Original Research Article

Anti-bacterial efficacy of Probiotics used in provisional polymethyl methacrylate crowns against *Porphyromonos gingivalis* – an In vitro study

Abstract

AIM: To evaluate the effect of Probiotic used in PMMA Temporary restorations on gingival inflammatory response by its action on the marginal gingival epithelial cells.

MATERIALS AND METHODS: This is an in vitro Interventional pilot study using discs of PMMA (Group A) and PMMA incorporated with Probiotics (Group B . The effect of probiotics incorporated PMMA on strains of Porphyromonos gingivalis grown on culture plates will be assessed using zone of inhibition test and minimum inhibitory concentration was assessed . Data was recorded, tabulated and statistically evaluated using SPSS software.

RESULT: The zone of Inhibition in the Group B was (Mean = 16.30mm) is comparatively higher than that of Group A with mean value 12.92mm. Results states that among the various concentration of Probiotic Lozenges (2.5, 5, 10, 15 and 20 µg/ml) used to determine the antibacterial activity , Highest mean zone of inhibition was observed with 20 µg/ml with 16 mm, followed by 15 µg/ml with 14mm , 10 µg/ml with 9mm, 5 µg/ml with 11 mm & with the lowest inhibition observed in 2.5 µg/ml with 8mm. There is significant correlation between concentration of probiotics and its antibacterial efficacy. The increase in concentration of probiotics in the PMMA discs is directly proportional to its antibacterial efficacy against P. gingivalis.

CONCLUSION : Thus the study proved a significant correlation between Probiotic used in PMMA Temporary crowns and reduction in gingival Inflammation at the margins of the restoration which will be beneficial when used in crowns used in patients undergoing Orthodontic treatment or Immediate Implant Loading with acrylic crowns.

Keywords : Porphyromonas gingivalis, Probiotics, antibacterial, Poly methyl methacrylate, Provisional crowns.

Comment [MO1]: Double check

Comment [MO2]: ?

Comment [MO3]: Italics

Comment [MO4]: Italics

INTRODUCTION:

In dentistry, Provisional restoration refers to a fixed or removable prosthesis designed to enhance aesthetics, stabilization and function for a limited period of time after which it is to be replaced by a definitive prosthesis.[1] Commonly used materials for provisional restorations includes Poly methyl methacrylate (PMMA) and Poly ether ether ketone (PEEK).

One of the major disadvantages of these provisional crowns is that it causes gingival inflammation/gingivitis around the soft tissue of the restoration. This ill effect is attributed to the inherent properties and surface of the PMMA material which harbours periodontal pathogens leading to biofilm formation and accumulation of plaque..[2]

Gingivitis and Periodontitis are multifactorial diseases that encompasses the hard and soft tissue through microbial colonization (with or without invasion) thereby leading to inflammatory and adaptive immune responses of the host.[3] Being a gram negative anearobe, P. gingivalis is one of the major pathogens associated with gingivitis. If left uninterveined, gingivitis can result in chronic periodontitis, a disease that initiates with inflammation of the soft tissue and progresses to destruction of alveolar bone, ultimately resulting in tooth loss.[5,6] Lipopolysaccharide (LPS), hemagglutinins, gingipains, and fimbriae accounts to the virulence factors of P. gingivalis. These factors plays a vital role in the induction of immune inflammatory responses and resorption of alveolar bone.[7]

Comment [MO5]: Italics

Comment [MO6]: Confirm spelling

Comment [MO7]: Italics

The term “Probiotic”, as opposed to “antibiotic”, was initially proposed by Lilley and Stillwell in 1965.[14] First probiotic species to be introduced in research was Lactobacillus acidophilus by Hull et al. in 1984;[14] followed by Bifidobacterium bifidum by Holcombh et al. in 1991.[14] In 1994, the World Health Organization deemed probiotics to be the next-most important immune defense system when commonly prescribed antibiotics are rendered useless by antibiotic resistance. According to WHO the probiotics are defined as the live microorganisms which when administered in adequate amounts confer a health benefit on the host.[8] Some of the most well-studied probiotics include Lactobacillus, Bifidobacterium, and Lactococcus species that colonize the gut. These probiotics act as an adjuvant in the treatment of celiac diseases, obesity associated irritable bowel syndrome, campylobacter jejuni infection, and infant sepsis.[9,10] Commonly found in yogurts and other fermented foods, many Lactobacillus strains are Generally Recognized As Safe (GRAS) by the U.S. Food and Drug Administration (FDA) for the use in specific food productions.

