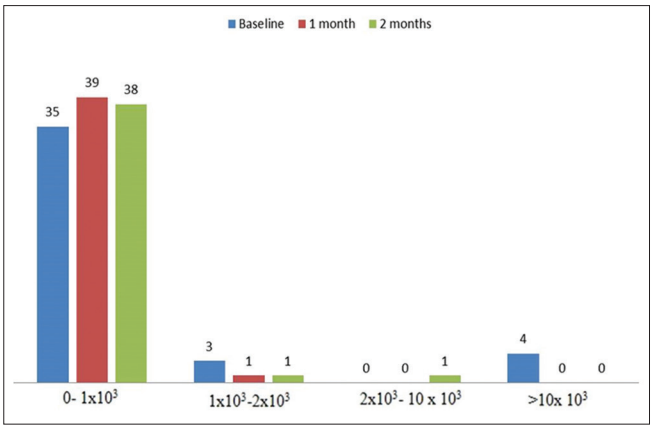
Paired proportion test. *A. actinomycetemcomitans=Aggregatibacter actinomycetemcomitans.* *$P \leq 0.001$ highly significant

Table 5: Polymerase chain reaction analysis of microbial samples for *Porphyromonas gingivalis*

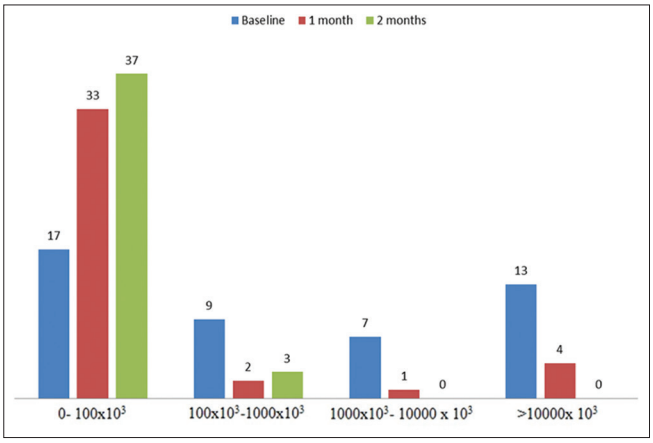
P. gingivalis	Baseline (%)	1 month (%)	2 months (%)	*P*
0-100×10^3	17 (40.5)	33 (82.5)	37 (92.5)	<0.001**
100-1000×10^3	9 (21.4)	2 (5)	3 (7.5)	0.045*
1000-10,000×10^3	7 (16.7)	1 (2.5)	0	0.004**
>10,000×10^3	13 (30.9)	4 (10.0)	0	<0.001**
Total	42 (100)	40 (100)	40 (100)	-
Lost to follow-up		2	2	-

Paired proportion test. *P. gingivalis=Porphyromonas gingivalis.* *$P \leq 0.001$ highly significant, **$P \leq 0.05$ highly significant

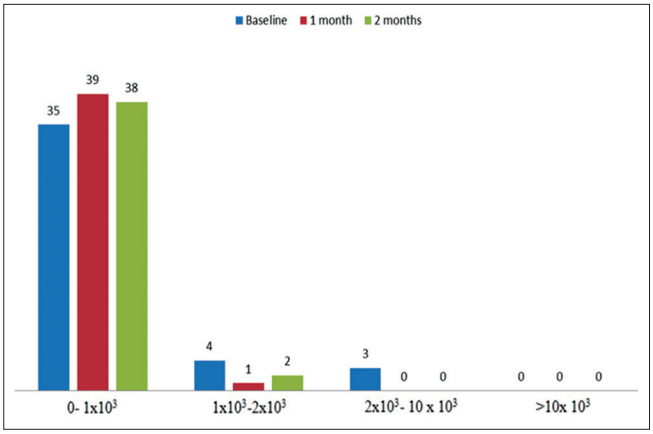
Microbiologic analysis of *P. intermedia* levels on comparison at baseline, 1 month, and 2 months showed no statistically significant results for levels at 0–1 × 10^3, 1–2 × 10^3. However, the microbial levels between 2 × 10^3 and 10 × 10^3 were observed to be moderately statistically significant. No patients had levels of the microorganism > 10 × 10^3 at baseline, 1 month or 2 months interval [Table 6 and Graph 3].



