Journal of Oral Biology and Craniofacial Research 14 (2024) 22–26

Contents lists available at ScienceDirect

Journal of Oral Biology and Craniofacial Research

journal homepage: www.elsevier.com/locate/jobcr

Comparative analysis of salivary microbiota in diabetic and non-diabetic individuals of North India using metagenomics

Jyotsana Agarwal [a], Pragya Pandey [a,*], Shailendra K. Saxena [b], Swatantra Kumar [b]

[a] Department of Conservative Dentistry & Endodontics, King George's Medical University, Lucknow, India
[b] Centre for Advanced Research, King George's Medical University, Lucknow, India

ARTICLE INFO

Keywords:
Diabetics
Metagenomics
Microbiota
Saliva
Dental diseases

ABSTRACT

Background: Saliva, an oral secretion is considered an essential biological modulator involved in maintaining oral homeostasis. Increased glucose levels in diabetic patients' saliva may have an impact on diversity of microbes. Comparing the salivary microflora of diabetic and non-diabetic cohorts will help in diagnosis and risk assessment of oral health complications. This will provide greater knowledge about the contribution of oral microbes to the development of oral illnesses. The association between salivary microbiota and diabetic state is less explored in the North Indian population, hence current observational study was performed to analyze the salivary microflora of diabetic and non-diabetic individuals using metagenomic analysis.

Materials and methods: This single-center non-randomized observational trial was conducted in Uttar Pradesh, India. Participants were enrolled into either diabetic ($n = 68$) or non-diabetic groups ($n = 68$) based on their diabetes status. Following saliva collection, DNA was extracted and metagenomic sequencing was performed.

Results: Phylum Bacteroidetes and Fusobacteria were significantly abundant in diabetic individuals ($p < 0.0001$), while Proteobacteria was significantly higher among non-diabetic individuals ($p < 0.0001$). No statistical difference in phylum Actinobacteria and Firmicutes among diabetics and non-diabetics. *Veillonella, Prevotella, Porphyromonas, Leptotrichia, Lactobacillus, and Streptococcus* were greater in diabetics whereas the abundance of *Capnocytophaga and Neisseria* was more among non-diabetics ($p < 0.05$).

Conclusions: The genera *Veillonella, Prevotella, Porphyromonas, Leptotrichia, Lactobacillus,* and *Streptococcus* were comparatively over the odds with the diabetics in India. The association between microbiota in diabetic population and risk related to increase in occurrence of caries, gingivitis, and periodontitis in diabetic population prevalence should be investigated.

1. Introduction

Diabetes Mellitus (DM) has become a considerable global burden, with nearly 537 million adults living with diabetes worldwide as per the latest statement released by "The International Diabetes Federation" (IDF). In India, the diabetic population is anticipated to increase to 80 million by 2025, transforming India into the 'Diabetes Capital of the world.[1]

Most of the diabetic patients (>90 %) develop oral ailments and complications like periodontal disease, Xerostomia, loss of a tooth, caries, burning mouth disorder, altered taste, dysfunction of the salivary gland, delay in wound healing, geographic tongue, lichen planus, and oral candidiasis.[2] For example, the incidence of Xerostomia among diabetics is 12.5 %, only 5 % among non-diabetics. It has been observed that increased glucose levels in blood and serum correlate to increased salivary gingival crevicular fluid (GCF) glucose levels.[2] Elevated salivary and GCF glucose levels succor the growth of many cariogenic bacteria,[2] resulting in an increased amount of organic acid production and increased tooth demineralization. Furthermore, the bacteria causing periodontal disease, *Porphyromonas gingivalis*, increases insulin resistance.[3]

Various published studies have confirmed that co-morbid conditions like diabetes are remarkably related to the degree of severity and advancement of periodontal disease[4] Various periodontal pathogens harboring in the oral mucosa of diabetic patients commonly cause responses to oxidative stress, damage to receptors for advanced glycation

