

ORIGINAL RESEARCH

Potential Antagonism Of Periopathogens: A Pilot Study To Decipher The Cryptic World Of Probiotics

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ABSTRACT:

Introduction: Several hypotheses have been proposed to elucidate the mechanism of action of probiotics. This study is aimed at validating these proposed hypotheses and in turn to determine the efficacy of three different commercially available probiotics and establish their plausible role as an adjunct to conventional periodontal therapy.

Methods: Subgingival plaque samples from periodontitis patients were collected using sterile paper points. These samples were anaerobically cultured and growth inhibition of the periodontopathogens by probiotics was assessed using disc diffusion method. Additionally, plaque biofilm formation in presence of probiotics was examined using scanning electron microscope (SEM).

Results: Evidence obtained in this in-vitro study validated two out of three hypotheses by which probiotics work. Validated hypotheses are 1) Probiotics adversely affecting growth of pathogens (by competitive inhibition) and 2) Probiotics actively limiting pathogens' ability of adhesion, colonization and biofilm formation on tissue surfaces. Probiotics demonstrated antagonistic effects towards gram-negative periodontopathogens. Additionally SEM study revealed plaque biofilm inhibition in presence of probiotics.

Conclusions: This in-vitro study indicated that growth of periodontopathogens can be restricted by probiotics and these observations corroborate the therapeutic potential of probiotics as an adjunct in treatment of periodontitis.

KEY WORDS: Antiplaque agent; Microbiology, Periodontitis; Probiotics

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INTRODUCTION

The term "probiotic" stems from the Greek word which means "for life."¹ During beginning of 20th century, Ukrainian bacteriologist and Nobel laureate Elie Metchnikoff laid down the scientific foundation for concept of probiotics. Since then probiotics are being explored extensively for its health benefits. Recently, in 2001 WHO defined "probiotics" as living organisms, principally bacteria which when administered in adequate amounts confer a beneficial health effect, beyond the basic nutrition to the host.⁴

Probiotics are broadly classified in two genera:

1. Lactobacillus:
 - *L. acidophilus*,
 - *L. casei*,
 - *L. rhamnosus*,
 - *L. reuteri* etc.
2. Bifidobacterium:
 - *B. bifidum*,
 - *B. longum*,
 - *B. infantis* etc.^{2,6}

Furthermore, certain strains of Saccharomyces, Streptococcus, Enterococcus and non-pathogenic strains of *E.coli*, *Clostridium butyricum* have also demonstrated probiotics properties.^{5,6} Natural sources of probiotics include raw milk and fermented dairy products such as cheese, yoghurt etc.⁶

Collectively the nutrients that probiotics require for “survival” are called prebiotics. The term synbiotic is used for the combination of probiotics and prebiotics.

Let us review the possible association between probiotics and periodontal health. Periodontitis is a multifactorial disease of tooth supporting apparatus caused due to polymicrobial colonization, release of inflammatory mediators and adaptive host immune responses. Socransky and Haffajee in 1992 and Wolff et al. in 1994 stated that mere presence of pathogens is not sufficient for occurrence and progression of disease activity. An active periodontal disease is characterised by three prime factors i.e. presence of susceptible host; presence of pathogenic bacteria; reduction in number of so called “beneficial” or “interacting” bacteria which impede disease progression.^{7,8}

Probiotics have already been proven advantageous in restoring natural microflora in treatment of several gastrointestinal diseases but their use in periodontal diseases is a relatively newer modality³. Adjunctive treatment of periodontal diseases has moved from elimination of non-specific bacteria by use of antibiotic/antimicrobial agents to maintenance of the health promoting bacterial ecology by probiotics. In recent times, where the overzealous use of antibiotics is leading to widespread emergence of bacterial resistance, the concept of probiotic therapy can provide a natural and safe option in combating periodontal diseases.

As far as probiotics are concerned, the core question is “how do they exert their beneficial effects?” Several hypotheses have been proposed pertaining to their mechanism of action.^{2,3,6,10,11}

1. Adversely affect growth of pathogens (by competitive inhibition)
2. Producing antimicrobial metabolites that would inhibit growth of pathogens
3. Actively limiting pathogens’ ability of adhesion, colonization and biofilm formation on tissue surfaces
4. Affecting pathogens’ ability to produce virulence factors
5. Modulating the host response

Aim of the present study is to validate these proposed mechanisms. In this in-vitro study, we have focused on first three hypothesis listed

above. Rest of the hypotheses are not in the scope. In conjunction with primary aim, we also tested the efficacy of three different commercially available probiotics products. This study was needed to understand the mechanism by which probiotics exert their therapeutic actions.