Graph 1: Polymerase chain reaction analysis of microbial samples for *Aggregatibacter actinomycetemcomitans*



Graph 2: Polymerase chain reaction analysis of microbial samples for *Porphyromonas gingivalis*



Graph 3: Polymerase chain reaction analysis of microbial samples for *Prevotella intermedia*

DISCUSSION

A particular concern when evaluating the probiotic effects on periodontal disease relates to the means of administration of these bacteria. Generally, probiotics are delivered in dairy products (mainly fermented milk), food supplements in tablet forms, or in soft drinks. However, these routes of administration cannot provide prolonged contact with oral tissues, facilitating probiotic adhesion to saliva-coated surfaces.

In our study, the probiotic drink was administered once daily, in the morning, half an hour after food intake. The

Table 6: Polymerase chain reaction analysis of microbial samples for *Prevotella intermedia*

P. intermedia	Baseline (%)	1 month (%)	2 months (%)	*P*
0-1×10³	35 (83.3)	39 (97.5)	38 (95)	0.285
1-2×10³	4 (9.5)	1 (2.5)	2 (0.5)	0.228
2-10×10³	3 (7.1)	0	0	0.044*
>10×10³	0	0	0	-
Total	42 (100)	40 (100)	40 (100)	-
Lost to follow-up	0	2	2	

Paired proportion test. *P. intermedia*=*Prevotella intermedia* *$P \leq 0.001$ highly significant

delay in the use of the probiotic drink after food intake was to facilitate the removal of any remnant adherent food particles by the washing off effect of the saliva and gingival crevicular fluid. The subjects were asked to swish the probiotic milk all around the mouth as much as possible before swallowing. This was done to facilitate contact of the probiotic drink with as many surfaces of the oral cavity, as much as possible.

To date, no studies have been performed to investigate the concentration of probiotic bacteria in the specific means of administration. It is generally acknowledged that to be effective in the gastrointestinal tract, the concentration of bacteria in the delivery system should not be lower than 10^6 colony forming units (CFU)/ml, but there are no studies to investigate the minimum concentration of probiotic microorganisms to be effective in the oral cavity.

In our study, the probiotic milk contained 6.5 billion/65 ml bottle (concentration of 10^8 CFU/ml) of *L. casei* strain *Shirota*, which is much higher than the minimum concentration required, for it to be effective in the gastrointestinal tract.

No statistically significant changes were observed in the GI and PI scores, during 1 month and 2 months intervals, when compared to baseline. This result is comparable to the outcome of a study conducted by Staab *et al.*,[25] but not in accordance to other previous studies, which showed statistically significant reductions in bleeding and plaque accumulation, after the administration of the probiotics, *Lactobacillus reuteri*,[6] and *L. reuteri prodentis* formulations.[26]

The reason why there was no reduction in the clinical parameters may be attributed to the study design, which was noninterventional. Further studies are required, using *L. casei*, with other conventional nonsurgical treatment modalities such as scaling and root planing, to check whether it may result in a clinical and statistically significant reduction of the clinical parameters, such as bleeding on probing and plaque reduction.

The microbial assessment of levels of *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* before, during and after the use of the probiotic drink was done using multiplex PCR.

The assessment of levels of *A. actinomycetemcomitans*, showed no statistically significant reduction when the levels of the microorganism were between 0×10^3 and 10×10^3. A moderately statistically significant reduction of the levels was seen, when the levels of the organism was $>10 \times 10^3$ ($P = 0.023$). These results are not concurrent with the previous studies, which showed statistically significant reduction in the levels of periodontopathic microorganisms after the use of probiotics. A study conducted by Mayanagi *et al.*, in Japan, using *Lactobacillus salivarius* WB21, showed that oral administration of probiotic lactobacilli reduced the numerical sum of live periodontopathic microorganisms and could contribute to the beneficial effects on periodontal conditions.[27] Another study by Matsuoka *et al.*, reported that various strains of lactobacilli, including *L. casei* and *L. salivarius*, have antimicrobial activity on a majority of the oral pathogens and the majority of lactobacilli suppressed the growth of *A. actinomycetemcomitans, P. gingivalis, P. intermedia*, and *S. mutans*.[16,17]