* Corresponding author. Conservative Dentistry & Endodontics, King George's Medical University, Shahmina Shah road, Chowk, Lucknow, 226003, India.
E-mail addresses: jyotsana.agar12@gmail.com (J. Agarwal), w.pragya78@gmail.com (P. Pandey), myedrsaxena@gmail.com (S.K. Saxena), swatantra15891@gmail.com (S. Kumar).

https://doi.org/10.1016/j.jobcr.2023.12.003
Received 1 May 2023; Received in revised form 28 October 2023; Accepted 5 December 2023

end products, and inflammatory cytokines, accentuating the fact that there is a significant elevation in the host hyper-inflammatory response to periodontitis in diabetes mellitus[5] These oral

Complications can unfavorably affect the QOL and due to the occurrence of these chronic and persistent oral infirmities, there is unfavorable altered control of blood glucose in diabetics. Hence, there is an alarming need to regularly assess microbiota-related oral complications in the diabetic population.

Modern headway in DNA sequencing techniques like metagenomics is the preferred approach for microbial analysis, compared to conventional culturing techniques. A significant problem with the conventional culture technique is maintaining the viability of microbes from the assortment, through transportation, to growth.

Despite investigations on microbiota-related impact in the European diabetic population, a paucity of research literature exists on the Indian population.[6] Assessing the same in the Indian population is an alarming requisite considering the rising diabetic population.

The present study aimed to analyze and compare the microbiota in the saliva of diabetic and non-diabetic individuals using metagenomic analysis.

2. Materials and method

2.1. Study setting

This is a single-center cross-sectional observational study. Patients aged 30–60 years with confirmed diabetes mellitus type II according to the diagnostic criterion followed by the American Diabetes Association,[7] with HbA1c between 6.4 and 8.9, and diabetic history of.

$\geq$ 5 years were included in the diabetic group. In addition, patients under insulin therapy were also included. Patients with Russel's Periodontal Index$\geq$0.9,[8] DMFT (Decayed missing filled Teeth) index$\geq$ 5,[9] HbA1c level $\geq$9, any other systemic disease/medical condition, or were on any other medication for the treatment of diabetes were excluded. The control/non-diabetic group included age and sex-matched healthy individuals without diabetic history and HbA1c.

$\leq$5.7. To eliminate bias, patients who underwent dental treatment in the past three months were not considered for enrollment in both groups.

The institutional ethical clearance was obtained (Ref code: 97th ECM II B-Thesis/P150), and the study was registered in CTRI (Clinical Trials Registry-India with registration number CTRI/2020/08/027,089). Before being enrolled in the study, all subjects provided their written, informed consent.

2.2. Sample size

The Sample size was calculated based on the prevalence of diabetes in Uttar Pradesh i.e., 8 %,[10] using the Daniel formula.

$$n = Z^2 \times P(1 - P)/d^2$$

Where the prevalence of diabetes in Uttar Pradesh is 8 % so, P = 0.08.

Z = 1.96 (for a 95 % level of confidence) and d = 0.05[11] The calculated sample size was found to be 112.

After assuming a 20 % drop-out rate, the sample size was identified as 136 between the groups.

2.3. Sample collection

Consecutive patients attending the diabetic clinic who have given consent were enrolled either in the diabetic or control group based on their diabetic status. In addition, data regarding age, sex, DMFT index, and Russel's index were recorded after oral examination using a plain mouth mirror and Williams periodontal probe. Oral samples were collected from the non-diabetic and diabetic individuals the next morning after overnight fasting. Before the sample collection, patients were instructed to avoid toothbrushing and consume only water. The participants were seated in coachman's position with their heads tilted down for saliva collection. To avoid contamination of the sample, the participants were instructed to avoid movement of the tongue and lips during sample collection. The saliva was accumulated gradually in the mouth for 2 min, followed by spitting the garnered saliva into the collecting vial containing 2 ml Xpress DNA storage buffer. The total sample collected was adjusted for a final volume of 4 ml by spitting. The process was repeated until the top fill mark indicated on the vial with liquid saliva, excluding the bubbles. Care was taken to complete the saliva collection process within 15 min to ensure the yield and DNA quality. The vials were capped vortexed for 10–20 s to ensure proper mixing and stored at 4 °C until further analysis.

