Assessment of *Filifactor alocis* and *Porphyromonas gingivalis* Levels in Subgingival Plaque of Patients with Type 2 Diabetes Mellitus with and without Periodontitis: A Case-control Study

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ABSTRACT

Introduction: Periodontitis, a chronic inflammatory condition, is influenced by microbial dysbiosis and the host's immune response. Type 2 Diabetes Mellitus (T2DM) represents a significant public health challenge in India and exacerbates periodontal inflammation due to hyperglycaemia, compromised immune regulation, and increased oxidative stress. Key pathogens such as *Porphyromonas gingivalis* and *Filifactor alocis*, an emerging anaerobe, are known to play a central role in disease progression, especially in diabetic individuals, where their coexistence appears more pronounced. Despite this, there is a significant gap in comparative data on the presence of these bacteria in the subgingival microbiome of diabetic patients with and without periodontitis.

Aim: This study investigates the levels of *Filifactor alocis* (*F. alocis*) and *Porphyromonas gingivalis* (*P. gingivalis*) in subgingival plaque samples from patients with T2DM with and without periodontitis.

Materials and Methods: This case-control study was conducted at the Departments of Periodontics and General Medicine, Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India from September to December 2022. Ethical clearance was obtained (IEC No: CSP/22/JUL/114/429). The study included 24 T2DM patients aged 30-60 years, with 12 having periodontitis and 12 without, based on the American Academy of Periodontology (AAP) 1999 criteria. Clinical parameters (Plaque Index, Bleeding on Probing (BOP), Periodontal Probing Depth (PPD), Clinical Attachment Loss (CAL), Periodontal Inflamed Surface Area (PISA), Periodontal Epithelial Surface Area (PESA), fasting/Postprandial Blood Sugar (PPBS), and subgingival levels of *P. gingivalis* and *F. alocis* (via species-specific qPCR) were evaluated. Data were analysed using SPSS v23 with the Mann-Whitney U test and Spearman correlation; significance was set at p-value ≤0.05.

Results: Periodontitis patients exhibited significantly higher mean values for BOP, plaque index, PPD, CAL, PISA, and PESA (p-value <0.05). Lower Ct values (indicative of higher bacterial loads) of both *P. gingivalis* and *F. alocis* were observed in the periodontitis group, although differences were not statistically significant. A significant positive correlation was found between *P. gingivalis* levels and Fasting Blood Sugar (FBS) (r=0.617, p-value=0.032). No significant correlations were found between *F. alocis* levels and clinical or glycaemic parameters.

Conclusion: These findings underscore the synergistic role of these pathogens in periodontal inflammation and their potential impact on glycaemic control, highlighting the importance of integrated oral and systemic care.

Keywords: Blood glucose, Dysbiosis, Microbiota, Periodontal pathogen, Real-time polymerase chain reaction

INTRODUCTION

Periodontitis is a complex, multifactorial, chronic inflammatory disease affecting the periodontium. Over the past 50 years, there has been a paradigm shift in the perception of the role of individual microorganisms, their interactions with each other, and the host's immune response in periodontitis. The established red-complex pathogens, primarily *Porphyromonas gingivalis* (*P. gingivalis*), promote periodontal destruction by enabling the biofilm to become more inflammophilic [1]. Conversely, newly identified species such as *Filifactor alocis* (*F. alocis*) emphasise an 'oxidative sink' that stabilises the microenvironment of the periodontal pocket, aiding the survival of *P. gingivalis* even under hydrogen peroxide-induced oxidative stress [2].

Alongside periodontitis, diabetes has emerged as a significant public health challenge in India, with the country housing one of the largest populations of diabetic patients globally, accounting for approximately 77 million individuals [3]. The bidirectional relationship between diabetes and periodontitis is mediated through the dysregulation of host-derived inflammatory mediators and oxidative stress, leading to impaired glycaemic control and periodontal tissue destruction [4]. This highlights the importance of integrated care, emphasising the need for individuals with diabetes to maintain good oral hygiene and for dental professionals to closely monitor the oral health of diabetic patients.

