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## A preliminary study on bacterial composition in the subgingival plaque of woman with periodontitis during pregnancy and menopause

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### Abstract

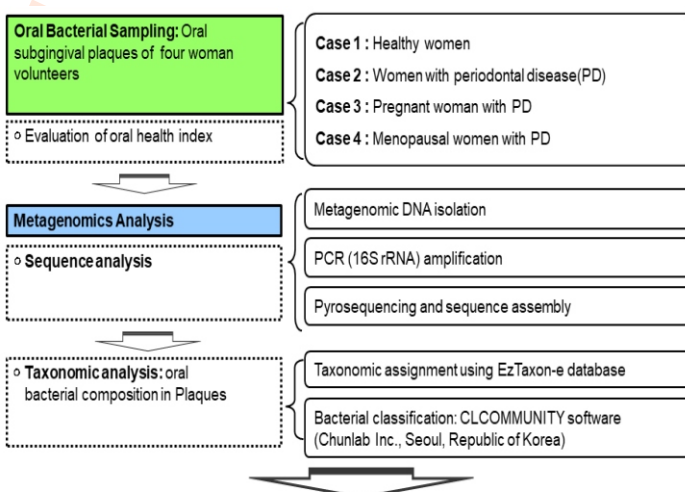
**Aim:** The aim of the present study was to explore the bacterial composition in subgingival plaque of females with periodontitis during pregnancy and menopause stages using 16S ribosomal RNA (rRNA) gene pyrosequencing approach.

**Methodology:** Subgingival plaque was collected from four woman volunteers (healthy, periodontitis, periodontitis at pregnancy and periodontitis at menopause). The microbial community composition was analyzed by 454/Roche GS FLX chemistry pyrosequencing approach using the variable (V1-V3) region of the 16S rRNA gene. Pyrosequencing reads were sorted to get the clean reads that were annotated against the EzBioCloud data base for taxonomic classification. Operational Taxonomic Units (OTUs) were assigned and shared, and subsequently identified using CLCOMMUNITY software.

**Results:** Pyrosequencing yielded 13,939 sequences comprising of 13 phyla, 124 genera, and 372 species. The predominant microbial phyla in subgingival plaque of all woman volunteers included *Firmicutes*, *Actinobacteria*, *Fusobacteria*, *Bacteroidetes*, and *Proteobacteria*. In the healthy volunteer, *Streptococcus* (52.4%) formed the predominant genus while in woman with periodontitis *Streptococcus* (24.6%) and *Fusobacterium* (11.7%) predominated. In the periodontitis volunteer with pregnancy, the predominant genus included *Streptococcus* (25.8%) and *Fusobacterium* (22.4%), whereas volunteer with menopause, the gingivitis was associated with genus *Alloprevotella* (19.5%), *Leptotrichia* (14.3%), *Fusobacterium* (12.3%), and *Porphyromonas* (12.0%).

**Interpretation:** This study proves on preliminary basis that the subgingival microbiome of woman with periodontitis at pregnancy or menopause tend to differ from that of healthy woman, and these species included certain periodontal pathogens such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis*.

**Key words:** 16S rRNA gene, Metagenomics, Oral microbiome, Periodontitis, Subgingival plaque



In women with periodontitis during pregnancy or menopause, the subgingival microbiome differed from that of healthy women, and these species included certain periodontal pathogens such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis*.