MATERIAL AND METHODS:

Patient Selection:

From December 2013- January 2014, patients who reported to the department of Periodontology at MGVS KBH Dental College and Hospital, Nashik and diagnosed with generalised chronic periodontitis were selected. Out of which twelve patients met the inclusion criteria (Fig.1). All subjects were in good medical health. None of them had received any antibiotic and/or probiotic therapy in the last three months prior to recruitment in the study. Those excluded were patients with diabetes mellitus or history of smoking. Patients who were immunocompromised, taking any steroids, chemotherapy, radiotherapy at the time of study were also not included.

Sample Collection

For anaerobic culture, 6ml sterile test tubes (six in number) were filled up to 3/4th of its volume with Robertson’s cooked meat (RCM) transport media[‡].

For biofilm assessment, fourteen freshly extracted human teeth were taken and scaled. Each one suspended using silk suture in 10 ml sterile test tubes (fourteen in number). They were filled up to 3/4th of its volume with Nutrient broth with 1% glucose[‡]. All these media were prepared according to manufacturer’s instructions and sterilized using steam under 15 psi pressure at 121° C for 20 minutes.

Informed consent was obtained from all the patients. Three sites with probing depth ≥6mm per patients were selected for sample collection. All the sites were scaled to remove supragingival deposits and isolated using sterile cotton roles. Subgingival samples of dental plaque were collected from the target sites using No. 30 sterile

paper points. The paper point was inserted into each periodontal pocket and kept approximately for twenty seconds.⁷ Upon removal, the samples from six patients were pooled in separate test tubes containing RCM media for anaerobic culture. Samples from remaining six patients were pooled in test tubes containing nutrient broth with 1% glucose for biofilm assessment of which six were done at 24 hours and six at 72 hours. RCM media and nutrient broth were used to transport respective samples to Dept. of

microbiology with minimal time lag and they were processed immediately.

Probiotic products

Three different commercially available probiotic products were chosen. They were labelled as Probiotic-1[§], Probiotic-2[‡] and Probiotic-3^{††}. Content of these probiotic samples are given in table 1.

Probiotic-1	Probiotic-2	Probiotic-3
<ul style="list-style-type: none"> × Lactobacillus acidophilus (0.48 billion) × Lactobacillus rhamnosus (0.48 billion) × Bifidobacterium longum (0.48 billion) × Bifidobacterium bifidum (0.48 billion) × Sacchromyces boulardii (0.10 billion) × Streptococcus thermophilus (0.48 billion) + Fructo-Oligo saccharides (300 mg) 	<ul style="list-style-type: none"> × Streptococcus faecalis T-110 JPC (30 million) × Clostridium butyricum TO-A (2 million) × Bacillus mesentericus TO-A JPC (1 million) × Lactobacillus sporogeneus (50 million) 	<ul style="list-style-type: none"> × Lactobacillus rhamnosus Gr-1 (1 million) × Lactobacillus reuteri RC-14 (1 million)

Table 1. Contents of three different commercially available probiotic products selected for the study

Sample inoculation(Fig 1)

For Hypothesis I (Adversely affecting growth of pathogens)