2.4. Extraction of DNA

Total genomic extraction of DNA was done after saliva lysate preparation, followed by binding and washing DNA. Later, DNA elution was performed by the standard technique. Next, DNA was subjected to PCR using primer sets & KAPA HiFi HotStartReadyMix. Later AMPure XP beads were used to decontaminate the 16S V3 and V4 amplicon from primer dimer species and free primers. Nextera XT Illumina kit was used for library preparation, and additional PCR

Clean-up was performed. Library preparation was run on Illumina MiSeq(Illumina Inc. California) for DNA analysis.

2.5. Statistical analysis

The statistical data analysis was done using IBM SPSS software 21 version. Descriptive statistics was represented as mean $\pm$ SD, wherever possible. Categorical data were summarized as percentages (%). The level of significance was p value < 0.05.

3. Results

One hundred thirty-six patients visiting the diabetic clinic were recruited into the diabetic and non-diabetic groups based on their diabetic status. Of those, an analyzable salivary sample could not be retrieved from 6 participants (4 in diabetic and 2 in healthy participants). To maintain uniformity between the groups, data were reported for 65 patients per group. Patient demographics are represented in Table 1.

Saliva was garnered and analyzed for microbiota from all the enrolled patients. 16 S RNA metagenomic analysis was performed to compare the salivary microbiota between the two groups. A total of 79,752 and 4,66,224 next-generation sequence (NGS) raw reads using metagenomic analysis were secured for samples taken from patients who were diabetic (n = 65) and those who were non-diabetic (n = 65), respectively. Raw reads were filtered as per parameters for quality control, resulting in 2,38,267 and 2,12,910 reads for diabetics and non-diabetics (Fig. 1). The filtered sequence length was in the range of 140–270 nucleotides.

Table 1
General characteristics and medical profile of Diabetic and Non-Diabetic Individuals.

Parameters	Diabetic	Non- diabetic
Number of participants	68	68
Male/female	38/30	35/33
Age (years ±S.D.)	46 ± 4.6	40 ± 5.7
Mean duration of diabetes mellitus (years ±S.D.)	14.37 ± 3.06	Not applicable
HbA1c percentage (±S.D.)	7.2 ± 0.43	5.2 ± 0.23

*S.D. = Standard Deviation.
Data was represented only for the salivary DNA analyzed participants only.