Comment [MO8]: Italics

Comment [MO9]: Italics

The use of probiotic Lactobacillus and Lactococcus species is just beginning to be explored in the oral cavity. [8,11,12] Metchnikoff was the first to state that probiotics could provide a health benefit. Based on his study among Bulgarian population, he stated that probiotics in the form of fermented milk containing viable bacteria had a positive effect on the lifespan of the individuals.[13] These incidences paved way for a new concept of probiotics in medicine and dentistry.[15]

Effect of probiotic against certain oral pathogens are well established by previous studies. Probiotics induce cidal or static inhibition of growth of pathogens through production of bacteriocins.[15] Probiotics also has the ability to modify the surrounding environment by altering the pH and/or the oxidation–reduction potential, which may compromise the ability of pathogens to become established. This anti-bacterial property of Probiotic is thus used in the current study to minimise the gingival pathogens around the provisional crowns. Thus, the aim of the study is to evaluate the Antibacterial efficacy of Probiotics incorporated in chemically activated Poly methyl methacrylate discs against the periodontal pathogen Porphyromonas gingivalis.

MATERIALS AND METHOD:

Acrylic discs of 6mm diameter and 2mm thickness according to ISO standardisation is fabricated using Chemically activated Poly methyl methacrylate [PMMA]. It is to be noted that the same material is used for fabrication of provisional crowns in our day to day practice. Commercially available probiotic lozenges were used in our the study. This contained Lactobacillus casei Shirota = 6.5x106, L.sporogenes =10x106 and Bifidobacterium species = 2x 106 according to the manufacturer. The discs were separated into two groups namely A and B with 12 samples in each group. Group A is the control group that consists of discs without probiotic coating whereas group B is the test group which is to be immersed in probiotic solution for 48 hrs at 37oC.

Comment [MO10]: Double check

Comment [MO11]: Double check

Comment [MO12]: ?

The study consists of two parts:

Comparing antibacterial efficacy between Group A (Control): Discs without Probiotic [12] + Group B (Test): Discs immersed in Probiotic lozenges [12] against P. gingivalis

Evaluation of Minimum inhibitory concentration of Probiotic against P. gingivalis among 5 different concentrations (2.5, 5, 10, 15 and 20 mg/mL)

The periodontopathic bacterial strains P. gingivalis ATCC33277 was grown in half-strength brain heart infusion (BHI) broth. The bacteria are grown at 37°C in an anaerobic medium. The disc diffusion method was used to determine the antibacterial activity. The antimicrobial activity was determined using BHI agar medium supplemented with 5% defibrinated sheep blood, and the optical density (OD) of the bacterial inoculum was adjusted to 0.1 at 600 nm (0.5 McFarland standard). On a blood agar plate, the bacterial inoculum

suspension (100 µL) was swabbed uniformly. This plate was then allowed to dry for 5 min. Various concentrations of extracts (2.5, 5, 10, and 20 mg/mL) at 20 µL were loaded onto a 6 mm sterile disc. The disc loaded with extracts in both the groups, A and B (3 each /petri dish) was placed on the surface of the medium., The compound loaded was then allowed to diffuse for 5 min, and the plates were incubated at 37°C for 48 h. The inhibition zones at the end of the incubation, formed around the loaded disc were measured with a transparent ruler(in mm). This experimental study was performed in 4 petri dishes to accommodate the sample size of 12 in each group. The probiotic extract prepared in five different concentrations with dilution as follows: 2.5, 5, 10, 15 and 20 µg/mL. The PMMA discs were made as explained earlier and immersed in different concentration of probiotic for 48 hrs at 37oC and these discs were inoculated into P. gingivalis cultured plates. Incubation of 48 hrs at 37oC was carried out. The zone of inhibition found around the discs was measured using a transparent ruler. The values thus obtained from all the discs are noted and tabulated. 96-well plate microdilution method using these 5 concentrations was used to assess the Minimal inhibitory concentration (MIC). The lowest concentration at which no growth was observed was defined as MIC (µg/mL). Acquired Data was tabulated and analysed statistically.