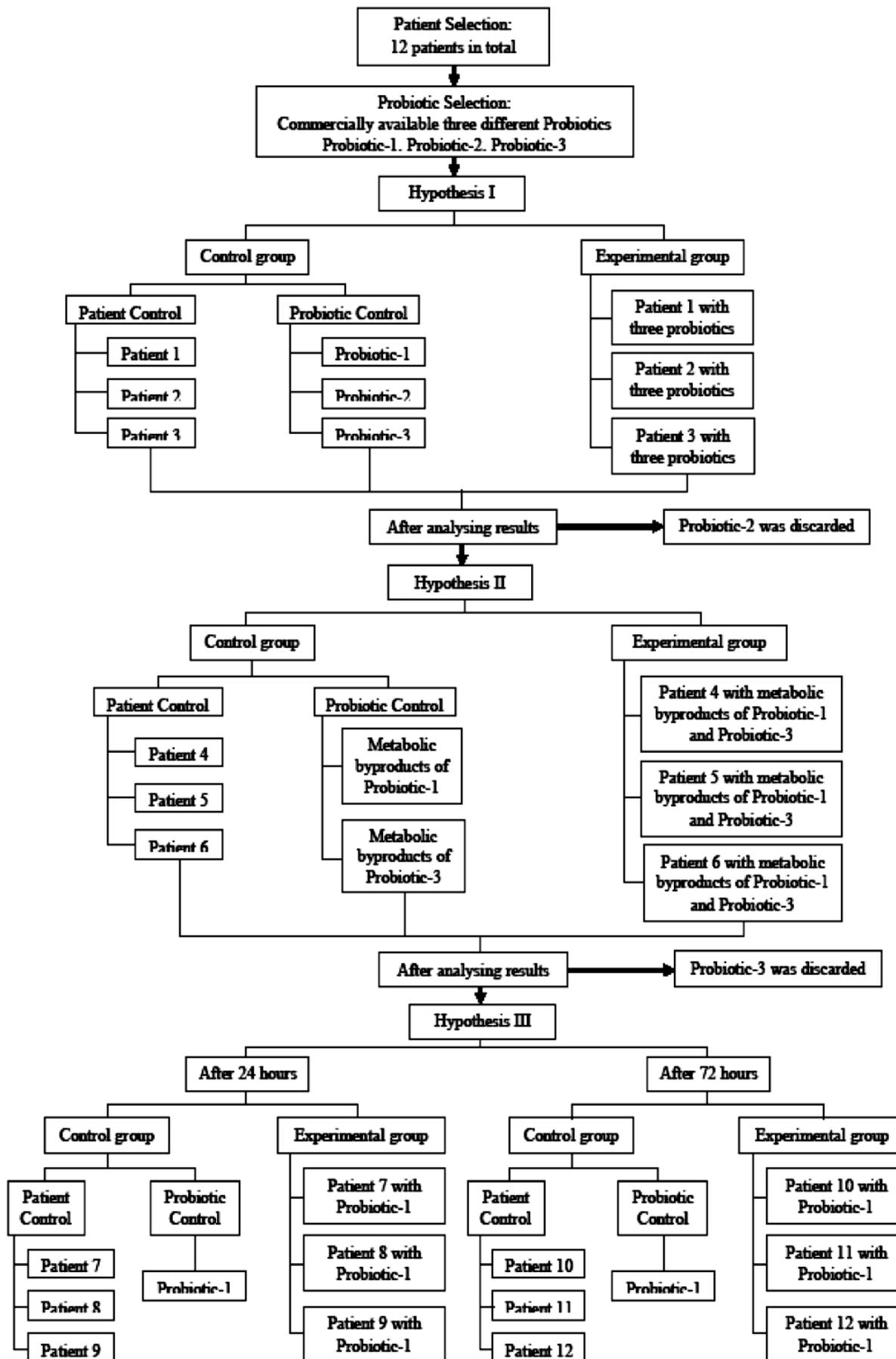
To check viability of samples collected, patients' samples were grown in RCM for 4-6 hours. Development of turbidity confirmed their viability. Five sterile blood agar plates were taken. Three blood agar plates were inoculated with three different patient samples by spread plate to obtain lawn culture. Simultaneously, capsules of selected probiotics were then dissolved in three separate test tubes containing 1 ml of sterile saline. To check the growth inhibition by diffusion method, three pre-sterilized non-impregnated paper discs[‡] were then immersed in each probiotic solution and placed over already inoculated blood agar plate. Procedure was repeated for remaining two plates. In total nine paper discs (three discs per plate)

were used. Additionally, two blood agar plates were used as control. One inoculated with patient's samples only and one with probiotic samples.

For Hypothesis II (Producing metabolic/enzymatic by-products that would passively inhibit growth of pathogens)

Probiotic-2 was dropped in testing this hypothesis as it was found to be comparatively ineffective during 1st of part of the study.

