International Journal of Research in Medical Sciences
Naushad AM et al. Int J Res Med Sci. 2023 Jun;11(6):2062-2067
www.msjonline.org
pISSN 2320-6071 | eISSN 2320-6012

DOI: https://dx.doi.org/10.18203/2320-6012.ijrms20231618

Original Research Article

An in vitro study of probiotic activity exhibited by *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* on oral isolates of *Streptococcus mutans* and *Candida albicans*

Ameena M. Naushad, Jenny M. Sunil, Harish Kumar K. S.*, Sajimi Sathar, Roopa Mathews, Anila Gopal

Department of Medical Microbiology, School of Medical Education, Centre for Professional and Advanced Studies, Kottayam, Kerala, India

Received: 24 March 2023
Revised: 17 April 2023
Accepted: 01 May 2023

***Correspondence:**
Dr. Harish Kumar K. S.,
E-mail: drharishkumarks@gmail.com

ABSTRACT

Background: Oral infections caused by microorganisms have led to increased risk of oral health problems like dental caries (DC). *Streptococcus mutans* and *Candida albicans* are the organisms responsible for DC. The goal of the presented study was to investigate the potential of probiotics to prevent and treat DC. An *in vitro* assay was developed to investigate several probiotic strains for their ability to inhibit the aforementioned oral pathogens.
Methods: 40 oral isolates of *Streptococcus mutans* and 51 oral isolates of *Candida albicans* were tested for probiotic activity against *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* using agar overlay interference technique as prescribed by Fleming et al.
Results: The zone of inhibition shown by *L. acidophilus* was higher than *L. rhamnosus* against *Streptococcus mutans* and *Candida albicans*.
Conclusions: In conclusion the two probiotic strains *L. acidophilus* and *L. rhamnosus* exhibited inhibitory activity on *S. mutans* and *C. albicans respectively in vitro*.

Keywords: Probiotic, Lactobacilli, *Candida albicans*, *Streptococcus mutans*, Caries, Agar overlay interference method

INTRODUCTION

The oral cavity houses a microbial ecosystem comprising more than 1000 bacterial species accountable for influencing oral health and disease. Dental caries results when there is imbalance within this oral ecosystem.[1,2]A large variety of microorganisms are associated with oral disease, however, the increased proliferation of *Streptococcus mutans* (MS), present in carious lesions, is the primary cause for the initiation and the progression of dental caries.[3,4] *Candida albicans* also play an important role in dental caries due to its aciduric nature and its abilities to develop thick biofilms.[5] Dental caries is the localized destruction of dental hard tissues (enamel and dentine) by acidic by-products from the bacterial fermentation of free sugars and host factors.[6,7] The host factors are the tooth, immune response of the oral cavity, and the amount, consistency, as well as buffering capacity of saliva. The presence of all these elements leads to the initiation of the disease and the disease process can be intercepted by elimination of any one factor.[8] Intensive investigations into the virulence of *S. mutans* has identified a number of properties of these organisms which are likely to be important in cariogenesis including: sucrose dependent biofilm formation, relatively high aciduricity and potent acidogenesis, resulting in demineralization of the tooth structure.[9] *C. albicans* has the ability to produce organic acids by the means of fermenting carbohydrates

and shows adherence to saliva-coated hydroxyapatite, dental hard tissues and dentinal collagen.[10-13] These above might implicate that *C. albicans* has the ability to destroy dental hard tissues, and can be proposed to act as a caries pathogen in dental caries.

Imbalance between the number of indigenous bacteria to that of pathogenic strains can be highlighted as one of the major causes of dental caries. The available pharmacological treatments are very effective but present some critical points, such as frequent side effects.[14] Therefore, it would appear critical to develop new prophylactic and complementary therapeutic strategies. The intake of probiotics seems a promising method in order to achieve these purposes. Probiotics are 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host, of which *Lactobacillus* and *Bifidobacterium* are the most commonly used.[15]

Briefly, the mechanisms proposed for probiotic actions consist of adhesion to the tooth surface and competition with cariogenic bacteria to reduce their growth[16] and the modulation of microbiota composition, thereby promoting oral health.[17] Beneficial effects include prevention of the growth of pathogens, competitive displacement of pathogens, and regulation of the microbial ecosystems. With this in mind, investigators have proposed probiotics, specifically *Lactobacilli*, for maintaining and regaining oral health.[18]

Lactobacilli have been used as probiotics for a number of disease applications, and are generally regarded as safe for human use, making them an ideal candidate for the development of a therapeutic.[19,20]

In this context, the current study focuses on the probiotic inhibitory effect of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* on oral isolates of *Streptococcus mutans* and *Candida albicans*.