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## Introduction

Periodontitis is an inflammatory condition that affects the gingiva, bone and other tissues that support the teeth. Periodontitis is responsible for tooth loss in adults, leading to nutritional disorders, reduced self-esteem, and socio-economic costs (Tonetti *et al.*, 2017). Periodontitis is caused by the shifts in the composition of bacterial communities on intraoral surfaces, which promote inflammation (Abusleme *et al.*, 2013). The pathogenesis of periodontitis is multi-factorial and may involve genetic and/or epigenetic factors (Luo *et al.*, 2018). The hormonal changes that occur during puberty, menstruation, pregnancy and menopause can increase the sensitivity of gums to bacterial plaque. At puberty, variations in the level of female sex hormones, estrogen and progesterone, lead to softer gums that are more sensitive to bleeding. During monthly cycle (menses), some woman experience swollen gums, lesions, swollen salivary glands, or bleeding gums. Pregnancy is associated with gingivitis, a condition associated with red, puffy or tender gums attributed to increased progesterone levels (Jafri *et al.*, 2015). Female-sex hormones impact the composition of oral microbiome, both qualitatively and quantitatively (Mariotti, 1994). This has been demonstrated in the gingiva; on periosteal fibers, scattered fibroblasts of the lamina propria, periodontal ligament fibroblasts, and osteoblasts (Mariotti, 1994). Further, during menopause, woman experience xerostomia (dry mouth), hyposalivation, and altered taste sensations, that influences the salivary microbiome to menopausal gingivostomatitis (Mahesh *et al.*, 2014). According to a report of American Academy of Periodontology, women are 26% more likely to have periodontitis than men with higher incidence noted in pregnant woman. Cultivation, microscopy, DNA-DNA checkerboard and quantitative real-time PCR approaches have been used to evaluate the association of periodontitis condition in pregnant woman (Gürsoy *et al.*, 2009). During pregnancy, subgingival periodontal pathogens such as *Porphyromonas gingivalis* and *Prevotella intermedia* promote inflammation. Moreover, the severity is correlated with maternal hormonal levels (Carrillo-de-Albornoz *et al.*, 2010). Black-pigmented *Bacteroides* (BPB) are abundant in pregnant woman with gingivitis, although it is unclear whether this is due to inflammation or imbalances in hormone levels (Kumar *et al.*, 2013).

High-throughput 16S rRNA sequencing facilitates an analysis of the relationships of the total bacterial diversity in the oral cavity (Park *et al.*, 2015). Pyrosequencing of the 16S rRNA amplicons from the subgingival space of periodontitis patients yielded ~700 species, including *P. gingivalis* and new species such as *Filifactor alocis* (Griffen *et al.*, 2011). In smokers with periodontitis, *Aggregatibacter actinomycetemcomitans* and *P. gingivalis* are reportedly the predominant bacteria (Shchipkova *et al.*, 2010). Further, bacteria at peri-implant and periodontal sites have been identified by amplification, sequencing and alignment of 16S rRNA genes (Schaumann *et al.*, 2014). However, application of metagenomics for the analysis of microbial

communities in the subgingival cavity of woman with periodontitis is limited. In this study, the subgingival microbiome of pregnant and menopausal woman volunteers with periodontitis were analyzed using 16S rRNA gene pyrosequencing approach.

## Materials and Methods

**Subject selection:** The study was approved by the Institutional Review Board of Soonchunhyang University (Approval number: 1040875-201409-BR-031). The woman volunteers (healthy, periodontitis, pregnant with periodontitis and menopausal with periodontitis) were recruited from the Soonchunhyang University Hospital, Asan, Republic of Korea. After obtaining affirmative consent from four woman volunteers, oral health status was evaluated by a dentist according to the Oral Health Survey Guidelines of World Health Organization (WHO) (Petersen and Baez, 2013). Oral health was evaluated considering three indexes. First, the Silness-Löe plaque index (PI) estimates the quantity of plaque in terms of tooth area covered. Second, the gingival index (GI) (Löe and Silness, 1963) assesses qualitative changes in the gingival condition, and third the periodontitis disease index (PDI) measures the periodontal status of an individual person (Ramfjord, 1967). All woman subjects showed signs of chronic periodontitis and had at least 24 teeth, including at least four molars. Further, it is noted that the periodontitis stage is more appropriate for examining the effect of female hormone on the subgingival microbiome than the gingivitis and advanced periodontitis stages (Table 1).

**Sampling:** Subgingival plaque samples were collected from right and left sides: 4 gingival sites (4 gingival sites/two teeth) from the first and second molar of mandible using Gracey curettes. Care was taken not to sample from infected sites such as deep caries, pulp necrosis, incomplete endodontic treatments, and secondary tooth decay. Further, samples were not taken from teeth with chronic apical periodontitis. The samples were lysed in 1ml of RNAzol solution and frozen at 70°C for later use.

**DNA extraction, PCR amplification and Pyrosequencing:** Bacterial DNA was extracted from oral plaque samples using Power Soil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's instructions. The purified DNA was quantified using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) and analyzed on an Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA).