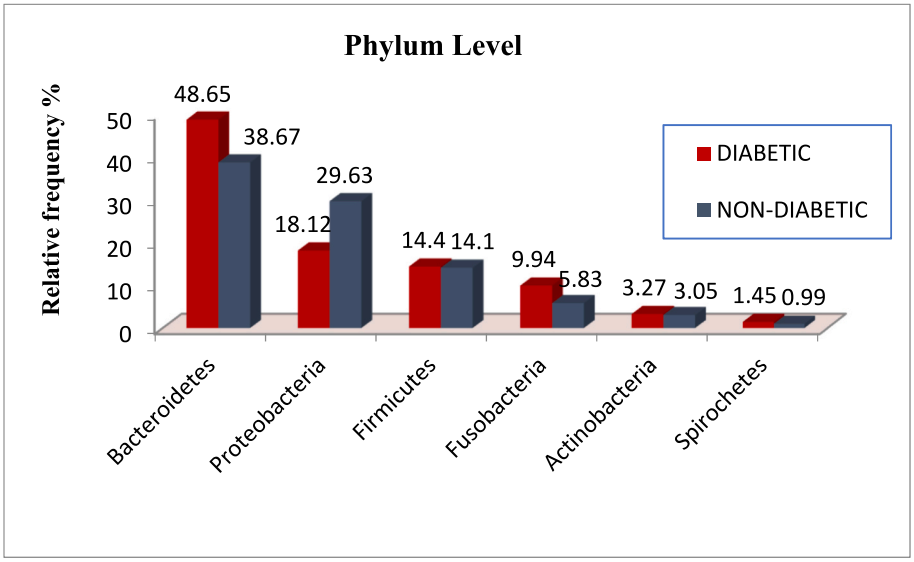

Fig. 1. Comparison of mean percentage of bacterial phyla between non-diabetic and diabetic groups.

3.1. Characterization of salivary bacterial communities

3.1.1. Diabetic group

Six major bacterial phyla were identified from the 2,38,267 high-quality 16S rRNA gene sequencing performed. Bacteroidetes were the most predominant phyla and were reckoned for 48.65 % of the bacterial sequences. Proteobacteria (18.12 %) and Firmicutes (14.4 %) were in line becoming the second and third most predominant phyla respectively. Other bacteria like Fusobacteria and Actinobacteria accounted for 9.94 % and 3.27 % of the bacterial sequences, respectively.

3.1.2. Non-diabetic group

A total of 2,12,910 high-quality 16S rRNA gene sequences were identified in the saliva of the non-diabetic group. Six phyla represented most of the bacterial population, with Bacteriodetes being the most abundantly observed phyla (38.7 %). Like the diabetic group, Proteobacteria.

(29.63 %) and Firmicutes (14.1 %) constituted the second and third considerable phyla observed (Fig. 2). In addition, Fusobacteria and Actinobacteria accounted for 5.8 %% and 3.05 % of the bacterial sequences, respectively. A small number of sequences represented minor phyla. Proteobacteria levels were observed to be considerably greater in non-diabetics ($p < 0.0001$) than in diabetics. Bacteriodetes were significantly higher in the diabetes group ($p < 0.0001$) (Fig. 1). There was no statistically significant difference noted for the phylum Actinobacteria. Actinobacteria, Firmicutes, and Spirochetes among diabetic and non-diabetic individuals. Genera like *Prevotella, Porphyromonas, Veillonella, Leptotrichia, Lactobacillus, and Streptococcus* were significantly elevated in people with diabetes compared to non-diabetics. However, the composition of *Neisseria and Capnocytophaga* was significantly higher in non-diabetics, as represented in Fig. 2. Bacterial species observed in both groups were represented in Fig. 3. *Prevotella melanogenic, Prevotella intermedia, and Porphyromonas endodontalis* were explicitly higher in saliva samples of the diabetes group. *Veillonella parvula and Campylobacter rectus* were present more in the non-diabetic sample ($p < 0.05$).

4. Discussion

The present study scrutinized the microflora in the saliva of non-diabetic and diabetic individuals using metagenomic sequencing. Saliva samples were analyzed to examine the bacterial count and bacterial diversity variation in both groups. In the samples obtained from the diabetic patients, the mean percentage of Bacteroidetes was higher indicating an association between Bacteroides and the incidence of periodontal diseases.[12] In the present study, phylum Proteobacteria was significantly lower ($p < 0.0001$) in diabetics by 1.63-fold in comparison to non-diabetics. In a recent study by **Yang *et al.* (2020),** Proteobacteria was the most abundant phylum in non-diabetic individuals, accounting for forty percent of the salivary microflora, whereas it was considerably lower in untreated diabetics.[13] Several reports have