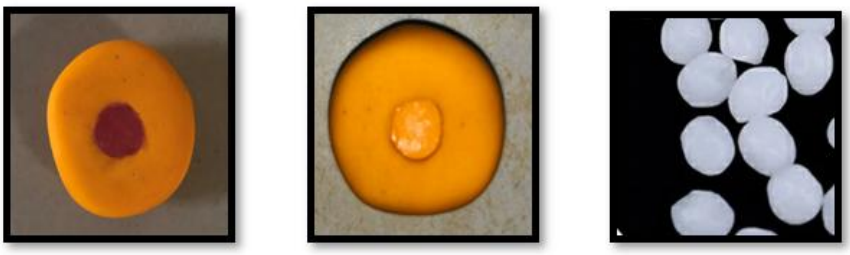


Figure 1.1 represents Procedure for fabrication of discs made up of Polymethyl methacrylate.

Figure 1.2 represents A. Culture plate with evident P. gingivalis growth and B. Acrylic disc (Both group A and B) incorporated into the culture plate.

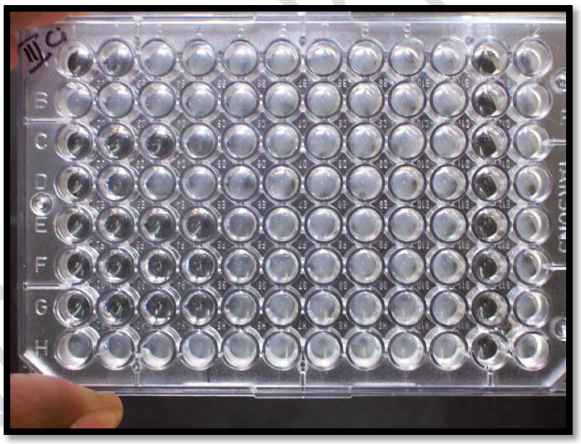


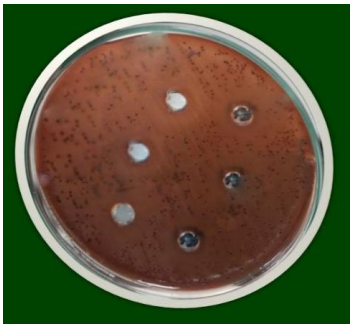
Figure 1.3 represents the 96 well Microdilution method used for assessing the minimum inhibitory concentration.

RESULTS:

Preliminary screening for antibiotic activity of probiotics against *P. gingivalis* was done using disc diffusion method. In this method, zone of inhibition represents the diameter of the area around the discs that is free of microbial growth of P. gingivalis measured in millimetres. The first part of the study results comparing the antimicrobial efficacy between two groups: One with probiotic discs and other without probiotic discs is stated in Table 1 and Figure 1.5. Results of microscopic evaluation comparing the Zone of inhibition in Group A (12) and Group B (12) suggests that the zone of inhibition around Group B mean value of 20.30mm is comparatively higher than that of Group A with mean value 12.92mm. There was growth of colonies close to the discs in Group A (PMMA discs without Probiotics) as evident in the Figure no.1.4 below.

The second part of the study compared antibacterial efficacy of various concentrations of probiotics against P. gingivalis. Results states that among the various concentration of probiotic lozenges (2.5, 5, 10, 15 and 20 µg/ml) used to determine the antibacterial activity , Highest mean zone of inhibition was observed with 20 µg/ml with 16 mm, followed by 15 µg/ml with 14mm , 10 µg/ml with 9mm, 5 µg/ml with 11 mm & with the lowest inhibition observed in 2.5 µg/ml with 8mm as shown in Figure 1.7 . There is significant correlation between concentration of probiotics and its antibacterial efficacy. The increase in concentration of probiotics in the PMMA discs is directly proportional to its antibacterial efficacy against *P. gingivalis*.