METHODS

The present cross-sectional study was conducted at School of Medical Education (SME), Centre for professional and advanced studies, Kottayam, Kerala between January 2022 and August 2022. 40 isolates of *Streptococcus mutans* were collected from dental plaques of the students in our department and 51 isolates of *Candida albicans* were collected from various diagnostic microbiology laboratories in central Kerala.

Inclusion and exclusion criteria

Streptococcus mutans

Only subjects in the 18- to 26-year-old range without any active carious lesions were selected as volunteers. Individuals who had undergone any antimicrobial therapy in the past three months was exclude from the study.

Candida albicans

Only *Candida albicans* isolated from symptomatic candidiasis was included in the present study. Patients who had undergone any antimicrobial therapy in the past three months was exclude from the study.

Bacterial strains for the study

Lactobacillus acidophilus MTCC 10307, *Lactobacillus rhamnosus* MTCC 1408, and *Streptococcus mutans* MTCC 495 were the bacterial strains used in the study that were procured from Institute of Microbial Technology (IMTECH) Chandigarh. *Streptococcus mutans* MTCC 495 was used as the quality control for probiotic activity.

Microbial growth medias

De Man, Rogosa and Sharpe Agar (MRS), Brain heart infusion broth (BHIB), Mitis-Salivaris agar (MSA), HiCrome™ Candida Differential Agar and Mueller Hinton Agar (MHA) was purchased from HiMedia Laboratories.

Microbiological sampling of the patient

Dental plaque samples from buccal, mesial, distal, lingual, and occlusal surfaces were collected using a sterile toothpick from the permanent first molar and transferred aseptically to sterile saline.

Microbiological methods

Streptococcus mutans

Dental plaque samples were collected from students using sterile toothpick and transferred to saline and vortexed for 1 minute and plated onto MSA [Hi-Media] and incubated at 37ºC for 24 hours in the presence of 5-10% CO_2. MS colonies were identified from the samples. MS strains produced "gum-drop" appearance with black centers. These colonies were sub-cultured onto blood agar for further identification.

Candida albicans

Isolates of *Candida albicans* were collected from various diagnostic microbiology laboratories in central Kerala. These isolates were reconfirmed by subculturing on to chromogenic media-HiCrome™ as light green coloured colonies, followed by gram staining and colonies were confirmed to be Gram positive yeast like budding cells.

Agar–overlay interference test

Agar overlay method were done as prescribed by Fleming et al.[21] The standard strains of *L. rhamnosus* and *L. acidophilus* were cultured initially on MRS (deMan, Rogosa, and Sharpe) agar for 16–20 hours. Transfer of a distinct colony of *L. rhamnosus* and *L. acidophilus* to a 4.5-ml MRS broth and was further incubated for another

16–20 hours. The next day, pure colonies of *L. rhamnosus* and *L. acidophilus* were obtained. They were then transferred to 2-ml trypticase soy broth and they were incubated for another 16–20 hours. The media were incubated at 37°C with 5–10% CO_2. Briefly, the surface of MRS agar was spot inoculated with 10 µL of an overnight culture of the *L. acidophilus* and *L. rhamnosus*. 2 spots of each of *L. acidophilus and L. rhamnosus* (4 spots per plate). The plates were incubated overnight at 37°C in 5-10% CO_2. Then the plates were overlaid with 7 ml of BHI soft agar (0.75%), which was seeded with 0.1 ml (100 µl) of MS to be tested after cooling to 50°C. Another sets of plates were overlaid with 7 ml BHIB soft agar which was seeded with 0.1 ml of *C. albicans*. After 48 hours of incubation at 37°C in 5-10% CO_2, a clear zone around the *Lactobacilli spp. was* recorded as positive inhibition and no zone was recorded as negative. Each experiment was done in duplicate and the average of the zones of inhibition was recorded. The study was approved by the institutional ethical committee at School of Medical Education. The data was analysed using Microsoft excel 2019.

RESULTS

The patient demographics and details of clinical isolates are exhibited in Table 1.

Table 1: Demographic characteristics of the sample.