Shown the presence of *Porphyromonas endodontalis* in the subgingival

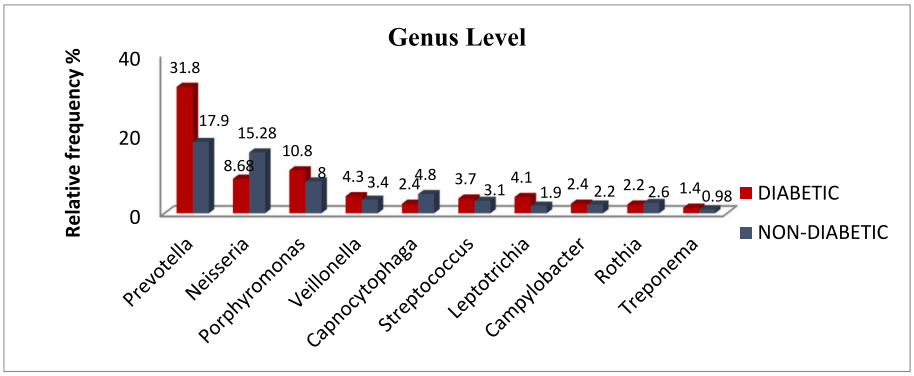

Fig. 2. Comparison of bacterial genera in diabetics and non-diabetic groups.

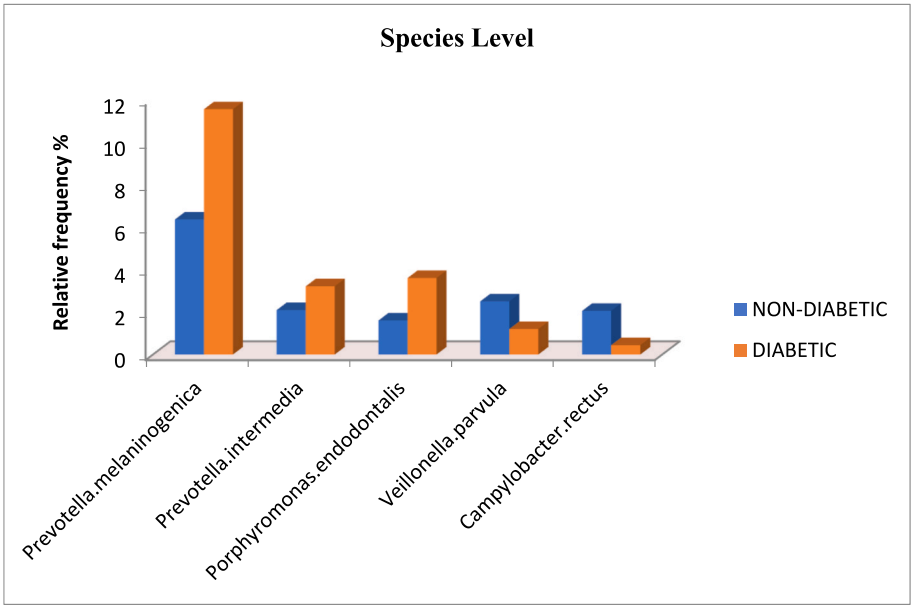

Fig. 3. Comparison of bacterial species in diabetics and non-diabetic groups.

microbiota and its association with chronic periodontitis.[14] Acidogenic bacteria mainly *Prevotella, Veillonella*, and *Leptotrichia* were found to be comparatively higher in the diabetic population of this study**.** The higher abundance of *Leptotrichia* in diabetics in this study can be compared with the results of a study by Wang et al. (2019), who found a significantly elevated level of *Leptotrichia* in elderly Chinese residents with diabetics.[15] The *Prevotella intermedia* was found to be significantly higher ($p < 0.012$) in the diabetic group, and we assume the same can be related to an increased intake of carbohydrates and retarded carbohydrate metabolism**.** Elevated levels of *Prevotella* species have been associated with periodontal diseases[16]

