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# Metagenome Analysis of Buccal Mucosal Biofilms to Identify Bacterial Prevalence in Subjects with and without type II Diabetes

Hari Priya A*, Kannan A, Krithika C L

Department of Oral Medicine and Radiology, SRM dental college, Bharathi Salai, Ramapuram, Chennai– 600089, Tamil Nadu, India

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ABSTRACT

To quantify the two most prevalent bacteria among Type II diabetic individuals and controls from the buccal mucosal biofilms using molecular methods. To compare the percent prevalence of Veillonella and Granulicetella bacteria in uncontrolled Type II diabetic individuals with a control group. Subjects selected randomly and categorized into two groups within the age range of 25 to 40 years diagnosed with and without Type II diabetes based on their HbA1c values. The samples of buccal mucosa biofilms are collected in sterile swabs and stored in bacterial lysis buffer, which was later subjected to quantification of DNA followed by 16S rRNA amplification and sequencing. The sequence obtained is then surveyed using BLAST Analysis to define the bacterial flora and two bacteria, namely Veillonella and Granulicatella are selected for further amplification and quantification by real-time PCR to express the bacteria in copy numbers. From the collected buccal mucosal biofilm samples (n=24), which was categorized into Type II diabetes (12) and non-diabetic (12). The sequence subjected to BLAST analysis gave a List of bacteria from which Veillonella sp. and Granulicatella sp. were selected and administered to real-time PCR for amplification and quantification, which revealed an increased bacterial prevalence in Type II diabetic subjects to non-diabetic subjects which was also proved statistically. Based on the results obtained, there is a significant prevalence of bacterial content in Type II diabetic subjects compared to non-diabetic subjects.

*Corresponding Author

Name: Hari Priya A
Phone: 9566157661
Email: dr.haripriya92@yahoo.com

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## INTRODUCTION

Diabetes mellitus, a pan- metabolic disorder, is characterized by chronic hyperglycemia. Diabetes a syndrome, rather than a disease entity, is classified according to whether hyperglycemia is the primary feature or is a part of some other disorder, such as those of pancreas, endocrine system, or some well defined genetic syndromes. The primary category of diabetes is presently differentiated into type 1 and type 2 depending on whether it emerges from subtotal destruction of $\beta$-cells of the pancreas with predominant absolute insulin deficiency (Type 1) or advances from a complex interaction between the deterioration of insulin action with relative insufficiency of insulin secretion (Type 2).

Amidst worldwide chronic diseases, diabetes and periodontitis seem to prevail the most with a bidirectional relationship, including their underlying regulatory mechanism. Hyperglycemic status has a direct effect on the microbial composition, col-

lagen metabolism and cellular function. By development of advanced glycation end-products (AGEs) contributing to the modification of the extracellular matrix with the binding to a cellular receptor, further exaggerating inflammation (Abass and Omer, 2011; Chang and Lim, 2012)

Various complications such as retinopathy with potential blindness, nephropathy leading to renal failure, neuropathy with increased risk of non-healing necrotic ulcers, cardiovascular, perivascular, cerebrovascular, autonomic dysfunction including sexual dysfunction may accompany diabetic individuals in the long run.

Periodontitis also is known as "the sixth complication of diabetes mellitus," is the most common oral disease associated with diabetes. Periodontitis is a multifactorial disease of the oral cavity as a consequence of local infections leading to irreversible destruction of the periodontia (Abass and Omer, 2011).

Specific protein-coding or gene structure are found using the phylogenetic markers. One of these markers is the 16S rRNA, it is the most widely used marker having a 1500 base pair gene coding for catalytic RNA that is part of the 30S ribosomal subunit making this the most advisable marker.

The bacterial microbes are site-specific relating to the vast surfaces present in the oral cavity, coated with a plethora of bacteria, the customary of bacterial biofilm, whereas the bacteria Veillonella and Granulicatella are most commonly evidenced bacterial microbe present in all sites of the oral cavity. Hence these both bacteria were selected for our study (Aas *et al.*, 2005).