Recent research has observed the coexistence of *F. alocis* with *P. gingivalis* in the gingival sulcus of patients with gingivitis and periodontitis [5]. In diabetes, elevated blood sugar levels create a more favourable environment for the growth of harmful bacteria, exacerbating periodontitis [6]. There exists a gap in knowledge regarding the relative proportions of these organisms and their correlation with clinical periodontal parameters in patients with type 2 diabetes, both with and without periodontitis. Thus, the objective of the present study was to quantitatively analyse the levels of *F. alocis* and *P. gingivalis* in

subgingival dental plaque from patients with type 2 diabetes, both with and without periodontitis.

MATERIALS AND METHODS

The present case-control study was conducted at the outpatient department of Periodontics and the main outpatient G-block of Sri Ramachandra Dental and Medical College and Research Institute, Chennai, Tamil Nadu, India. The present study was approved by the Institutional Scientific and Ethical Review Board (IEC Number: CSP/22/JUL/114/429) before the commencement of the study. Patients attending the outpatient department of Periodontics and the main outpatient department of the medical hospital were recruited for the study. Written informed consent was obtained, and the study was carried out from September 2022 to December 2022.

Inclusion criteria: Inclusion criteria comprised patients aged 30-60 years diagnosed with T2DM based on ICMR 2018 guidelines, which include Fasting Plasma Glucose (FPG) ≥126 mg/dL, 2-hour post-load glucose (2-h PG) ≥200 mg/dL, HbA1c ≥6.5%, or random plasma glucose level ≥200 mg/dL with classic hyperglycaemic symptoms [7]. They had been diagnosed with chronic periodontitis in accordance with the criteria established by the American Academy of Periodontology (AAP) in the 1999 classification system [8].

Exclusion criteria: Exclusion criteria included patients with current or past tobacco use, a history of orthodontic treatment or maxillofacial surgery, recent antibiotic or analgesic use, pregnancy or lactation, systemic diseases other than diabetes, and prior surgical periodontal therapy. Cases and controls were matched based on age (30-60 years), gender, and glycaemic status to minimise confounding factors. All participants were type 2 diabetics. The key differentiating factor between the groups was the presence or absence of periodontitis.

Sample size calculation: The sample size for this matched case-control study was calculated based on Gogeneni H et al., (2015) [9]. Using an expected exposure proportion of 0.10 in the control group, a 90% confidence level (α=0.10), and 80% power (β=0.20), the calculated minimum sample size was 12 participants per group, resulting in a total sample size of 24 participants (12 cases and 12 controls), which was considered adequate to detect a statistically significant association between the exposure and outcome.

saliva, blood, or surrounding tissues. Saliva ejectors were also used when necessary to maintain a dry field. The area was gently air-dried, and supragingival plaque was carefully removed using sterile instruments to avoid interference with subgingival sampling. This isolation process ensured that the collected plaque reflected only the subgingival sample and not extraneous material, thus maintaining the integrity and reliability of the microbial analysis.

Following isolation, two sterile paper points were inserted into the base of the deepest pocket. After a 30-second interval, the paper points were immediately transferred to a microcentrifuge tube and placed on ice [16]. This preserved the integrity of the microbial DNA and prevented enzymatic degradation, thereby ensuring the sample's viability. The samples were then transported to the microbiological laboratory for storage according to the manufacturer's guidelines and subsequent analysis.

Bacterial DNA isolation: An enzyme solution containing Tris-HCl, EDTA, proteinase K, and lysozyme (20 mg/sample) was added (1 mL) to the subgingival plaque samples and incubated at 37°C for 30 minutes. This was followed by the addition of 20 μL of 10% Sodium Dodecyl Sulfate (SDS) and a second 30-minute incubation. Samples were then treated with equal volumes of phenol-chloroform, centrifuged (10,000 rpm for 10 minutes), and the supernatant was mixed with chloro-isoamyl alcohol and centrifuged again. Sodium acetate (1/10th volume) and 300 μL of absolute ethanol were added, and samples were stored overnight at -50°C. After final centrifugation and drying, the DNA pellet was resuspended in 30-50 μL of RNA/DNase-free distilled water [17].