PCR amplification was performed using primers targeting the V1 to V3 regions of 16S rDNA: Universal primers 27F (5'-CCTATCCCCTGTGTGCCTTGGCAGTC-TCAG-AC-AGAGTTTGATCMTGGCTCAG-3' (underlining primer-target sequence) and 518R (5'-CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-X-AC-CAGCAGCCGCGGTAATACG-3'); where 'X' indicates a unique barcode. PCR involved an initial denaturation of 95°C for 5 min; followed by 30 cycles of denaturation at 95°C for

**Table 1** : Clinical and demographic characteristics of four woman volunteers

Case	Woman participants	Age (year)	Oral Health Index		
			Plaque Index (PI) <sup>a</sup>	Gingival Index (GI) <sup>b</sup>	Periodontal Disease Index (PDI) <sup>c</sup>
1	Healthy woman	26	1	0	0
2	Woman with periodontal disease	25	3	2	5
3	Pregnant woman with periodontal disease	35	3	2	5
4	Menopausal woman with periodontal disease	51	3	3	6

<sup>a</sup>Plaque Index(PI): tooth is assigned a score of 0 to 3 (Løe and Silness, 1963). <sup>b</sup>Gingival index (GI): marginal and interproximal tissues are separately scored from 0 to 3(Løe and Silness, 1963). <sup>c</sup>Periodontal Disease Index(PDI): Six teeth are examined and scored. The higher the PDI score, greater the severity of periodontitis(Ramfjord, 1967)

30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and a final elongation at 72°C for 5 min. PCR products were resolved in 2% agarose gel electrophoresis and visualized using the Gel Doc system (BioRad, Hercules, CA). Amplicons were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Subsequently, equimolar concentrations of amplicons were prepared, clonally amplified on capture beads by emulsion PCR and the enriched DNA beads were deposited into a PicoTiter plate. Pyrosequencing was carried out at Chunlab Inc., (Seochogu, Seoul), using the GS Junior Sequencing System (Roche, Branford, CT) according to the manufacturer's instructions.

**Bioinformatics analysis:** The obtained reads were sorted according to their barcodes. The barcode, linker, and primer sequences were trimmed from the original sequencing reads. Further, reads containing ambiguous nucleotides, those with low quality scores (average < 25), and reads shorter than 300 bp were discarded from the analysis. Potential chimera sequences were detected by the Bellerophon method and removed from the analysis. We used the EzBioCloud Database (<https://www.ezbiocloud.net>) for taxonomic classification of reads (Yoon et al., 2017).

The richness and diversity of samples were determined by abundance-based coverage estimation and by calculating the Shannon diversity index at 3% distance. A revised complete sequence dataset was clustered and assigned to Operational Taxonomic Units (OTUs). CLCOMMUNITY software (Chunlab Inc., Seoul, Republic of Korea) was used to identify shared OTUs for comparison purposes. The species, genus, family and phylum levels were defined at 3%, 5%, 10% and 20% dissimilarities, respectively (Schloss and Handelsman, 2005). For taxonomy-based analysis, ARB-SILVA database 114 was used with an 80% confidence threshold (Quast et al., 2013). Rarefaction curves and richness estimates were analyzed using R VEGAN (Oksanen et al., 2013) and MOTHUR (Schloss et al., 2009) packages, respectively.

## Results and Discussion

The oral microbiome is an indicator of total body wellness and is influenced by factors such as sex hormone, diet, temperature and pH. The oral microbial communities can

modulate the outcome of diseases of other organs, e.g., association between periodontitis and lung diseases (Raghavendran et al., 2007), and cancer (Sabbah et al., 2019). Periodontitis (gingivitis) is an inflammatory condition of the gums that presents initially as bleeding gums, followed by plaque formation. The succession of bacterial communities during different phases of woman's growth (including puberty) has been reported (Contreras et al., 2006). However bacterial composition of subgingival plaque in females with periodontitis during pregnancy and menopause stages is not clear.