In this study, we focused on utilizing culture-independent molecular techniques for understanding the breadth of the bacterial variance of Veillonella and Granulicatella in the oral cavity of Type II diabetic subjects with non-diabetic subjects.

The study was reviewed and approved by the SRM Dental College Institutional Review Board and Ethical Committee, IRB approval number SRMDC/IRB/2016/MDS/No.901. All the study participants provided informed, written and signed consent before study enrollment.

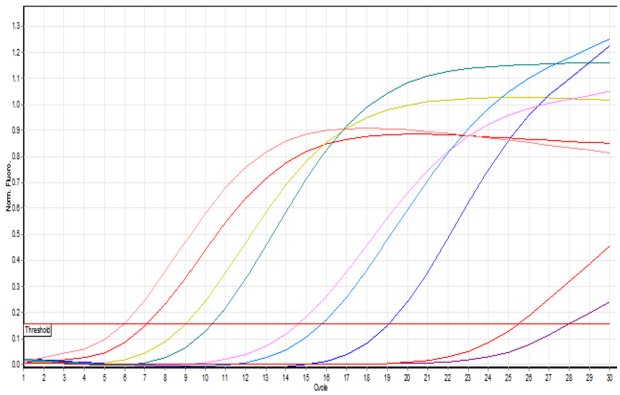
## MATERIALS AND METHODS

### Selection Criteria

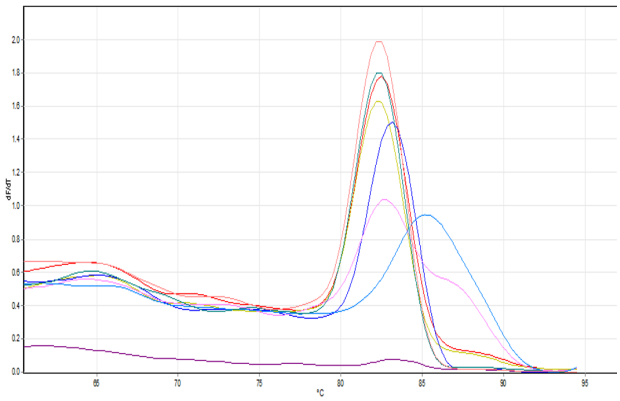
Twenty four subjects(n=24) were included in this study, comprising of both male and female genders, with an age group ranging from 25 years to 40years. They were divided into Twelve uncontrolled types II diabetic subjects and twelve non-diabetic subjects as a control based on their HbA1c value.

Subjects included should not suffer from any oral lesions and having less than 5mm deep periodontal pockets evaluated using the CPITN Index and subjects presented with neither redness nor inflammation of the gums were selected.

Subjects with caries on the teeth were excluded based on their DMFT Index. The subjects under any other drugs except antidiabetic medications for the last 6 months are excluded from this study.




Graph 1: Amplification Curves of *Veillonella* in Diabetic Samples 1 to 8




Graph 2: Melt Curves of *Veillonella* in Diabetic Samples 1 to 8

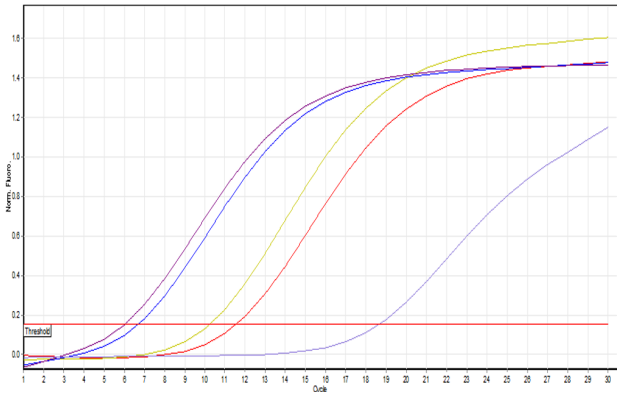
### Sample collection and DNA extraction

Buccal mucosal samples were collected with sterile swabs in universal bacterial lysis buffer containing 2% SDS (SIGMA-ALDRICH, Cat# 71736) and 10% Triton-X100 (SRL Fine Chemicals, Cat#64518).