Real-time PCR technique: Real-time PCR was employed to quantify the presence of two specific oral pathogens, *Fusobacterium alocis* and *Porphyromonas gingivalis*, in subgingival plaque samples. The reaction mixture, with a final volume of 20 μL, was prepared using species-specific primers targeting the 16S rRNA gene of each organism [Table/Fig-1]. Each sample was analysed in duplicate to ensure accuracy and reproducibility. The reaction mix was dispensed into an optical 96-well plate, sealed with an optical adhesive film to prevent evaporation, and subjected to thermocycling.

The thermocycling protocol began with an initial denaturation at 50°C for two minutes, followed by enzyme activation at 95°C for 10 minutes.

Target gene	Primer sequences (5'-3')	
	Forward	Reverse
Filifactor alocis	5'-CAGGTGGTTTAACAAGTTAGTGG-3'	5'-CTAAGTTGTCCTTAGCTGTCTCG-3'
Porphyromonas gingivalis	5'-AGG CAG CTT GCC ATA CTG CG-3'	5'-ACT GTTAGCAACTACCGATGT-3'

[Table/Fig-1]: Primers of the microorganisms.

A total of 24 individuals were enrolled, comprising 12 T2DM patients without periodontitis (controls) and 12 with periodontitis (cases).

Clinical examination: Prior to sample collection, a range of clinical and laboratory parameters were assessed, including Bleeding on Probing (BOP) [10], Plaque Index [11], Periodontal Probing Depth (PPD) [12], Clinical Attachment Loss (CAL) [13], Periodontal Inflamed Surface Area (PISA) [14], and Periodontal Epithelial Surface Area (PESA) [14]. In addition, fasting blood sugar (FBS) and postprandial blood sugar (PPBS) levels were recorded.

Study Procedure

Subgingival plaque sample collection for real-time PCR analysis: Subgingival plaque samples were obtained from the tooth with the deepest pocket for microbial analysis under proper isolation [15]. The deepest periodontal pocket was identified by measuring the Periodontal Probing Depth (PPD) at six sites around each tooth using a calibrated periodontal probe. The site with the greatest depth, indicating the most advanced tissue destruction and inflammation, was selected for sampling.

Before sampling, the tooth with the deepest periodontal pocket was isolated using sterile cotton rolls to prevent contamination from

This was followed by 40 amplification cycles, each consisting of denaturation at 95°C for 15 seconds and extension at 60°C for one minute. Fluorescence signals were measured during each cycle to determine the Cycle Threshold (CT) values, which indicate the point at which the fluorescence exceeds the background signal. Data were analysed using the RQ Manager software, which interprets CT values to assess the relative quantity of bacterial DNA present in each sample.

STATISTICAL ANALYSIS

All analyses were performed using Statistical Package for the Social Sciences (SPSS) version 23. The Mann-Whitney U test was employed to compare the estimates of study variables between the two groups. To assess association, the Spearman correlation test was utilised. The statistical significance was set at p≤0.05.

RESULTS

The mean age of participants in the periodontitis group was 52.00±8.21 years, while in the non periodontitis group it was 46.83±8.85 years; this age difference was not statistically significant (p=0.193). Both groups included an equal distribution of five males and seven females, ensuring gender balance in the study population.

A comparative evaluation of baseline periodontal parameters between diabetic patients with and without periodontitis is shown in [Table/Fig-2]. The periodontitis group demonstrated significantly higher levels of Bleeding on Probing (BOP) (p=0.008), plaque accumulation (p=0.001), Probing Pocket Depth (PPD) (p=0.001), and Clinical Attachment Loss (CAL) (p=0.001), indicating more severe periodontal tissue damage. Inflammatory burden, as measured by Periodontal Inflamed Surface Area (PISA) and Periodontal Epithelial Surface Area (PESA), was also substantially greater in the periodontitis group, with p-values of 0.005 and 0.009, respectively. These findings highlight a significantly elevated periodontal disease burden in diabetic individuals with periodontitis compared to those without.