The minimum inhibitory concentration (MIC) was determined by using various concentrations ranging from 7.5 to 100 µg/ml by microdilution method and the results stated that the MIC of Probiotic against P. gingivalis was found to be 25 µg/ml.



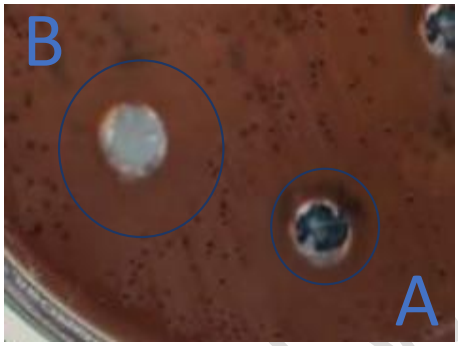


Figure 1.4 represents a. Culture plate with discs of both group A and B incorporated into it after incubation.

b. Zone of Inhibition around the Discs of Group A (without Probiotics) and Group B (With Probiotics)

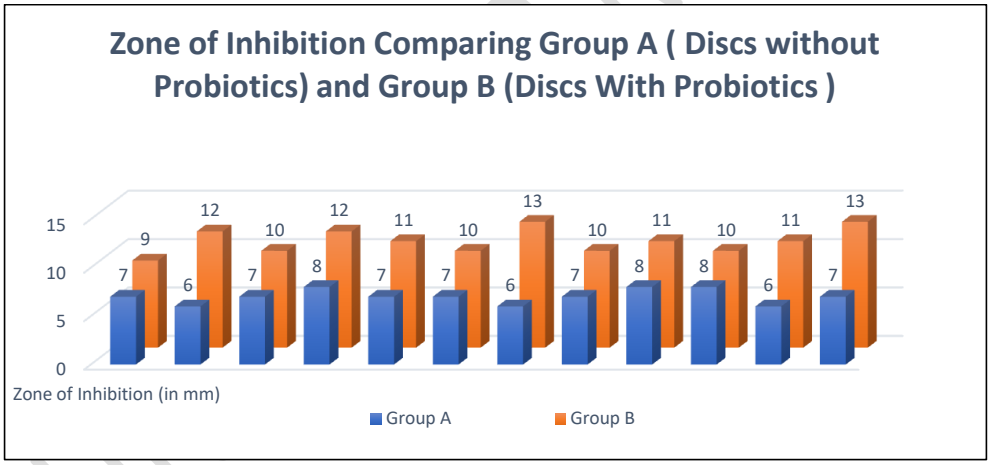


Figure 1.5 represents graphical representation of comparison of Antibacterial efficacy of Group A (discs without probiotics) and B (Discs with Probiotics) against P. gingivalis.

TABLE 1:

ZONE OF INHIBITION AROUND THE DISC (DIAMETER IN mm)													
	Disc 1	Disc 2	Disc 3	Disc 4	Disc 5	Disc 6	Disc 7	Disc 8	Disc 9	Disc 10	Disc 11	Disc 12	Mean
Group A	7	6	7	8	7	7	6	7	8	8	6	7	12.92308

Group B	9	12	10	12	11	10	13	10	11	10	11	13	20.30769

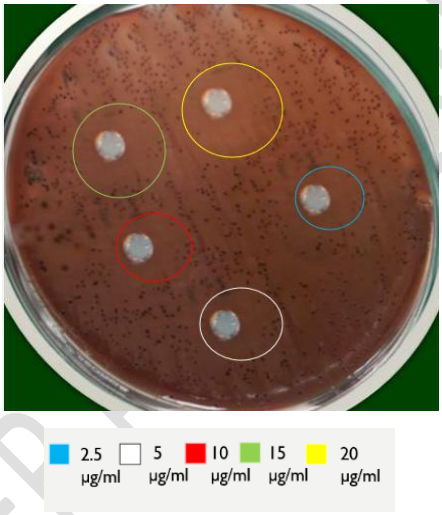


Figure 1.6 represents zone of inhibition of Probiotics in various concentrations against P. gingivalis.