Probiotic samples 1 and 3 were grown in two separate test tubes containing 40ml of nutrient broth with 1% glucose for 72 hours. After 72 hours; they were filtered using Milipore membrane filter (0.45 μ)[‡] to collect their metabolites. For this experiment, instead of probiotics, their culture filtrates were used. Two pre-sterilized non-impregnated paper discs were then immersed in each probiotic filtrate solution



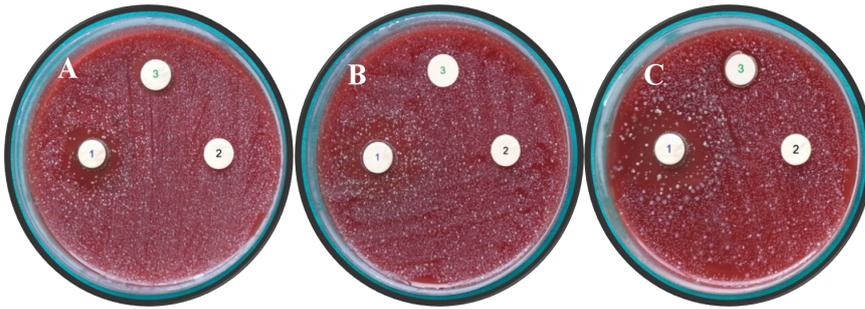


Figure - 2: Growth inhibition around probiotic samples using disc diffusion method. A) Patient sample 1, B) Patient sample 2, C) Patient sample 3 and 1) Disc impregnated with Probiotic-1, 2) Disc impregnated with Probiotic -2 and 3) Disc impregnated with Probiotic-3

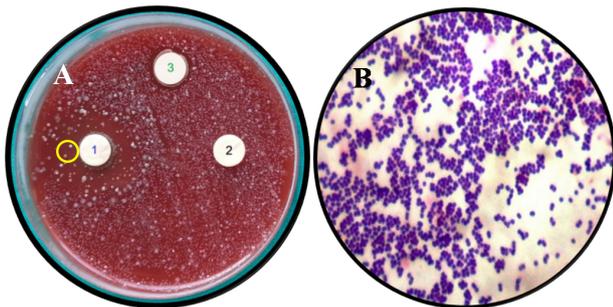


Figure-3: A) Smear obtained around Probiotic-1 indicated by yellow circle B) Smear area under light microscope (100X) after gram staining. No gram negative rod shaped bacteria were observed around

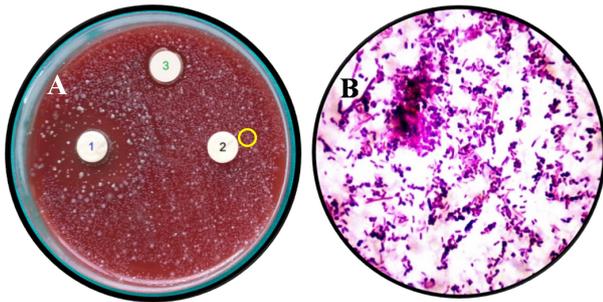


Figure-4: A) Smear obtained around Probiotic-2 indicated by yellow circle B) Smear area under light microscope (100X) after gram staining.

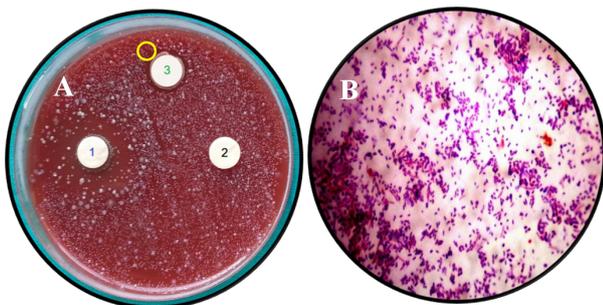


Figure-5: A) Smear obtained around Probiotic-3 indicated by yellow circle B) Smear area under light microscope (100X) after gram staining

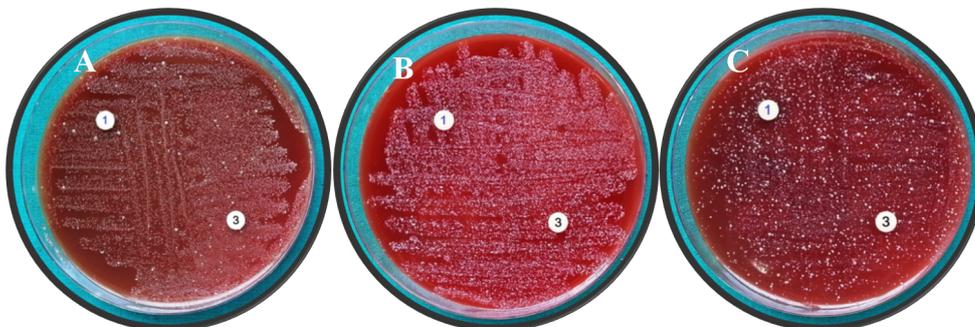


Figure-6: A) Patient sample 1, B) Patient sample 2, C) Patient sample 3 and 1) Disc impregnated with metabolite filtrate of Probiotic-1, 3) Disc impregnated with metabolite filtrate of Probiotic -3

and placed over already inoculated blood agar plate with patient's sample. Separate plate was used for three patients. Additionally, two blood agar plates were used as control. One inoculated with patient's samples to check viability and one with probiotic filtrate samples to confirm absence of bacteria.