Parameters	Diabetes with periodontitis	Diabetes without periodontitis	p-value
Age	52.00±8.21	46.83±8.85	0.193
BOP	0.61±0.05	0.55±0.040	0.008*
Plaque index	1.42±0.29	0.71±0.38	0.001*
PPD	2.97±0.81	2.00±0.38	0.001*
CAL	4.52±0.91	0.30±0.48	0.001*
PESA	1443.84 mm²±348.95 mm²	1085.87 mm²±226.17 mm²	0.009*
PISA	625.41 mm²±155.83 mm²	419.39 mm²±109.5 mm²	0.005*
FBS mg/dL	103±15.56	109±17.56	0.435
PPBS mg/dL	174.16±44.79	156.9±21.94	0.402

[Table/Fig-2]: Comparison of descriptive data in individual diabetes mellitus with and without periodontitis.

Quantitative real-time PCR (qPCR) revealed that in individuals with diabetes, the mean Cycle Threshold (CT) value for *P. gingivalis* was lower in the periodontitis group, suggesting a higher bacterial load; however, the difference was not statistically significant (p=0.621). Similarly, *F. alocis* also showed a lower mean CT value in the periodontitis group, but this difference was likewise not significant (p=0.885) [Table/Fig-3]. These findings indicate a possible increase in subgingival colonisation of both species in the presence of periodontitis, though the variation was not statistically meaningful.

CT values	Diabetes with periodontitis	Diabetes without periodontitis	p-value
P. gingivalis	13.28±9.51	15.23±11.26	0.621
F. alocis	16.15±3.68	16.98±3.15	0.885

[Table/Fig-3]: Comparison of CT values of *P. gingivalis* and *F. alocis* of the diabetic groups with and without periodontitis.

Correlation analysis was conducted to explore the relationship between the CT values of *Porphyromonas gingivalis* and *Filifactor alocis* and various periodontal clinical parameters, as well as glycaemic indices, among diabetic individuals with and without periodontitis. For *P. gingivalis*, no significant correlations were observed with BOP, plaque index, PPD, CAL, PISA, or PESA in either group (p>0.05). However, a significant positive correlation was found between Fasting Blood Sugar (FBS) and the CT value of *P. gingivalis* in the periodontitis group (r=0.617, p=0.032). No significant correlations were observed for Postprandial Blood Sugar (PPBS) with *P. gingivalis* in either group.

For *F. alocis*, similar results were found, with no significant correlations between its CT values and clinical parameters such as BOP, plaque index, PPD, CAL, PISA, or PESA in either group (p>0.05). Although it did not reach statistical significance, moderate but non-significant correlations were observed between *F. alocis* CT values and probing pocket depth (r=0.133) and clinical attachment level (r=0.283) in the diabetic group with periodontitis. A borderline inverse correlation was found with postprandial blood glucose levels in the diabetic group with periodontitis (r=-0.538, p=0.071). These findings suggest a possible link between elevated fasting glucose and the subgingival abundance of *P. gingivalis* in individuals with periodontitis, while the associations for *F. alocis* remain inconclusive and warrant further investigation [Table/Fig-4].

Parameters		*Porphyromonas gingivalis*		*Filifactor alocis*	
		DM with periodontitis	DM without periodontitis	DM with periodontitis	DM without periodontitis
		CT value	CT value	CT value	CT value
BOP	r value	-0.057	-0.060	0.176	0.078
	p-value	0.860	0.852	0.583	0.810
Plaque index	r value	0.018	-0.153	-0.346	0.124
	p-value	0.956	0.635	0.270	0.702
PPD	r value	0.350	0.120	0.133	0.179
	p-value	0.265	0.710	0.680	0.578
CAL	r value	0.369	-0.415	0.283	0.479
	p-value	0.238	0.180	0.373	0.115
PESA	r value	0.275	0.211	0.301	-0.315
	p-value	0.388	0.510	0.342	0.319
PISA	r value	0.211	0.113	0.238	-0.308
	p-value	0.510	0.727	0.457	0.331
FBS mg/dL	r value	0.617	-0.127	-0.056	-0.063
	p-value	**0.032***	0.695	0.862	0.846
PPBS mg/dL	r value	-0.134	0.394	-0.538	-0.357
	p-value	0.678	0.205	0.071	0.255

[Table/Fig-4]: Correlation between *P. gingivalis* and *F. alocis* and clinical parameters of the diabetic groups with and without periodontitis.