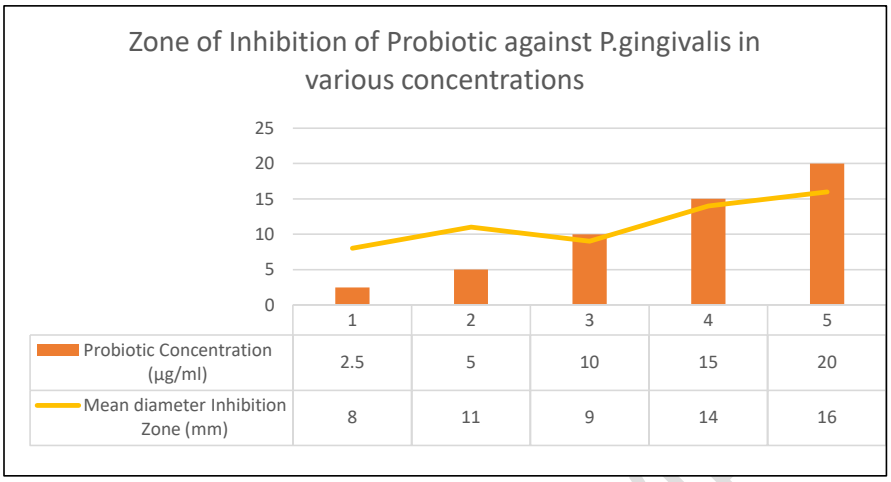


Figure 1.7 represents graphical correlation between zone of inhibition and Increasing concentration of Probiotic.

DISCUSSION:

Probiotic has been used for its gastro intestinal benefits for a long time. The effect of probiotics in promoting general health was extensively studied over the past few years. The mechanism of action of probiotic species against several other pathogens is by one or more of the following mechanisms:

a. Exclusion and competition with potential pathogens for nutrients and epithelial cell adhesion,

b. Production of antimicrobial substances against periodontal pathogenic organisms,

c. Immune-modulations, and

d. Enhancement of the mucosal barrier function.

e. Immunomodulatory action of the probiotics regulates anti-inflammatory and proinflammatory cytokine production.[16]

P. gingivalis is a Red complex pathogen and the most common bacterium involved in the onset of gingivitis and chronic periodontitis. P. gingivalis causes a microbial shift of the oral cavity, allowing for uncontrolled growth of the commensal bacterium.[17] To evade the host

immune response, P. gingivalis has many ways. These include invasion by using gingipain proteases, which is a capsular polysaccharide responsible for neutrophil recruitment.

There is evidence of gingival inflammation around the crevice of the temporary crowns made up of PMMA when they are used for more than 7 to 10 days. Goug et al., [15] in his study, found that the microbial analysis of the long-standing temporary crowns showed presence of periodontal pathogens with P. gingivalis being predominant bacterium.

There are only fewer experimental evidences exploring probiotic incorporated in PMMA temporary crown material in preventing gingival inflammation caused by P. gingivalis. Probiotic lozenges were selected as the material of choice in the present study as it contains Lactobacillus casei Shirota, Lactobacillus sporogenes and Bifidobacterium spp as the main constituents. The effect of probiotic tablets on gingivitis and different grades of periodontitis were studied by Grudianov et al., [18]. His study results suggested that probiotic treatment resulted in better microbiota normalization than control group. Krasse et al, [19] through his study suggested that, patients with moderate to severe gingivitis who were given L. reuteri formulations of probiotics, had reduced plaque and gingivitis scores compared to a placebo group. also confirmed the plaque inhibitory effect of probiotics was also proved by Vivekananda et al [20].