As shown in Table 2, a total of 14,048 reads passed quality control. The sequence comprised ~17,900 amplicons from V1 to V3 hypervariable regions of the 16S rRNA gene. Most of the high-quality reads showed 97% nucleotide similarity, indicating species - level phylotypes. The 3% sequence dissimilarity cut-off value is used in most microbial dynamics and diversity studies to profile species-level phylotypes. The reads showing a greater sequence variation were determined at the genus, family and order levels. The greatest proportion of reads with species-level phylotypes was registered from the sequence of healthy woman volunteer, followed by periodontitis, periodontitis and pregnant and periodontitis and menopausal subjects. Pyrosequencing identified 13 phyla, 124 genera and 372 species. The bacterial communities identified belonged to phyla Firmicutes, Bacteroidetes, Fusobacteria, Proteobacteria, Actinobacteria and Spirochaetes. Firmicutes predominated in the healthy and periodontitis subjects and Bacteroidetes in the pregnant and menopausal woman volunteer with periodontitis. Fusobacterium was also detected in abundance in all the woman volunteers. These data suggest a shift in the bacterial community under periodontitis condition from Firmicutes and Proteobacteria to Bacteroidetes and Fusobacteria. Spirochaetes were also detected in the pregnant and menopausal woman volunteers with periodontitis. At genus-level (Table 3), *Streptococcus* was the dominant bacteria in healthy woman(50.5%) and women with periodontal disease (25.9%). In pregnant and menopausal periodontitis woman, the dominant genera were *Alloprevotella* and *Prevotella*, respectively.

**Table 2** : Sequencing information and taxonomic assignments of plaque bacterial communities

Classification	Similarity range	No. of reads (%)			
		Healthy woman	Periodontitis woman	Pregnant Woman	Menopausal woman
Species	$x \geq 97\%$	4920 (98.73)	3233 (96.50)	2550 (95.93)	2895 (94.70)
Genus	$97 > x \geq 94.5\%$	52 (1.04)	93 (2.77)	79 (2.97)	96 (3.14)
Family	$94.5 > x \geq 86.5\%$	10 (0.20)	22 (0.65)	27 (1.01)	50 (1.63)
Order	$86.5 > x \geq 82\%$	1 (0.02)	0 (0.00)	2 (0.07)	14 (0.45)
Class	$82 > x \geq 78.5\%$	0 (0.00)	2 (0.05)	0 (0.00)	2 (0.06)
Phylum	$78.5 > x \geq 75\%$	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Unidentified	$75\% > x$	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Total	<b>14,048 reads</b>	4983 (100)	3350 (100)	2658 (100)	3057 (100)