In both the procedures, following inoculation, all the plates were incubated in an anaerobic jar at 37° C for 48 hours.

For Hypothesis III (Actively limiting pathogens' ability of adhesion, colonization and biofilm formation on tissue surfaces)

Probiotic-3 was eliminated from validation of Hypothesis III since Probiotic-1 was consistently more effective than Probiotic-3 in both earlier tests.

Probiotic-1 was dissolved in 4ml of normal saline. This solution was then distributed using graduated pipette in four test tubes. These test tubes contained nutrient broth with 1% glucose solution along with a human tooth. Further three out of four were inoculated with patients' sample. Remaining one test tube was used as control (to assess the biofilm formation by probiotic only). Simultaneously, three test tubes with nutrient broth with 1% glucose solution were inoculated with patients' sample without Probiotic-1. These test tubes were incubated at 37° C for 24 hours. Similar procedure was repeated for all the other samples and this set of test tubes was then incubated at 37° C for 72 hours.

After incubation all the teeth specimens were retrieved and kept in sterile container containing 1% glutaraldehyde and 4% formaldehyde at 4°C for 24 hours. Post 24 hours fixation, all the specimens were washed with phosphate buffer saline (PBS), dried and dehydrated in an increasing concentration of ethanol (50% followed by 100%) at 10 minutes interval. Each specimen was then removed and air-dried at room temperature. This was done as per the procedure followed in an earlier study.¹² Later all the specimen were examined using scanning electron microscope^v for biofilm formation.

Microbial analysis

For microbial analysis, sites next to Probiotic-1, 2 and 3 and probiotic filtrate-1 and 3 were selected to confirm Hypothesis I and II respectively. Smears were prepared using sterile wire loop and gram staining was performed. Samples from all the control blood agar plates were also collected in similar fashion.

pH analysis

Probiotic-1 provided compelling evidence in validating all three hypotheses, hence pH analysis was carried out using Probiotic-1. A capsule of probiotic-1 was dissolved in 14ml of sterile saline. This solution was equally distributed to 14 sterile test tubes, each containing 40ml of nutrient broth with 1% glucose. All these test tubes were kept in an incubator till further analysis. The pH was checked for each of the sample at a 2 hour interval (approximately) over the period of 24 hours using a calibrated pH meter[#].

RESULTS

For Hypothesis I

Probiotic-1 showed maximum inhibition, 2 showed none while 3 showed mild inhibition (Fig.2). Hence Probiotic-2 was excluded from further analysis. Microbial analysis also confirmed the above observations.

Microscopy revealed even distributions of gram positive coccoid shaped microorganisms and significant reduction in number of gram negative rod shaped bacteria (short and long bacilli suggestive of fusobacterium) around Probiotic-1 (Fig. 3). Reduction in gram-negative rod shaped bacteria was not significant for rest two probiotic samples (Fig 4,5).

Thus this strongly indicates that Hypothesis I holds true and Probiotic sample-1 is most effective in antagonising periodontopathic bacteria.

For Hypothesis II

Culture filtrates, when tested for antimicrobial activity did not show any inhibition of growth

(Fig 6). On microbial analysis, there was no appreciable reduction in gram-negative rod shaped bacteria (Fig 7). This contradicts a premise that metabolic/enzymatic by products passively inhibit the growth of periodontopathic bacteria.

For Hypothesis III

Periopathogens in presence of probiotics were unable to form plaque biofilm either at 24 or at 72 hours.

At 24 hours, in absence of probiotics, plaque microorganisms formed a 5.005μ thick biofilm at 1000X magnification (Fig 8). Contrastingly in the presence of probiotics, at 1000X magnification, specimens displayed relative clean surface without any biofilm formation (Fig 9).