The anti-inflammatory effects of probiotics were also suggested by Riccia and colleagues [21] through a study. It evaluated the effect of Lactobacillus brevis in a group of patients with chronic periodontitis. A significant reduction in salivary levels of prostaglandin E2 (PGE2) and matrix metalloproteinases (MMPs) was observed in their results. The authors suggested that the beneficial anti-inflammatory effects of L. brevis is attributed to its ability to prevent the production of nitric oxide and PGE2. [21] Socransky et al. [22] stated that Lactobacillus probiotic strain has been used for its inhibitory action against periodontal pathogens through acid release. Another study by Silva et al. [23] suggests that Lactobacillus has the ability to produce different antimicrobial components. As stated by the author, these components include organic acids, hydrogen peroxide, low molecular weight antimicrobial substances, adhesion inhibitors and bacteriocins. Recently, Kõll et al. [24] characterized 22 strains of orally isolated lactobacilli to prove its antimicrobials activities on oral pathogens including pathogenic bacteria. This is in accordance with our in vitro study that suggests that, Probiotic at 20 µg/ml concentration has shown maximum antibacterial activity. Even at 2.5 µg/ml the current study proves inhibition in growth of P. gingivalis. Hence, it is clearly evident that

probiotics can serve the purpose of being an adjuvant in PMMA crowns to increase its antibacterial efficacy against P. gingivalis thus preventing gingival inflammation.

TABLE 2:

AUTHOR	YEAR	PROBIOTICS USED IN THE STUDY	STUDY STATEMENT	IN ACCORDANCE / NOT IN ACCORDANCE WITH PRESENT STUDY
Socransky *et al* [22]	1998	*Lactobacillus*	*Lactobacillus* is one of the important probiotic strain that has been used and the inhibitory action against periodontal pathogens by the production of acid.	Yes
Silva *et al* [23]	1987	Lactobacillus	*Lactobacillus* can produce different antimicrobial components including organic acids, low molecular weight antimicrobial substances, hydrogen peroxide, bacteriocins, and adhesion inhibitors.	Yes
Kõll *et al* [24]	2008	22 Strains of Orally active probiotic	*Lactobacillus salivarius* were shown to suppress the growth of *Aggregatibacter*	Yes

			actinomycetemcomitans, *P. gingivalis*, and *Prevotella intermedia*	
Matsuoka *et al* [25]	2006	L. salivaris	The oral administration of probiotic tablets containing *L. salivarius* to healthy subjects significantly reduced the number of *P. gingivalis* in the saliva and subgingival plaque.	Yes
Irshad et al [17]	2012	Bifidobacterium	*In vitro* invasion and survival of *Porphyromonas gingivalis* not inhibited by oral Bifidobacterium tablets in gingival fibroblasts cell lines	No
Vivekananda *et al* [20]	2010	Prodentis Lozenges	The usage of Prodentis in chronic periodontitis patients had a reduction in a number of *P. gingivalis*, A. actinomycetemcomitans, and P. intermedia.	Yes

CONCLUSION:

The present *in vitro* study confirms that probiotic used with Chemically activated PMMA has a significant antimicrobial effect against *P. gingivalis.* The minimum inhibitory concentration

of probiotic against *P. gingivalis* is found to be 20µg/ml according to the present study. Hence, probiotics incorporated in PMMA is found to reduce the gingival inflammation by acting against *Porphyromonas gingivalis. Limitations* of the study includes, this being an in vitro study it cannot replicate the natural oral environment and its exact complex micro flora. Also, cytotoxicity and half-life of probiotics has to be considered when it is used in oral environment. Further clinical trials are required to provide a strong evidence for the use of probiotics in PMMA as a constituent.

REFERENCES:

1. Gegauff AG, Holloway JA. Provisional restorations. Contemporary fixed prosthodontics. 3rd ed. Missouri: Mobsy Inc.; 2001. pp. 380–416.

2. Gough M. A review of temporary crowns and bridges. Dent Update. 1994;21:203–207.

3. Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C. & Kent, R. L. Jr. Microbial complexes in subgingival plaque. J Clin Periodontol 25, 134–144 (1998).