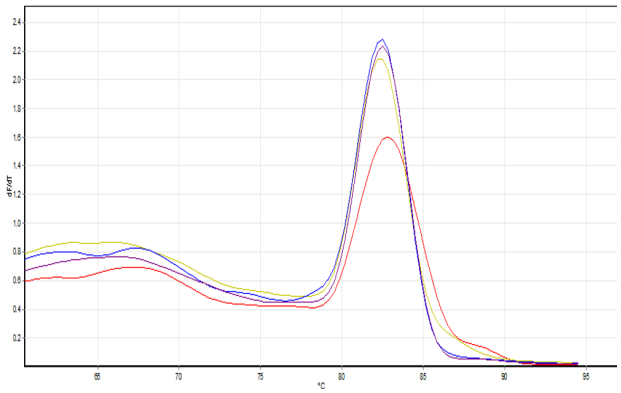
Bacterial cells were lysed by heating the samples at 95°C for 10 minutes.

### Quantification of DNA

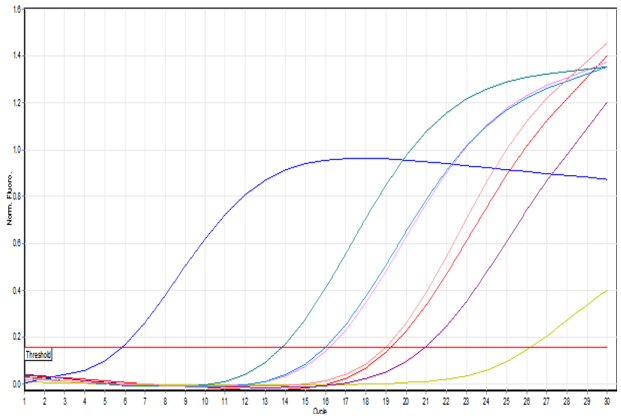
The DNA extracted from samples were quantified by QUBIT Fluorometer to determine the total DNA concentration.



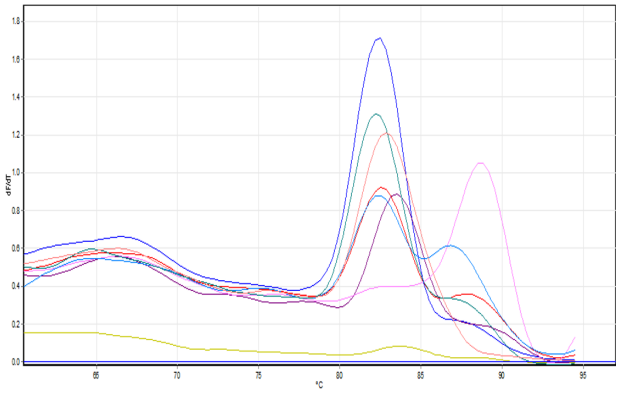
Graph 3: Amplification Curves of *Veillonella* in Diabetic Samples 9 to 12



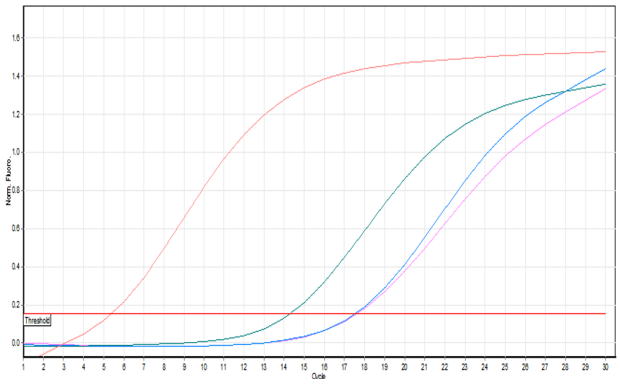
Graph 4: Melt Curves of *Veillonella* in Diabetic Samples 9 to 12