**Table 3** : Relative genus-level bacterial composition in the oral plaque

Rank of genus	Healthy women (26 years old)			Women with periodontal disease (25 year old)			Pregnant woman with periodontal disease (35 year old)			Menopausal women with periodontal disease (51 year old)		
	Genus name	%	No.	Genus name	%	No.	Genus name	%	No.	Genus name	%	No.
1	<i>Streptococcus</i>	52.4	2612	<i>Streptococcus</i>	25.9	866	<i>Alloprevotella</i>	19.5	518	<i>Prevotella</i>	25.4	776
2	<i>Haemophilus</i>	15.6	779	<i>Fusobacterium</i>	22.4	749	<i>Leptotrichia</i>	14.3	379	<i>Fusobacterium</i>	13.9	426
3	<i>Veillonella</i>	14.0	697	<i>Prevotella</i>	6.6	222	<i>Fusobacterium</i>	12.3	326	<i>Streptococcus</i>	9.4	287
4	<i>Actinomyces</i>	5.0	248	<i>Parvimonas</i>	4.5	149	<i>Porphyromonas</i>	12.0	319	<i>Atopobium</i>	6.6	203
5	<i>Lautropia</i>	3.8	188	<i>Alloprevotella</i>	4.2	141	<i>Treponema</i>	9.6	256	<i>Treponema</i>	6.5	198
6	<i>Rothia</i>	2.9	146	<i>Treponema_g1</i>	4.0	134	<i>Treponema_g1</i>	4.2	111	<i>Dialister</i>	3.6	110
7	<i>Fusobacterium</i>	1.9	95	<i>Actinomyces</i>	3.2	108	<i>Peptostreptococcus</i>	3.1	81	<i>Shuttleworthia</i>	3.1	94
8	<i>Prevotella</i>	1.2	59	<i>Leptotrichia</i>	2.9	96	<i>Prevotella</i>	2.7	71	<i>Megasphaera</i>	2.4	74
9	<i>Neisseria</i>	0.8	41	<i>Selenomonas_g1</i>	2.7	90	<i>Mycoplasma_g4</i>	2.6	70	<i>Alloprevotella</i>	2.0	61
10	<i>Capnocytophaga</i>	0.7	35	<i>Fretibacterium</i>	1.7	58	<i>Selenomonas</i>	2.1	57	<i>Olsenella</i>	1.8	54
11	<i>Aggregatibacter</i>	0.2	11	<i>Corynebacterium</i>	1.6	54	<i>Catonella</i>	2.1	56	<i>Eubacterium_g10</i>	1.7	51
12	<i>Campylobacter</i>	0.2	9	<i>Campylobacter</i>	1.6	52	<i>Fretibacterium</i>	1.8	49	<i>Porphyromonas</i>	1.6	50
13	<i>Kingella</i>	0.1	7	<i>Treponema</i>	1.4	47	<i>Johnsonella</i>	1.8	47	<i>Treponema_g1</i>	1.5	46
14	<i>Cardiobacterium</i>	0.1	6	<i>Saccharimonas</i>	1.4	46	<i>Selenomonas_g1</i>	1.7	45	<i>Eggerthia</i>	1.3	39
15	<i>Corynebacterium</i>	0.1	5	AF432141_g	1.3	43	<i>Streptococcus</i>	0.9	25	<i>Parvimonas</i>	1.2	36
16	<i>Granulicatella</i>	0.1	5	<i>Johnsonella</i>	1.2	41	<i>Parvimonas</i>	0.9	24	<i>Fretibacterium</i>	1.1	34
17	<i>Actinobaculum</i>	0.1	5	<i>Veillonella</i>	1.0	34	<i>Phocaeicola</i>	0.7	19	<i>Cryptobacterium</i>	1.0	30
18	<i>Leptotrichia</i>	0.08	4	<i>Gemella</i>	1.0	34	GQ422727_g	0.7	19	<i>Campylobacter</i>	0.9	28
19	<i>Veillonellaceae_uc</i>	0.08	4	<i>Aggregatibacter</i>	1.0	33	<i>Actinomyces</i>	0.6	17	<i>Actinomyces</i>	0.9	27
20	ETC	0.54	27	ETC	10.5	353	ETC	6.4	169	ETC	14.2	433

In healthy volunteer, *Streptococcus pseudopneumoniae* predominated, followed by *Haemophilus parainfluenzae*, *Streptococcus sanguinis* and *Veillonella dispar*. *Streptococcus* sp. predominated in the plaque microbiome of healthy woman volunteer; the species detected were *S. gordonii*, *S. tigurinus*, *S. mitis* and *S. dentisani*. In the periodontitis woman volunteer, *Fusobacterium nucleatum* and *S. pseudopneumoniae* were the predominant species (Table 4). Further, the other *Fusobacterium* and *Streptococcus* species that were represented in the subgingival plaque of periodontitis woman volunteer included *S. cristatus*, *F. periodonticum*, *F. canifelinum*, *S. oralis* and *S. mitis* (data not shown). *Alloprevotella tanneriae* and *F. nucleatum* also predominated in the pregnant and menopausal woman volunteers with periodontitis, respectively, followed by *P.*

*gingivalis* and *Prevotella multiformis*, respectively. The bacterial communities at genus and species levels in the pregnant and menopausal woman volunteers with periodontitis disease were considered closer to the bacterial community in the woman subject with periodontitis. Further, the abundance of *Veillonella* and *Streptococcus* is associated with health-associated biofilms (Bernardi et al., 2020; Sanz et al., 2017; Naginyte et al., 2019). In recent studies, *P. gingivalis* (Bacteroidetes), *Treponemas denticola* (Spirochaetes) and *F. nucleatum* (Fusobacteria) were major bacterial species causing periodontal disease (Pérez-Chaparro et al., 2014; Choi et al., 2018). In our study, while *F. nucleatum* predominated in woman volunteers with periodontitis, *P. gingivalis* was detected only in pregnant woman volunteers with periodontitis (Table 4). *F. nucleatum* is a species involved in