Variables	N (%)
Streptococcus mutans	40
Sex	
Male	11 (27.5%)
Female	29 (72.5%)
Age (years)	22 (18-26)
Duration	8 months
Candida albicans	51
Sex	
Male	25 (49%)
Female	26 (50.98%)
Age (years)	64 (59-72%)
Duration	8 months

Characterization of Streptococcus mutans

The first 40 strains of MS were isolated from the plaque samples. MS strains produced a "gum-drop" appearance with black centers.

Characterization of Candida albicans

C. albicans strains produced light green-coloured colonies on HiCrome differential agar.

Growth inhibition of Streptococcus mutans

S. mutans was inhibited by *L. acidophilus* and *L. rhamnosus* as visible by zone of inhibition using agar overlay interference technique (Figure 1). The result showed that all the 40 clinical strains of MS exhibited inhibition with the probiotic strains of *L. rhamnosus* and *L. acidophilus* (Figure 2). The mean zone of inhibition of clinical MS strains by *L. rhamnosus* was 17.12. The mean zone of inhibition of clinical strains of MS by *L. acidophilus* was 18.71. The zone of inhibition shown by *L. acidophilus* was higher than *L. rhamnosus* and was statistically significant (p value <0.05).

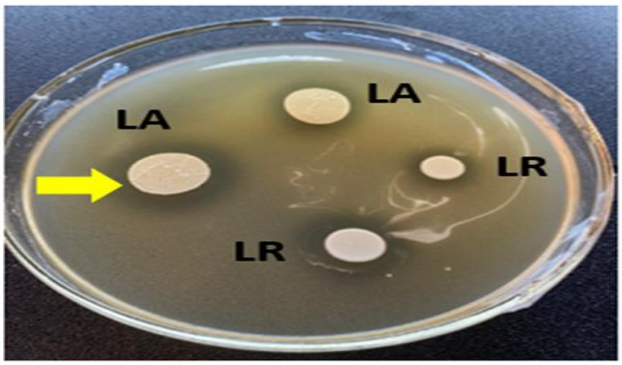

Figure 1: Inhibition of Mutans streptococci by *L. acidophilus* (L.A) and *L. rhamnosus* (L.R) as visible by zone of inhibition using agar overlay interference technique.

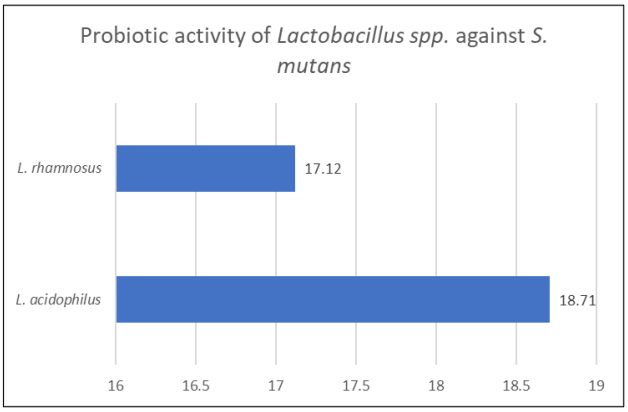

Figure 2: Probiotic activity of *Lactobacillus spp.* against *S. mutans* isolates.

Comparative activity of L. acidophilus and L. rhamnosus against S. mutans by using ANOVA single factor

The difference of probiotic activity between *L. acidophilus* and *L. rhamnosus* was analyzed using ANOVA one-way method and was found significant that is p value is <0.05, probiotic activity of *L. acidophilus* is better than *L. rhamnosus* against *S. mutans* (Table 2).

Growth inhibition of Candida albicans

C. albicans was inhibited by *L. acidophilus* and *L. rhamnosus* as visible by zone of inhibition using agar overlay interference technique (Figure 3). The result showed that all the 51 clinical strains of *C. albicans* exhibited inhibition with the probiotic strains of *L.*

rhamnosus and *L. acidophilus* (Figure 4). The mean zone of inhibition of clinical *C. albicans* strains by *L. rhamnosus* was 0.73. The mean zone of inhibition of clinical strains of *C. albicans* by *L. acidophilus* was 0.82. The zone of inhibition shown by *L. acidophilus* was higher than *L. rhamnosus*.