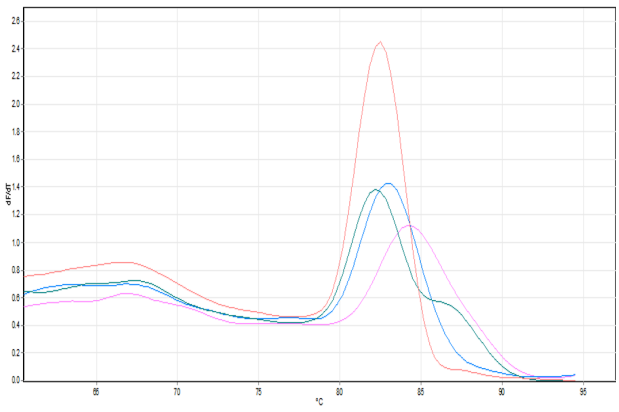
Graph 5: Amplification Curves of *Veillonella* in Control Samples 1 to 8



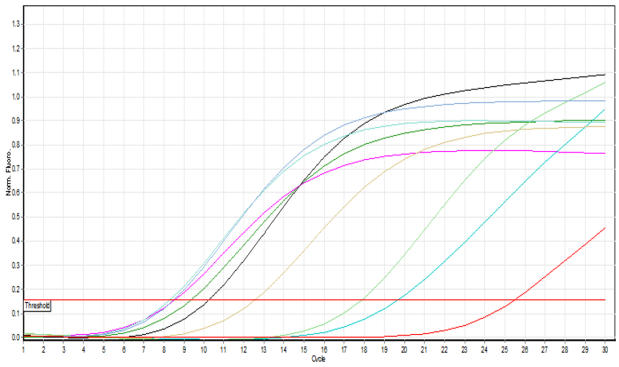
Graph 6: Melt Curves of *Veillonella* in Control Samples 1 to 8



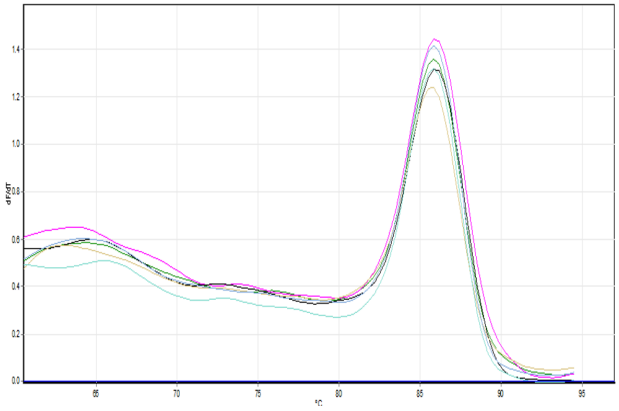
Graph 7: Amplification Curves of *Veillonella* in Control Samples 9 to 12



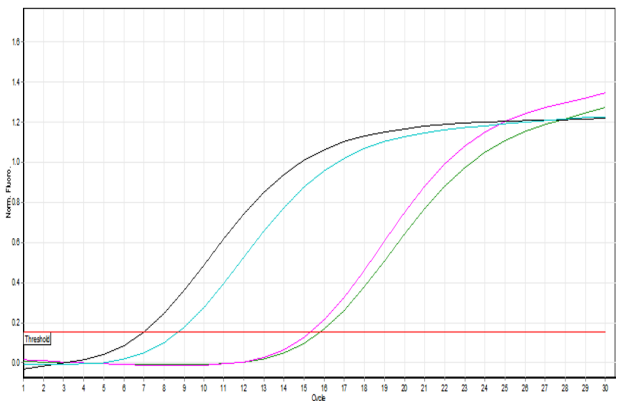
Graph 8: Melt Curves of *Veillonella* in Control Samples 9 to 12