4.1. Genera Lactobacillus and Streptococcus were significantly higher in diabetics. Streptococcus

3.7 vs. 3 % in diabetics and non-diabetics, respectively. These results were compared with another Asian study from South Thailand.[17] Several researchers have demonstrated a notable relationship between *Streptococcus mutans* salivary levels and initiation of caries[18–20] *Lactobacillus* is also a distinguished organism in dental caries, and comparatively higher levels are discovered in the cavitated tooth. This reflects that they are predominantly associated with and responsible for the progressive and advanced status of caries rather than in the initiation of the disease.[21] Genera *Capnocytophaga* was observed to be significantly elevated in non-diabetics and causes periodontal diseases and dental caries in immunocompromised patients[22]

The present study supports the idea that hyperglycemia in Type 2 diabetes mellitus results in escalated salivary glucose levels, which decreases the salivary pH, thereby promoting the growth of aciduric bacteria. This is related to an increased risk of caries, gingivitis, and periodontitis.[23] As a pioneer study on the Indian population, the current study established the comparative microflora at the pilot level. The results could be instrumental in monitoring oral health hygiene in diabetes patients. Furthermore, the results observed in the diabetic population in India revealed the presence of pathogenic bacteria higher than in the European population, and the same could be considered for healthcare decision-making.

There are some limitations to this study. To begin, the study used a limited sample size, and the results should merit additional research with a larger sample size. In addition, the sample population considered in the study represents only the northern part of India and could not be extended to the Nation's characteristics. Furthermore, the study only included those individuals who were normally healthy without any local or systemic illness (except Type 2 diabetes). However, the polarity in innate immunity that is conditioned on the patient's age, gender, socioeconomic status ethnic diversity, and living habitat cannot be ignored. Lastly, diabetic participants included in our study were controlled diabetics, and differential results concerning microbiological profiles can be expected among patients with uncontrolled diabetes.

5. Conclusion

Bacterial sequences of phylum Bacteriodetes and Fusobacteria were copious existence in the oral milieu of diabetic individuals, while Proteobacteria existed significantly among non-diabetic individuals. In addition, notable inhabitants of the genera *Veillonella, Prevotella, Porphyromonas, Leptotrichia, Lactobacillus,* and *Streptococcus* was observed in the Indian population with diabetes. The observed microbiota population and its association with oral hygiene and diseases will be investigated in future studies.

Source of support

Nil.

Declaration of competing interest

None.

References

1. http://www.diabetesfoundationindia.org/about.htm.
2. Hintao J, Teanpaisan R, Chongsuvivatwong V, Ratarasan C, Dahlen G. The microbiological profiles of saliva, supragingival and subgingival plaque and dental caries in adults with and without type 2 diabetes mellitus. *Oral Microbiol Immunol.* 2007 Jun;22(3):175–181. https://doi.org/10.1111/j.1399-302X.2007.00341.x.
3. Blasco-Baque V, Garidou L, Pomié C, et al. Periodontitis induced by Porphyromonas gingivalis drives periodontal microbiota dysbiosis and insulin resistance via an impaired adaptive immune response. *Gut.* 2017 May 1;66(5):872–885.
4. Graves DT, Ding Z, Yang Y. The impact of diabetes on periodontal diseases. *Periodontol.* 2000;82(1):214–224. https://doi.org/10.1111/prd.12318, 2020 Feb.
5. Luong A, Tawfik AN, Islamoglu H, et al. Periodontitis and diabetes mellitus comorbidity: a molecular dialogue. *J Oral Biosci.* 2021 Dec 1;63(4):360–369. https://doi.org/10.1016/j.job.2021.10.006.