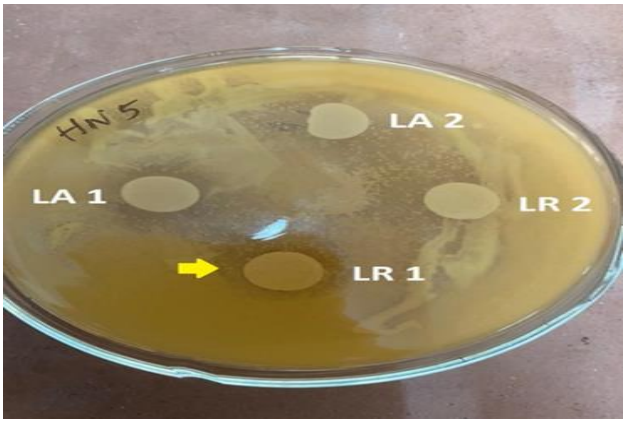

Figure 3: Inhibition of *C. albicans* by *L. acidophilus* (L.A) and *L. rhamnosus* (L.R) as visible by zone of inhibition using agar overlay interference technique.

14 strains of *C. albicans* was inhibited by the probiotic activity of *L. acidophilus* and *L. rhamnosus* and 37 strains of *C. albicans* was not inhibited by the probiotic activity of *L. acidophilus* and *L. rhamnosus* and was statistically not significant (p value is significant only if <0.05).

Comparative activity of L. acidophilus and L. rhamnosus against C. albicans by using ANOVA single factor

The difference of probiotic activity between *L. acidophilus* and *L. rhamnosus* was analysed using ANOVA –one-way method and was found not significant, that is p value is <0.05, so both *L. acidophilus* and *L. rhamnosus* have similar activity against *C. albicans* (Table 3).

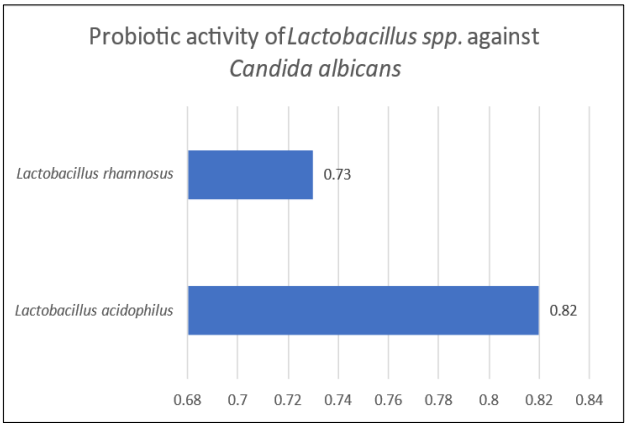

Figure 4: Probiotic activity of *Lactobacillus spp* against *C. albicans* isolates.

Table 2: Comparative activity of *L. acidophilus* and *L. rhamnosus* against *S. mutans*.

Groups	Count	Sum	Average	Variance		
Column 1	40	748.5	18.7125	3.485737		
Column 2	40	685	17.125	5.637821		
ANOVA						
Source of variation	**SS**	**df**	**MS**	**F**	**P value**	**F crit**
Between groups	50.40312	1	50.40312	11.04901	0.001354	3.963472
Within groups	355.8188	78	4.561779			
Total	406.2219	79				

ANOVA: Single factor

Table 3: Comparative activity of *L. acidophilus* and *L. rhamnosus* against *C. albicans*.

Groups	Count	Sum	Average	Variance		
Column 1	51	41.85	0.820588	0.618218		
Column 2	51	37.15	0.728431	0.655825		
ANOVA						
Source of variation	**SS**	**df**	**MS**	**F**	**P value**	**F crit**
Between groups	0.216569	1	0.216569	0.339971	0.561159	3.936143
Within groups	63.70216	100	0.637022			

ANOVA: Single factor; p value significant if <0.05

DISCUSSION

The most common microorganism associated with dental caries is *Mutans streptococci. C. albicans* is also capable to play a significant role in dental caries progression. They have essential cariogenic traits like glucan formation as well as aciduric and acidogenic properties. They also colonize on the surface of the tooth.[22]

Probiotic bacteria have been used to modify microfloral ecosystems, and have already shown some success as a therapeutic for oral diseases.[23] For the development of a

more efficacious therapeutic, optimization and selection of probiotic strains need to be undertaken. The goal of the presented research was to investigate the potential of probiotics to inhibit two prominent oral pathogens *S. mutans* and *C. albicans.* The beneficial effects of probiotic *Lactobacillus spp.* on general health are very well known.[24] Hence, in the present study, *Lactobacilli spp*. were selected as they are generally regarded as safe. In a healthy mouth, they generate organic acids from carbohydrate fermentation, which could interfere, *in vivo*, with the growth of surrounding microorganisms by lowering the pH of the oral cavity. Additionally, some strains produce hydrogen peroxide or bacteriocins, which results in bacterial antagonism.[25] They strengthen innate and acquired immunity as well as help in pro-inflammatory mediator's inhibition.[26,27] The capability of adhesion of *Lactobacilli* strains on oral surfaces has been reported in a number of studies.[28]