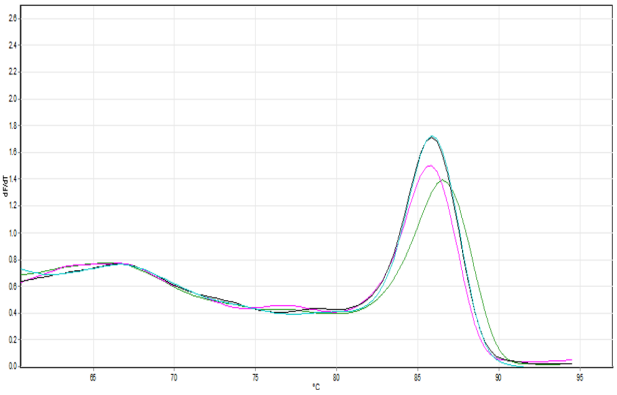
Graph 9: Amplification Curves of *Granulicatella* in Diabetic Samples 1 to 8



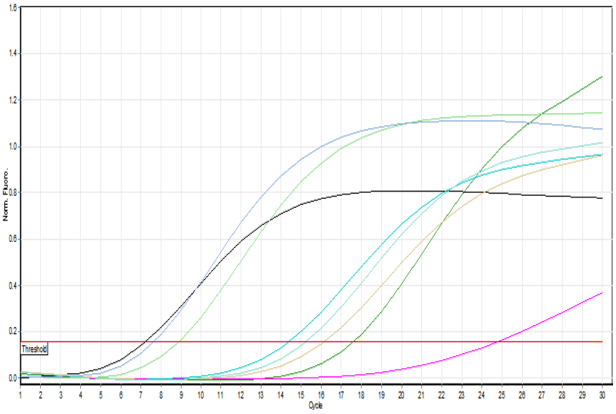
Graph 10: Melt Curves of *Granulicatella* in Diabetic Samples 1 to 8



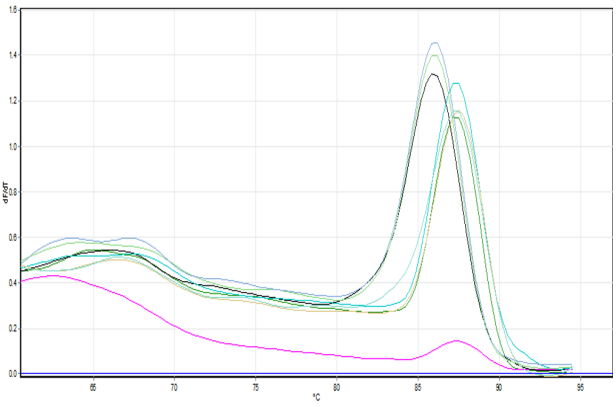
Graph 11: Amplification Curves of *Granulicatella* in Diabetic Samples 9 to12



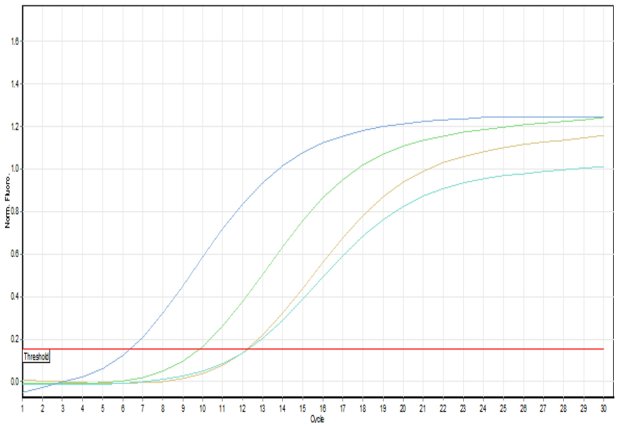
Graph 12: Melt Curves of *Granulicatella* in Diabetic Samples 9 to 12



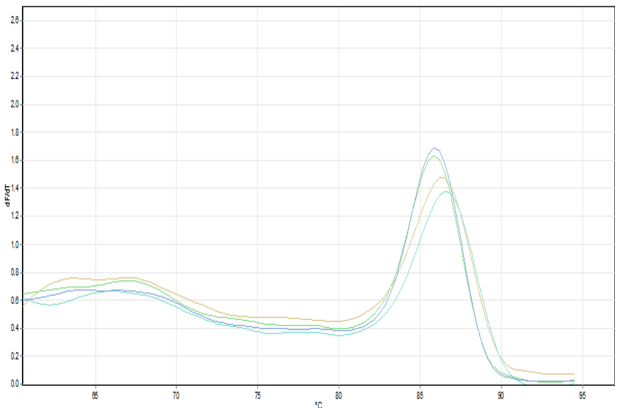
Graph 13: Amplification Curves of *Granulicatella* in Control Samples 1 to 8