The assessment of levels of *P. gingivalis* showed a highly statistically significant reduction in the levels of the microorganism after use of the probiotic drink, during the 1 month and 2 months recall, compared to baseline levels, in all the groups ($0–100 \times 10^3$, $100–1000 \times 10^3$, $1000–10,000 \times 10^3$, $>10,000 \times 10^3$). These results are comparable to the previous studies, which showed statistically significant reduction in the levels of periodontopathic microorganisms after the use of probiotics.[16,17,27]

The assessment of levels of *P. intermedia* showed no statistically significant reduction when the levels of the microorganism were between 0×10^3 and 2×10^3. Moderately statistically significant reduction in the levels were seen, when the levels of the organism were $2–10 \times 10^3$. These results are not in accordance to the previous studies, which showed statistically significant reduction in the levels of periodontopathic microorganisms after the use of probiotics.[16,17,27]

Apart from the parameters checked for our study, a few of the study subjects also reported an improvement in appetite as well as bowel movements, during the study period. This may be attributed to the effects of *L. casei* on the gastrointestinal flora. No adverse reactions were reported by any patient or observed by the clinician during the study period. This has once again proved the safety of the probiotic drink Yakult®™, Yakult Danone India Pvt. Ltd., containing *L. casei* strain *Shirota*.

Considering the beneficial effects of probiotics, use of this probiotic drink for therapy could serve as a useful adjunct/

alternative along with conventional periodontal therapy. Further studies are required in this direction.

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Nil.

Conflicts of interest
There are no conflicts of interest.