**Table 4** : Relative species-level bacterial composition in the oral plaque.

Rank of species	Healthy woman (26 year old)	%	Woman with periodontal disease (25 year old)	%	Pregnant woman with periodontal disease (35 year old)	%	Menopausal woman with periodontal disease (51 year old)	%
1	<i>Streptococcus pseudopneumoniae</i>	21.3	<i>Fusobacterium nucleatum</i>	19.0	<i>Alloprevotella tanneriae</i>	18.8	<i>Fusobacterium nucleatum</i>	13.6
2	<i>Haemophilus parainfluenzae</i>	15.3	<i>Streptococcus pseudopneumoniae</i>	13.0	<i>Porphyromonas gingivalis</i>	12.0	<i>Prevotella multiformis</i>	7.8
3	<i>Streptococcus sanguinis</i>	13.1	<i>Parvimonas micron</i>	4.5	<i>Fusobacterium nucleatum</i>	11.4	<i>Streptococcus anginosus</i>	6.3
4	<i>Veillonella dispar</i>	10.0	<i>Alloprevotella tanneriae</i>	3.9	AY134896_s	10.6	<i>Atopobium rimae</i>	6.3
5	<i>Streptococcus gordonii</i>	6.5	<i>Streptococcus cristatus</i>	3.4	<i>Peptostreptococcus stomatis</i>	3.0	<i>Prevotella nigrescens</i>	5.9
6	<i>Streptococcus tigurinus</i>	5.6	<i>Actinomyces cardiffensis</i>	2.6	<i>Mycoplasma faucium</i>	2.6	<i>Shuttleworthia satelles</i>	3.1
7	<i>Lautropia mirabilis</i>	3.8	<i>Treponema socranskii</i>	2.0	JN713365_s	2.2	<i>Dialister invisus</i>	2.6
8	<i>Streptococcus mitis</i>	2.5	<i>Prevotella intermedia</i>	1.8	<i>Leptotrichia wadei</i>	2.0	<i>Prevotella intermedia</i>	2.16
9	<i>Veillonella parvula</i>	2.4	<i>Fusobacterium periodonticum</i>	1.7	4P000005_s	1.8	AF385554_s	1.9
10	<i>Actinomyces viscosus</i>	2.3	<i>Prevotella baroniae</i>	1.7	AF287774_s	1.8	<i>Alloprevotella tanneriae</i>	1.9
11	<i>Streptococcus dentisani</i>	1.5	<i>Corynebacterium matruchotii</i>	1.5	<i>Fretibacterium fastidiosum</i>	1.8	<i>Anaeroglobus geminatus</i>	1.8
12	<i>Rothia dentocariosa</i>	1.5	<i>Fusobacterium canifelinum</i>	1.4	<i>Catonella morbi</i>	1.7	<i>Eubacterium infirmum</i>	1.6
13	<i>Fusobacterium nucleatum</i>	1.5	AY349371_s	1.4	<i>Selenomonas sputigena</i>	1.7	<i>Treponema medium</i>	1.6
14	<i>Rothia aera</i>	1.3	<i>Streptococcus oralis</i>	1.3	<i>Treponema medium</i>	1.6	<i>Treponema denticola</i>	1.5
15	<i>Actinomyces oris</i>	1.2	<i>Streptococcus mitis</i>	1.3	<i>Treponema socranskii</i>	1.2	<i>Eggerthia catenaformis</i>	1.3
16	Etc	10.0	Etc	39.5	Etc	25.7	Etc	40.8
Total		100		100		100		100

dental plaque development and is found in the oral tissue of woman with normal or high-risk pregnancies (Tateishi *et al.*, 2012). It is also closely associated with periodontal attachment loss and has local immunosuppressive activity (Silva *et al.*, 2015). Similarly, *P. gingivalis* is abundant in gingival sulcus during early and middle stages of pregnancy (Fujiwara *et al.*, 2017). This bacterium modulates the host inflammatory response directly or indirectly and destroys periodontal tissue (How *et al.*, 2016; Fujiwara *et al.*, 2017). Subject with periodontitis had greater taxonomic diversity than healthy subjects in agreement with a prior study (Griffen *et al.*, 2012).