Graph 14: Melt Curves of *Granulicatell*a in Control Samples 1 to 8




Graph 15: Amplification Curves of *Granulicatella* in Control Samples 9 to 12



Graph 16: Melt Curves of *Granulicatella* in Control Samples 9 to 12

### 16S rRNA amplification and sequencing

Equal concentration of genomic DNA samples was subjected to a polymerase chain reaction (PCR) for amplification of the 16S rRNA gene hypervariable regions V1 to V9 with the following set of primers: Forward: AGTTTGATCCTGGCTCAG, and Reverse: TACCTTGTTACGACTT under the following conditions. After an initial denaturation at 94°C for 4 minutes, the samples were subjected to 40 cycles of 94°C for 45 seconds, 48°C for 45 seconds, 72°C for 2 minutes, with a final extension at 72°C for 5 minutes. The PCR amplified products were cleaned with QIA quick PCR purification kit (Cat# 28104) to remove primer dimers, which otherwise may interfere with sequencing reactions. The purified PCR amplicons were then subjected to sanger sequencing (also called direct sequencing) to determine the bacterial species present in the samples (Table 1).

### BLAST Analysis

The nucleotide sequences identified by sanger sequencing were compared to the oral microbiome database to determine the presence of bacterial species that are known to be present in the oral cavity. The oral microbiome database is a publicly accessible free database and is available at http://www.homd.org/.

### Amplification and quantitation of Veillonella and Granulicatella by Real-time PCR

To identify the quantitative presence of the above two bacteria (Table 2 & Table 3), the DNA samples obtained from the patients were subjected to real-time PCR analysis to determine their quantitative presence. 2ng of total DNA was subjected to polymerase chain reaction (PCR) amplification with species-specific primers in the rotor gene Q real-time PCR unit. The following set of primers that are present within the 16S rRNA gene were used for each of the species:

**Veillonella**

Forward: CCGTGATGGGATGGAAACTGC

Reverse: CCTTCGCCACTGGTGTTCTTC

**Granulicatella**

Forward: GGCGCTAACAGTTACTCTAGCAC

Reverse: ACTCCTACGGGAGGCAGC

10$\mu$M of each of the above primers were added to BRYT-green RT-Master Mix (Cat# A6001, Promega Corporation, USA) in 20$\mu$l reaction, and samples were analyzed in Rotor-Gene Q real-time PCR equipment (Qiagen, Germany). The following amplification condition was used: after an initial denaturation at 95°C for 10 min, samples were amplified for 25 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s. The following six standards were used to identify the copy number of amplicons in each of the sample - 106, 105, 104, 103, 102 and 101. Upon completion of the real-time run, the samples were quantified with in-system software about standards, and the presence of bacteria was expressed as copy numbers.

### STATISTICAL ANALYSIS

The descriptive statistical analysis obtained for Veillonella and Granulicatella bacteria in both Type II Diabetic and Control groups is demonstrated in the (Table 4 & Table 5). The Paired T-Test obtained for both Veillonella and Granulicetella bacteria in Both Diabetic and Control groups is demonstrated in the (Table 6 & Table 7). Based on the statistical analysis obtained we reject the null hypothesis

The result shows us a significant increase in bacterial content of Type II Diabetic subject's oral cavity in comparison to non-diabetic subjects with a p-value <0.05.