The current study is in accordance with an *in vitro* study by Hasslof et al, according to them the selected probiotic strains showed inhibitory action on *S. mutans* and *C. albicans*.[29] They performed agar overlay interference technique using different concentrations of *Lactobacillus spp*. They used *L. plantarum, L. rhamnosus, L. paracasei, L. reuteri* and *L. acidophilus.* All *Lactobacilli* strains inhibited the growth of the MS strains, *L. acidophilus* executed more inhibition on *Mutans streptococci*, while only a slight inhibition was seen for MS strains by *L. rhamnosus*. In our study, same results were obtained. *L. acidophilus* shown more inhibitory activity than *L. rhamnosus* against mutans *Streptococci*. All tested *Lactobacilli* strains reduced *C. albicans* growth. So, *L. rhamnosu*s and *L. acidophilus* showed similar activity against *C. albicans*. In our study, the same results were obtained.

In another study, Kõll et al used the deferred antagonism method to test the inhibitory capacity against mutans streptococcus and *C. albicans.*[30] They used *L. plantarum, L. rhamnosus, L. rhamnosus, L. paracasei, L. reuteri* and *L. acidophilus* strains. With the exception of *L. acidophilus*, other probiotic strains displayed inhibitory capacities against *Mutans streptococci*, this particular system not is optimal for *L. acidophilus.* The inhibitory potential may be different, for better or for worse, in other test system or *in vivo*. There was a larger variation of *Lactobacilli* inhibition on *C. albicans*. The above study was in concordance with the results of *L. rhamnosus* but was contrary to *L. acidophilus*.

The use of bacteriotherapy using probiotics came as a unique concept for prevention of dental caries and they also improve oral flora. The use of probiotics for dental caries is also non-invasive. The results from our present study as well as all the available literature support that probiotics can be an effective means to combat dental caries.[31] However, it is necessary to create more awareness among the general population, so that we can utilize bacteriotherapy that is a novel concept and can help to reduce dental caries.

The main limitation of the present study is that; it is an *in-vitro* study which does not mimic complex microbiota found in the oral cavity. As probiotic activity is strain dependent, a study with different strains of *L. acidophilus* and *L. rhamnosus* can be done.

CONCLUSION

In conclusion, the two probiotic strains *L. acidophilus* and *L. rhamnosus* exhibited inhibitory activity on *S. mutans and C. albicans* respectively *in vitro*. To answer more definitively the probiotic activity against *S. mutans* and *C. albicans* should be tested in suitable animal models. So, there is a need for detailed conclusive research *in vivo, in vitro* and clinical trials. Additional studies and research data is required to clarify relevant questions such as efficacy of single versus a mix of probiotic species or strains, determination of the most successful probiotic strains, use of postbiotics, duration of treatments, optimum dosage regimens, risk benefit potential, for the prevention of *S. mutans* and *C. albicans* infections, and effectiveness of cost.

ACKNOWLEDGEMENTS

Authors would like to thank Mrs. Rajumol B. Zacharia for her technical assistance.

Funding: No funding sources
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

REFERENCES

1. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, et al. The human oral microbiome. J Bacteriol. 2010;192(19):5002-17.
2. Samot J, Badet C. Antibacterial activity of probiotic candidates for oral health. Anaerobe. 2013;19:34-8.
3. van Houte J. Role of micro-organisms in caries etiology. J Dent Res. 1994;73:672-81.
4. Banas JA. Virulence properties of Streptococcus mutans. Front Biosci. 2004;9:1267-77.
5. Pereira D, Seneviratne CJ, Koga-Ito CY, Samaranayake LP. Is the oral fungal pathogen Candida albicans a cariogen? Oral Dis. 2018;24(4):518-26.
6. Fejerskov O, Nyvad B, Kidd E. Dental caries: the disease and its clinical management. Copenhagen: Blackwell Monksgaard. 2003.
7. Selwitz RH, Ismail AI, Pitts NB. Dental caries. Lancet. 2007;369:51-9.
8. Cagetti MG, Mastroberardino S, Milia E, Cocco F, Lingström P, Campus G. The use of probiotic strains in caries prevention: a systematic review. Nutrients. 2013;5(7):2530-50.