Likewise after 72 hours, in absence of probiotics, plaque microorganisms formed 13.08μ thick

biofilm when viewed at 1000X (Fig 10). The higher SEM magnification (3000X) revealed that these microorganism colonies accumulated in multiple layers covering most of the specimen surfaces. The bacteria were found to be clustered as towers of cocci indicating a possible corn-cob appearance. Few independent rod shaped bacteria could also be visualized (Fig 11). However in presence of probiotics, SEM revealed significant reduction in biofilm formation at both lower and higher magnifications. At few sites, isolated flecks of coccoid shaped bacteria could be seen on the surface (Fig 12). This is strong evidence in favour of Hypothesis III.

For pH transition

pH analysis revealed that it gradually decreased over the period of 24 hours. It reached the critical value of 5.5 in 10.5 hours (Fig 13).

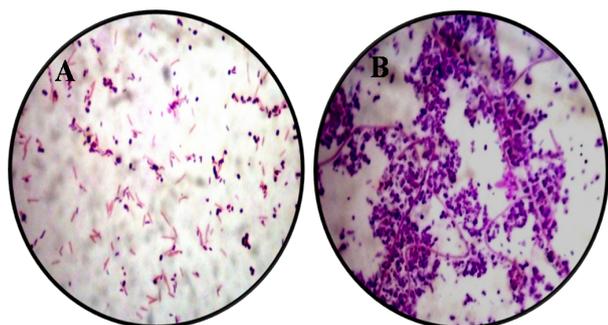


Figure 7: A) Smear around filtered metabolites of Probiotic-1 under light microscope (100X) after gram staining B) Smear around filtered metabolites of Probiotic-3 under light microscope (100X) after gram staining.

DISCUSSION

Teughels W et al. in 2011 reviewed 14 different studies (3 animal and 11 in vivo human studies). Most of these demonstrated varying results, since different strains of probiotics were used without determining their efficacy. Many of these were pilot studies and with low quality. Thus authors recommended that properly conducted clinical trials should be carried out using probiotic strains with in-vitro proven beneficial periodontal effects.¹³ There is a very limited evidence-based data as far as postulated mechanism by which probiotics function. Therefore, prior to conducting a clinical trial, we decided to carry an in-vitro study to test the validity of proposed mechanisms and evaluate strains of the probiotics that are best suited for oral use.

In periodontal diseases, there is an increase in obligate and facultative anaerobic gram-negative rod-shaped bacteria. *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans* etc. are few examples of gram-negative bacteria commonly associated with periodontal diseases.^{14,15,16,19} In this study we have demonstrated an active inhibition conferred by probiotics towards these type of periodontal pathogens and the results are in agreement with the previous studies.^{17,18} On microbial analysis, Probiotic-1 showed a significant reduction in gram-negative rod shaped bacteria as compared to rest of the two products. The difference in inhibitory action could be due to difference in

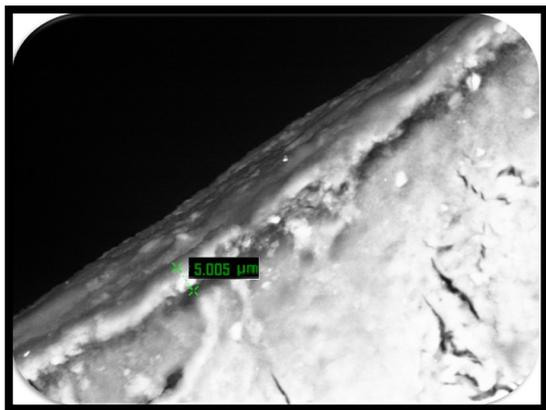


Figure-8: 5.005μ thick plaque biofilm formation in absence of probiotics post 24 hours (1000X magnification)

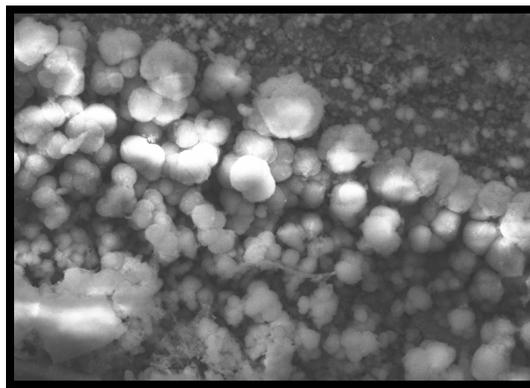


Figure-11: Coccoid and rod shaped bacteria within the biofilm formed in absence of probiotics post 72 hours (3000X magnification)