6. Vangipurapu J, Fernandes Silva L, Kuulasmaa T, Smith U, Laakso M. Microbiota-related metabolites and the risk of type 2 diabetes. *Diabetes Care*. 2020 Jun 1;43(6): 1319–1325. https://doi.org/10.2337/dc19-2533.
7. https://www.diabetes.org/diabetes/a1c/diagnosis.
8. Deepa D, Jain G. Assessment of periodontal health status in postmenopausal women visiting dental hospital from in and around Meerut city: cross-sectional observational study. *J Midlife Health*. 2016 Oct;7(4):175. https://doi.org/10.4103/0976-7800.195696.
9. Moreno-Quispe LA, Espinoza-Espinoza LA, Bedon-Pajuelo LS, Guzmán-Avalos M. Dental caries in the peruvian police population. *J Clin Exp Dent*. 2018 Feb;10(2): e134. https://doi.org/10.4317/jced.54265.
10. Singh PS, Sharma H, Zafar KS, et al. Prevalence of type 2 diabetes mellitus in rural population of India-a study from Western Uttar Pradesh. *Int J Res Med Sci*. 2017 Apr; 5(4):1363–1367. https://doi.org/10.18203/2320-6012.ijrms20171227.
11. Daniel W. *Biostatistics: A Foundation for Analysis in the Health Sciences*. seventh ed. New York: Wiley; 1999.
12. Bui FQ, Almeida-da-Silva CL, Huynh B, et al. Association between periodontal pathogens and systemic disease. *Biomed J*. 2019 Feb 1;42(1):27–35.
13. Yang Y, Liu S, Wang Y, et al. Changes of saliva microbiota in the onset and after the treatment of diabetes in patients with periodontitis. *Aging (Albany NY)*. 2020 Jul 7; 12(13), 13090. https://doi.org/10.18632/aging.103399.
14. Lombardo Bedran TB, Marcantonio RA, Spin Neto R, et al. Porphyromonas endodontalis in chronic periodontitis: a clinical and microbiological cross-sectional study. *J Oral Microbiol*. 2012 Jan 1;4(1), 10123.
15. Wang RR, Xu YS, Ji MM, et al. Association of the oral microbiome with the progression of impaired fasting glucose in a Chinese elderly population. *J Oral Microbiol*. 2019 Jan 1;11(1), 1605789. https://doi.org/10.1080/20002297.2019.1605789. PMID: 31069021; PMCID: PMC6493323.
16. Dayakar MM, Bhat S, Lakshmi KN. Prevotella intermedia-An overview and its role in periodontitis. *J. adv. clin. res.* 2021 Jul 1;8(4):79–82. https://doi.org/10.15713/ins.jcri.336.
17. Kampoo K, Teanpaisan R, Ledder RG, McBain AJ. Oral bacterial communities in individuals with type 2 diabetes who live in southern Thailand. *Appl Environ Microbiol*. 2014 Jan 15;80(2):662–671. https://doi.org/10.1128/AEM.02821-13.
18. Dasanayake A, Caufield P, Cutter G, Roseman J, Köhler B. Differences in the detection and enumeration of mutans streptococci due to differences in methods. *Arch Oral Biol*. 1995;40(4):345–351. https://doi.org/10.1016/0003-9969(94)00164-7.
19. Forssten SD, Björklund M, Ouwehand AC. Streptococcus mutans, caries and simulation models. *Nutrients*. 2010 Mar 2;2(3):290–298. https://doi.org/10.3390/nu2030290.
20. Thibodeau EA, O'Sullivan DM. Salivary mutans streptococci and caries development in the primary and mixed dentitions of children. *Community Dent Oral Epidemiol*. 1999;27(6):406–412. https://doi.org/10.1111/j.1600-0528.1999.tb02039.x.
21. Ahirwar SS, Gupta MK, Snehi SK. Dental caries and lactobacillus: role and ecology in the oral cavity. *Int J Pharma Sci Res*. 2019;11:4818–4829.
22. Chen C, Hemme C, Beleno J, et al. Oral microbiota of periodontal health and disease and their changes after nonsurgical periodontal therapy. *ISME J*. 2018 May;12(5): 1210–1224.
23. Rohani B. Oral manifestations in patients with diabetes mellitus. *World J Diabetes*. 2019 Sep 9;10(9):485. https://doi.org/10.4239/wjd.v10.i9.485.