4. Hajishengallis, G. et al. Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. Cell Host Microbe 10, 497–506 (2011).

5. Darveau, R. P., Hajishengallis, G. & Curtis, M. A. Porphyromonas gingivalis as a potential community activist for disease. J Dent Res 91, 816–820 (2012).

6. Lamont, R. J. & Jenkinson, H. F. Life below the gum line: pathogenic mechanisms of Porphyromonas gingivalis. Microbiol Mol Biol Rev 62, 1244–1263 (1998).

7. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME. 2014. Expert consensus document: the international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 11:506–514. https://doi.org/10.1038/nrgastro.2014.66.

8. Moayyedi P, Ford AC, Talley NJ, Cremonini F, Foxx-Orenstein AE, Brandt LJ, Quigley E. 2010. The efficacy of probiotics in the treatment of irritable bowel syndrome: a systematic review. Gut 59:325–332. https://doi.org/ 10.1136/gut.2008.167270.

9. Delzenne NM, Neyrinck AM, Bäckhed F, Cani PD. 2011. Targeting gut microbiota in obesity: effects of prebiotics and probiotics. Nat Rev Endocrinol 7:639–646.

10.Brown AC, Valiere A. Probiotics and Medical Nutrition Therapy. Nutr Clin Care; 2004;7:56-68.

11. Patil MB, Reddy N. Bacteriotherapy and probiotics in dentistry. KSDJ; 2006; 2:98-102.

12. Elisa KB, Scott BS. Regulatory T cells in IBD. Curr Opin Gastroenterol; 2008; 24:733-41.

13. Manisha N, Ashar, Prajapathi JB. Role of probiotic cultures and fermented milk in combating blood cholesterol. Indian J Microbial; 2001; 41:75-86.

14.Gegauff AG, Holloway JA. Provisional restorations. Contemporary fixed prosthodontics. 3rd ed. Missouri: Mobsy Inc.; 2001. pp. 380–416.

15.Gough M. A review of temporary crowns and bridges. Dent Update. 1994; 21:203–207.

16. Ince G, Gürsoy H, Ipçi SD, Cakar G, Emekli-Alturfan E, Yilmaz S. Clinical and biochemical evaluation of lozenges containing *Lactobacillus reuteri* as an adjunct to non-surgical periodontal therapy in chronic periodontitis. J Periodontol. 2015; 86:746–54.

17. Irshad M, van der Reijden WA, Crielaard W, Laine ML. *In vitro* invasion and survival of *Porphyromonas gingivalis* in gingival fibroblasts; role of the capsule. Arch Immunol Ther Exp (Warsz) 2012; 60:469–76.

18. Grudianov AI, Dmitrieva NA, Fomenko EV. Use of probiotics Bifidumbacterin and Acilact in tablets in therapy of periodontal inflammations. Stomatologiia Mosk; 2002; 81:39-43.

19. 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.

20. Vivekanada MR, Vandana KL, Bhat KG. Effect of probiotic lactobacilli reuteri (Prodentis) in the management of periodontal disease: a preliminary randomized clinical trial. J Oral Microbiol.; 2010;2:5344

21. Riccia DN, Bizzini F, Perilli MG, Polimeni A, Trinchieri V, Amicosante G, Cifone MG. Antiinflammatory effects of Lactobacillus brevis (CD2) on periodontal disease. Oral Dis.; 2007;13(4):376-85.

22. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL., Jr Microbial complexes in subgingival plaque. J Clin Periodontol. 1998; 25:134–44.

23. Silva M, Jacobus NV, Denice C, Orbach SL. Antimicrobial substance from a human *Lactobacillus* strain. Antimicrob Agents Chemother. 1987; 31:1231–3.

24. Kõll P, Mändar R, Marcotte H, Leibur E, Mikelsaar M, Hammarström L. Characterization of oral lactobacilli as potential probiotics for oral health. Oral Microbiol Immunol. 2008; 23:139–47.

25. Matsuoka T, Sugano N, Takigawa S. Effect of oral *Lactobacillus salivarius* T1 2711 (LS1) administration on periodontopathic bacteria in subgingival plaque. J Jpn Soc Periodontol. 2006; 48:315–24.