Next-generation sequencing technologies facilitate analysis of microbial diversity. 16S rRNA pyrosequencing was utilized to profile the subgingival microbiome of woman with periodontitis. 454 pyrosequencing has been applied to profile the bacterial communities in throat, stomach and fecal samples of humans (Andersson *et al.*, 2008), and soil sediments samples (Pramanik *et al.*, 2015). All taxonomic classifications were assigned using Bayesian algorithm developed for Ribosomal Database Project (RDP) classifier and Ez Bio Cloud database. Pyrosequencing reads were utilized using the Ez Bio Cloud database which contains the phylotypes of cultured and uncultured microorganisms. A total of 195-300 OTUs per sample were detected in the periodontitis subject. OTUs are clustered 16S rRNA sequences and are used as proxies for species

classification (Choi *et al.*, 2018). Typically, 97% and 95% sequence identities are cut-offs for species and genera, respectively (de Gannes *et al.*, 2013), including in oral biofilms (Pushalkar *et al.*, 2011). But this is an approximate measure as two different species may have identical 16S rRNA sequences and/or a single species may have two copies of 16S rRNA gene that could differ by more than 97%. In this study, 94% of pyrosequencing reads were classified as species. The sequences were taxonomically classified by comparison with the SILVA database (at a threshold of 97% identity).

The total plaque bacterial community richness was estimated by rarefaction. To determine richness and diversity, OTUs were identified at genetic distances of 3%. In this study, the maximum 300 and 389.31 OTUs were observed and estimated in the woman subject with periodontitis condition. The ecological organization of communities was evaluated by calculating Shannon's diversity and Simpson's evenness indices. Shannon index values were 3.63, 4.32 and 4.35 in healthy, pregnant and periodontitis woman, respectively. The Simpson's index values were 0.053, 0.036 and 0.047 in the healthy, periodontitis, and pregnant woman volunteers with periodontitis, respectively (Table 5). Thus, bacterial diversity was higher in the volunteers with periodontitis compared to healthy volunteer. In subgingival plaques of healthy humans, four bacterial species dominated (60% of the total bacteria) whereas woman with periodontal

**Table 5** : Species richness, diversity and evenness of oral plaque. Estimated statistical values obtained from the Mothur program

Case	Read length (bp)		Observed OTUs	Estimated OTUs(Chao 1)	Simpson Evenness	Shannon diversity index
	Mean	Maximum				
Healthy woman	439.03	511	148	179.32	0.053	3.63
Periodontitis woman	449.11	511	300	389.31	0.036	4.35
Pregnant Woman	438.9	513	258	370.86	0.047	4.32

\*OTUs: Operational Taxonomic Unit

disease composed of 15 bacterial species (Table 4). This diversity of bacterial composition is for the following reasons: the microbial communities of oral cavity are in symbiosis with the host. However, in the presence of stressors, such as imbalance of hormone levels that can perturb this homeostasis, several oral infectious diseases may appear, including periodontitis (Sanz *et al.*, 2017). In healthy individuals, the oral bacterial microbiome encompasses approximately 700 commonly occurring phylotypes, approximately half of which can be present at any time in any individual (Gao *et al.*, 2018). These bacterial compositions of pregnancy and menopause women can be dynamic and adapt to a wide range of environments and interactions with other microbial species. Several evidences have been found that influence microbial communities in postmenopausal women, such as decrease of estrogen receptor-beta in oral mucosa, exfoliation of age-related normal buccal mucosa and increase in surface keratinization and lipid lamellae in the epithelial samples (Donald *et al.*, 2013). In pregnancy, the literature describes at least some reasons that may contribute to the exacerbation of periodontal inflammation in the presence of high levels of estradiol and progesterone (Massoni *et al.*, 2019). Some reasons are the vasodilatory effect of estrogen, suppression of the immune system, and quantitative and qualitative alterations of bacterial flora (Carrillo-de-Albornoz *et al.*, 2010).

The results of this preliminary study suggest that the subgingival microbial communities in woman with periodontitis during pregnancy or menopause tend to differ from that of healthy woman, and these species included certain periodontal pathogens *F. nucleatum* and *P. gingivalis*. The study of microbial community dynamics in women with periodontitis would be crucial to establish pathogenesis of periodontal diseases correlating it to pregnancy and menopausal stages. Additionally, the microbial species can act as biomarkers of hormonal changes in women.

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