9. Hoare A, Marsh PD, Diaz PI. Ecological therapeutic opportunities for oral diseases. Microbiol Spectr. 2017;5(4).
10. Samaranayake LP, Hughes A, Weetman DA, MacFarlane TW. Growth and acid production of Candida species in human saliva supplemented glucose. J Oral Pathol. 1986;15:251-4.
11. Cannon RD, Nand AK, Jenkinson HF. Adherence of Candida albicans to human salivary components adsorbed to hydroxylapatite. Microbiol. 1995;141:213-9.
12. Sen BH, Safavi KE, Spångberg LS. Colonization of Candida albicans on cleaned human dental hard tissues. Arch Oral Biol. 1997;42:513-20.
13. Makihira S, Nikawa H, Tamagami M. Bacterial and Candida adhesion to intact and denatured collagen in vitro. Mycoses. 2002;45:389-92.
14. Sardi JCO, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini MJS. Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J Med Microbiol. 2013;62(Pt 1):10-24.
15. Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. World health organization and food and agriculture organization of the United Nations. Cordoba, Argentina. 2001.
16. Comelli EM, Guggenheim B, Stingele F, Neeser JR. Selection of dairy bacterial strains as probiotics for oral health. Eur J Oral Sci. 2002;110(3):218–24.
17. Näse L, Hatakka K, Savilahti E, Saxelin M, Pönkä A, Poussa T, et al. Effect of longterm consumption of a probiotic bacterium, Lactobacillus rhamnosus GG, in milk on dental caries and caries risk in children. Caries Res. 2001;35(6):412-20.
18. Haukioja A. Probiotics and oral health. Eur J Dent. 2010;4:348-55.
19. Prakash S, Tomaro-Duchesneau C, Saha S, Cantor A. The gut microbiota and human health with an emphasis on the use of microencapsulated bacterial cells. J Biomed Biotechnol. 2011;981214.
20. Tomaro-Duchesneau C, Saha S, Malhotra M, Coussa-Charley M, Al-Salami H, et al. Lactobacillus fermentum NCIMB 5221 has a greater ferulic acid production compared to other ferulic acid esterase producing Lactobacilli. Int J Probio Prebio. 2012;7.
21. Fleming HP, Etchells JL, Costilow RN. Microbial inhibition by an isolate of Pediococcus from cucumber brines. Appl Microbiol. 1975;30(6):1040-2.
22. Simark-Mattsson C, Emilson C-G, Grahn Hakansson E. Lactobacillus-mediated interference of mutans streptococci in cariesfree vs. caries-active subjects. Eur J Oral Sci. 2007;115(4):308-14.
23. Saha S, Tomaro-Duchesneau C, Tabrizian M, Prakash S. Probiotics as oral health biotherapeutics. Expert Opin Biol Ther. 2012;12:1207-20.
24. Saraf K, Shashikanth MC, Priy T, Sultana N, Chaitanya NC. Probiotics--do they have a role in medicine and dentistry? J Assoc Physicians India. 2010;58:488-90.
25. Sookkhee S, Chulasiri M, Prachyabrued W. Lactic acid bacteria from healthy oral cavity of Thai volunteers: Inhibition of oral pathogens. J Appl Microbiol. 2001;90(2):172-9.
26. Hedayati-Hajikand T, Lundberg U, Eldh C, Twetman S. Effect of probiotic chewing tablets on early childhood caries – a randomized controlled trial. BMC Oral Health. 2015;15(1):1-5.
27. Haukioja A, Loimaranta V, Tenovuo J. Probiotic bacteria affect the composition of salivary pellicle and streptococcal adhesion in vitro. Oral Microbiol Immunol. 2008;23(4):336-43.
28. Samot J, Badet C. Antibacterial activity of probiotic candidates for oral health. Anaerobe. 2013;19:34-8.
29. Hasslof P, Hedberg M, Twetman S, Stecksen-Blicks C. Growth inhibition of oral mutans Streptococci and Candida by commercial probiotic Lactobacilli-- an in vitro study. BMC Oral Health. 2010;10:18.
30. 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.
31. Anderson MH, Shi W. A probiotic approach to caries management. Pediatr Dent. 2006;28(2):151-3.

Cite this article as: Naushad AM, Sunil JM, Kumar HKS, Sathar S, Mathews R, Gopal A. An in vitro study of probiotic activity exhibited by *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* on oral isolates of *Streptococcus mutans* and *Candida albicans*. Int J Res Med Sci 2023;11:2062-7.