REFERENCES

1. Metchnikoff II. The Prolongation of Life: Optimistic Studies. New York: Springer Publishing Company; 2004. p. 97-132.
2. Goldman E, Green LH. Practical Handbook of Microbiology. 2nd ed. Florida: CRC Press; 2009. p. 309-38.
3. Schrezenmeir J, de Vrese M. Probiotics, prebiotics, and synbiotics – Approaching a definition. Am J Clin Nutr 2001;73 2 Suppl: 361S-4S.
4. Fuller R. Probiotics in man and animals. J Appl Bacteriol 1989;66:365-78.
5. Näse L, Hatakka K, Savilahti E, Saxelin M, Pönkä A, Poussa T, *et al.* Effect of long-term consumption of a probiotic bacterium, *Lactobacillus rhamnosus* GG, in milk on dental caries and caries risk in children. Caries Res 2001;35:412-20.
6. Caglar E, Cildir SK, Ergeneli S, Sandalli N, Twetman S. Salivary mutans streptococci and lactobacilli levels after ingestion of the probiotic bacterium *Lactobacillus reuteri* ATCC 55730 by straws or tablets. Acta Odontol Scand 2006;64:314-8.
7. Twetman S, Derawi B, Keller M, Ekstrand K, Yucel-Lindberg T, Stecksen-Blicks C. Short-term effect of chewing gums containing probiotic *Lactobacillus reuteri* on the levels of inflammatory mediators in gingival crevicular fluid. Acta Odontol Scand 2009;67:19-24.
8. Cutler CW, Jotwani R. Dendritic cells at the oral mucosal interface. J Dent Res 2006;85:678-89.
9. Grimaudo NJ, Nesbitt WE. Coaggregation of *Candida albicans* with oral *Fusobacterium* species. Oral Microbiol Immunol 1997;12:168-73.
10. Kang MS, Kim BG, Chung J, Lee HC, Oh JS. Inhibitory effect of *Weissella cibaria* isolates on the production of volatile sulphur compounds. J Clin Periodontol 2006;33:226-32.
11. Iyer C, Kosters A, Sethi G, Kunnumakkara AB, Aggarwal BB, Versalovic J. Probiotic *Lactobacillus reuteri* promotes TNF induced apoptosis in human myeloid leukemia-derived cells by modulation of NF-kappaB and MAPK signalling. Cell Microbiol 2008;10:1442-52.
12. Yan F, Polk DB. Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. J Biol Chem 2002;277:50959-65.
13. Mennigen R, Nolte K, Rijcken E, Utech M, Loeffler B, Senninger N, *et al.* Probiotic mixture VSL#3 protects the epithelial barrier by maintaining tight junction protein expression and preventing apoptosis in a murine model of colitis. Am J Physiol Gastrointest Liver Physiol 2009;296:G1140-9.
14. Ishikawa H, Aiba Y, Nakanishi M, Ohhashi Y, Koga Y. Suppression of periodontal pathogenic bacteria in the saliva of humans by the administration of *Lactobacillus salivarius* TI 2711. J Jpn Soc Periodontol 2003;45:105-12.
15. Krasse P, Carlsson B, Dahl C, Paulsson A, Nilsson A, Sinkiewicz G. Decreased gum bleeding and reduced gingivitis by the probiotic *Lactobacillus reuteri*. Swed Dent J 2006;30:55-60.
16. Matsuoka T, Sugano N, Takigawa S, Takane M, Yoshimura N, Ito K, *et al*. Effect of oral *Lactobacillus salivarius* TI 2711 (LS1) administration on periodontopathic bacteria in subgingival plaque. J Jpn Soc Periodontol 2006;48:315-24.
17. Teughels W, Newman MG, Coucke W, Haffajee AD, Van Der Mei HC, Haake SK, *et al.* Guiding periodontal pocket recolonization: A proof of concept. J Dent Res 2007;86:1078-82.
18. Shimauchi H, Mayanagi G, Nakaya S, Minamibuchi M, Ito Y, Yamaki K, *et al.* Improvement of periodontal condition by probiotics with *Lactobacillus salivarius* WB21: A randomized, double-blind, placebo-controlled study. J Clin Periodontol 2008;35:897-905.
19. Devine DA, Marsh PD. Prospects for the development of probiotics and prebiotics for oral applications. J Oral Microbiol 2009;1. Available from: http://www.journaloforalmicrobiology.net/index.php/jom/article/view/1949. [Last accessed on 2013 Oct].
20. Loe H, Silness J. Periodontal disease in pregnancy. I. Prevalence and severity. Acta Odontol Scand 1963;21:533-51.
21. Silness J, Loe H. Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal condtion. Acta Odontol Scand 1964;22:121-35.
22. Santangelo R, D'Ercole S, Graffeo R, Marchetti S, Deli G, Nacci A, *et al.* Bacterial and viral DNA in periodontal disease: A study using multiplex PCR. New Microbiol 2004;27:133-7.
23. D'Ercole S, Piccolomini R, Capaldo G, Catamo G, Perinetti G, Guida L. Effectiveness of ultrasonic instruments in the therapy of severe periodontitis: A comparative clinical-microbiological assessment with curettes. New Microbiol 2006;29:101-10.
24. Suresh K, Chandrashekara S. Sample size estimation and power analysis for clinical research studies. J Hum Reprod Sci 2012;5:7-13.
25. Staab B, Eick S, Knöfler G, Jentsch H. The influence of a probiotic milk drink on the development of gingivitis: A pilot study. J Clin Periodontol 2009;36:850-6.
26. Vicario M, Santos A, Violant D, Nart J, Giner L. Clinical changes in periodontal subjects with the probiotic *Lactobacillus reuteri* Prodentis: A preliminary randomized clinical trial. Acta Odontol Scand 2013;71:813-9.
27. Mayanagi G, Kimura M, Nakaya S, Hirata H, Sakamoto M, Benno Y, *et al.* Probiotic effects of orally administered *Lactobacillus salivarius* WB21-containing tablets on periodontopathic bacteria: A double-blinded, placebo-controlled, randomized clinical trial. J Clin Periodontol 2009;36:506-13.