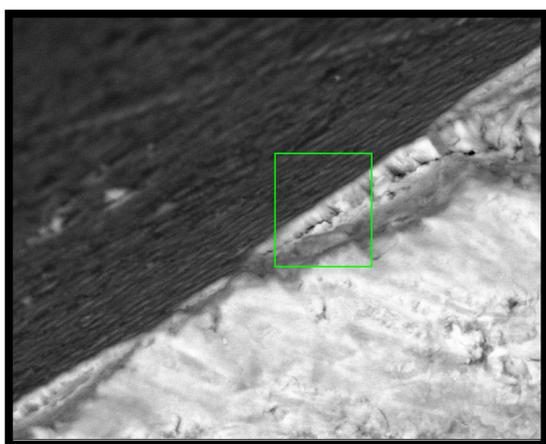


Figure-9: No visible plaque biofilm in presence of probiotics post 24 hours (1000X magnification)

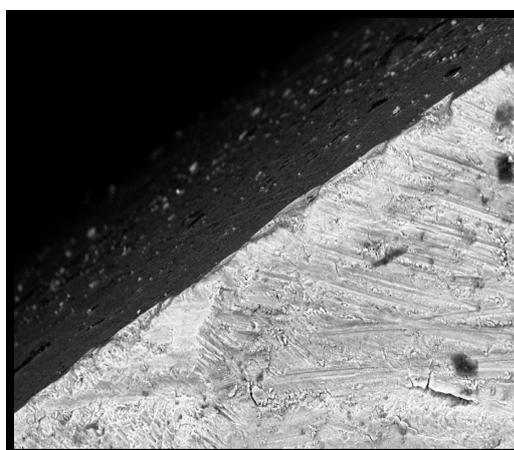


Figure-12: Isolated flecks of coccoid shaped bacteria seen on the surface of teeth in presence of probiotics post 72 hours (1000X magnification)

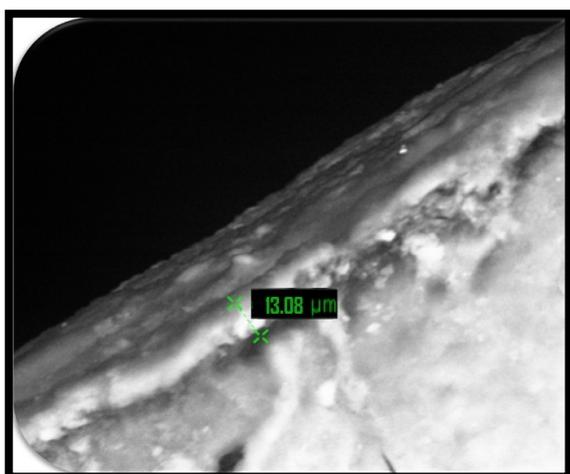


Figure-10: 13.08 μ thick plaque biofilm formation in absence of probiotics post 72 hours (1000X magnification)

composition of the probiotics. There was no evident growth inhibition of periodontal pathogens by the metabolic by-products of probiotics. This is not in agreement with the other studies conducted.^{7,19,20,25} However most of these studies were conducted on gastrointestinal bacteria. There are very few studies which target periodontal pathogens.²¹ The plausible explanation to this is a likely cell-to-cell, biochemical or molecular level interaction between probiotics and pathogens. Nevertheless this needs to be investigated further at molecular level using more superior and sophisticated techniques. If proven correct, the extracted by-products of probiotics can themselves be used instead of probiotics, proving to be a novel and safe approach bypassing the side effects (pH variation) of probiotics. The most interesting aspect of the study is inhibition of plaque biofilm