DISCUSSION

Periodontitis is a chronic inflammatory disease of the gums, primarily caused by an imbalance in the oral microbiome, known as dysbiosis, which can lead to tissue destruction, bone loss, and other severe oral health problems. While traditional models focus on keystone pathogens like *P. gingivalis*, which play a central role in initiating and maintaining periodontitis, recent research has highlighted the role of newer pathogens that favour dysbiosis without being classified as keystone pathogens [18,19]. These newer microaerophilic organisms, such as *Fretibacterium humanum*, *F. alocis*, and members of the Synergistetes phylum, contribute to the development and progression of periodontitis. Their ability to survive and thrive in low-oxygen, inflammatory environments makes them key contributors to the disease. Evidence from the literature suggests a symbiotic association of these newer species, particularly *F. alocis*, with keystone pathogens like *P. gingivalis* [20]. In diabetic patients, the presence of these pathogens together can exacerbate the already impaired immune response, increasing susceptibility to periodontal disease.

F. alocis contributes to the production of reactive oxygen species, restricts the growth of gingival fibroblasts, and induces apoptosis and autophagic cell death in gingival epithelial cells [21]. The results of the present study are in alignment with the well-established bidirectional relationship between diabetes mellitus and periodontitis. Studies conducted by Löe H (1993) and Taylor GW and Borgnakke WS (2008) observed that poorly controlled diabetes exacerbates periodontal inflammation, resulting in increased CAL and pocket depth [21,22]. The significantly higher clinical indices in the diabetic group with periodontitis in this study are in accordance with these observations.

The microbial analysis performed in the present study, although not statistically significant, indicates that the lower mean CT values of *P. gingivalis* and *F. alocis* in the periodontitis group suggest a trend toward higher bacterial load. This trend is biologically plausible, since both organisms are considered key periodontal pathogens, often more prevalent in sites with active periodontal destruction, as observed in studies by Aruni AW et al., (2015) and Griffen AL et al., (1998) [19,23]. The present study also observed comparatively higher levels of *P. gingivalis* and *F. alocis* in diabetic patients with periodontitis than in those without periodontitis, aligning with studies

conducted by Pandian DS et al., (2023) and Cheng H et al., (2015) [18,24]. Furthermore, the positive correlation between fasting blood glucose levels and *P. gingivalis* abundance in the periodontitis group supports previous reports by Nishimura F et al., (2003), linking hyperglycaemia to increased periodontal pathogen colonisation [25]. Hyperglycaemic conditions can impair host immune function and enhance bacterial proliferation in the periodontal environment.

Despite these observations, the lack of statistically significant differences in the CT values of the microorganisms challenges the assumption that microbial burden directly mirrors clinical disease severity in all cases. These findings contradict those of Kumar PS et al., (2006) and Schlafer S et al., (2010) [26,27], where increased levels of *P. gingivalis* and *F. alocis* were significantly associated with clinical measures of periodontal disease. However, the lack of statistical significance may be attributed to the limited sample size. Thus, while the data suggest a potential increase in the subgingival colonisation of *P. gingivalis* and *F. alocis* in periodontitis patients, larger studies are needed to confirm these observations and assess their clinical relevance.

Limitation(s)

The limitations of the present study include its cross-sectional design, which restricts the ability to draw causal inferences. Future research should focus on longitudinal studies to better establish causality, investigate the underlying mechanisms through which these pathogens influence glycaemic control, and assess the impact of periodontal treatment on improving both oral and systemic health in diabetic patients.

CONCLUSION(S)

In conclusion, this study provides valuable insights into the relationship between *P. gingivalis* and *F. alocis* in diabetic patients with periodontitis. The results demonstrate that these bacteria, although statistically insignificant, are present at comparatively higher levels in diabetic patients with periodontitis compared to those without, highlighting their potential role in the progression and severity of the disease. Furthermore, the observed significant correlation between the levels of these pathogens and FBS levels in diabetic patients suggests a bidirectional relationship between periodontitis and diabetes.

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