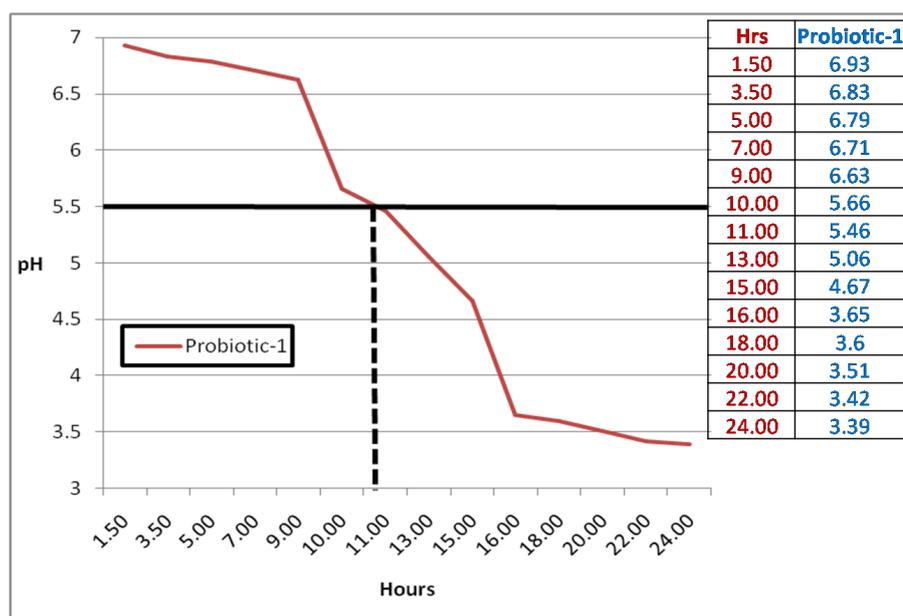


Figure-13: This is a graph of pH transition with respect to time. X-axis is showing hours at approximately 2 hours interval and Y-axis is depicting pH.

formation. In our study we were able to demonstrate the beneficial aspect of probiotics as an anti-plaque agent. So far to our best knowledge there are no related studies which evaluate the biofilm-inhibitory action of probiotics towards periodontal pathogens in particular.

Challenges in future in-vivo study:

Usually probiotics are delivered systemically as dairy products (mainly fermented milk) or in tablet forms. However these routes of administration cannot provide prolonged contact with oral tissues, hampering its adhesion to desired sites. Thus designing an appropriate route of administration as a local drug delivery system in the form of lozenge or chewing gum or mouthwash is a need of hour. Indeed property of substansivity for probiotics to be developed.

Probiotics are viable organisms, and therefore it is feasible to think that they could infect the host. However when ingested orally, probiotics are generally considered safe and well tolerated.^{7,10} Recently, major and minor risk factors for probiotics-associated sepsis have been identified.^{22,27} Thus it is recommended that probiotics should be used cautiously in patients with one major risk factor or more than one minor risk factor.²⁷

Another challenge to be addressed is potential

transition of pH from basic to acidic by probiotic bacteria. A number of probiotic lactobacilli and bifidobacteria have shown to produce acid from fermentation of dietary sugars in vitro.^{23,24} Production of this acid is often thought to be an important component of their protection against pathogenic colonisation. However the same acid can initiate demineralization of tooth substance which manifest clinically as enamel/root caries.^{23,24} To assess the time required for the pH of probiotic solution to drop below the critical level of pH (5.5)²⁶ pH analysis was performed. pH was falling below the critical value (5.5) after around 10.5 hours (Fig. 13). It is crucial in the context that modes of delivery are to be developed to provide sufficient retention and exposure time in the mouth with an appropriate clearance time. However in a dynamic oral environment, one has to take into consideration few other factors like salivary buffers, cleansing action of saliva and neutralizing products from other bacteria.

Limitations:

Sample size of the present study was small. Results obtained by this in-vitro study need to be tested and validated with larger sample size.

Isolation and identification of periodontopathogenic and probiotic bacteria was not possible due to inaccessibility to such facility.

It is an in-vitro study which is carried out in a static environment. Results might defer during clinical trial using same probiotic strains under dynamic oral conditions.

CONCLUSIONS

Within the limitation of study, preliminary data showed that probiotics do have a potential to antagonize the growth of periodontal pathogenic bacteria. However their metabolic by-products demonstrated no inhibition towards periodontal pathogens. This simply means probiotics need to have cell-to-cell molecular level interaction with pathogenic bacteria to confer their beneficial effects. Inhibition of plaque biofilm by probiotics was the most striking finding among all. It opens up a whole new avenue for probiotics as an anti-plaque agent.

These beneficial effects of probiotics on periodontal health and its maintenance including modes of administration, dosage and safety aspects need to be assessed through numerous randomized long-term clinical trials.

FOOTNOTES

‡ HiMedia Laboratories Pvt. Ltd., Mumbai, India

§ Bion, Merck KGaA, Darmstadt, Germany

¥ Bifilac, TOA Pharmaceutical Co. Ltd., Tokyo, Japan

†† Ecoflora, Chr. Hansen, DK-2970 Hørsholm, Denmark

ψ EVO 50, Zeiss, Oberkochen, Germany

HI 3512, Hanna Instruments, Mumbai, India

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