**Table 1: List of Oral Bacterial Flora Obtained from Blast Analysis**

| S.No | Description | Max Score | Total Score | Query Cover | E value | Ident |
|---|---|---|---|---|---|---|
| 1 | Uncultured Granulicatella sp. partial 16S rRNA gene, isolate W438T_4032 | 226 | 226 | 78% | 8e-55 | 68% |
| 2 | Granulicatella adiacens gene for 16S rRNA, strain: GF02 | 856 | 856 | 100% | 0.0 | 95% |
| 3 | Granulicatella para-adiacens strain C593 16S rRNA gene, partial sequence | 856 | 856 | 100% | 0.0 | 95% |
| 4 | Granulicatella elegans gene for 16S rRNA, partial sequence, strain: PAGU 792 | 856 | 856 | 100% | 0.0 | 95% |
| 5 | Uncultured Sphingomonas sp.clone EHFS1_S03b 16S rRNA gene, partial sequence | 854 | 854 | 100% | 0.0 | 95% |
| 6 | Granulicatella adiacens strain 04356101 16S rRNA gene, partial sequence | 852 | 852 | 100% | 0.0 | 95% |
| 7 | Gemella haemolysans clone IC039 16S rRNA gene, partial sequence | 62.6 | 62.6 | 22% | 5e-06 | 71% |
| 8 | Gemella taiwanensis strain NTUH_5572 16S rRNA, partial sequence | 392 | 392 | 71% | 2e-105 | 76% |
| 9 | Gemella parahaemolysans strain NTUH_1465 16S rRNA, partial sequence | 392 | 392 | 71% | 2e-105 | 76% |
| 10 | Gemella sanguinis strain 2045-94 16S rRNA gene, p. sequence | 392 | 392 | 71% | 2e-105 | 76% |
| 11 | Gemella morbillorum gene for 16S rRNA, p. sequence, strain: JCM 12968 | 387 | 387 | 71% | 1e-103 | 75% |
| 12 | Oceanobacillus iheyensis partial 16S rRNA gene, strain Marseille-P1365 | 340 | 340 | 69% | 1e-89 | 74% |
| 13 | Granulicatella adiacens strain 04356101 16S rRNA gene, p. sequence | 340 | 340 | 69% | 1e-89 | 74% |
| 14 | Bacillus asahii strain FJAT-28834 16S rRNA gene, p. sequence | 338 | 338 | 72% | 4e-89 | 74% |
| 15 | Oceanobacillus kimchii partial 16S rRNA gene, strain Marseille-P393 | 336 | 336 | 69% | 1e-88 | 74% |
| 16 | Oceanobacillus iheyensis strain AM9 16S rRNA gene, p. sequence | 336 | 336 | 69% | 1e-88 | 74% |
| 17 | Haloalkaliphilic bacterium Ve2-10-82 16S rRNA gene, partial sequence | 336 | 336 | 69% | 1e-88 | 74% |

*Continued on next page*

*Table 1 continued*

| S.No | Description | Max Score | Total Score | Query Cover | E value | Ident |
|---|---|---|---|---|---|---|
| 18 | Uncultured Firmicutes bacterium clone QZ-J41 16S rRNA gene, partial sequence | 331 | 331 | 69% | 6e-87 | 74% |
| 19 | Bacillus okhensis strain Kh 10-101 16S rRNA gene, partial sequence | 322 | 322 | 69% | 3e-84 | 74% |
| 20 | Corynebacterium singulare strain C2E1 16S rRNA gene, partial sequence | 71.6 | 71.6 | 14% | 1e-08 | 73% |
| 21 | Uncultured Alcaligenes sp. clone JLAF 16S rRNA gene, partial sequence | 57.2 | 57.2 | 9% | 2e-04 | 76% |
| 22 | Uncultured Ruminococcaceae bacterium clone S1_B41 16S rRNA gene, partial sequence | 51.8 | 51.8 | 11% | 0.010 | 73% |
| 23 | Actinobacterium CA10 16S rRNA gene, p. sequence | 51.8 | 51.8 | 9% | 0.010 | 77% |
| 24 | Propionibacterium sp. CA7 16S rRNA gene, p. sequence | 51.8 | 51.8 | 9% | 0.010 | 77% |
| 25 | Candidatus Methylopumilus planktonicus partial 16S rRNA gene, strain MMS-14A-134 | 48.2 | 48.2 | 8% | 0.12 | 75% |
| 26 | Oscillibacter velericigenes strain AGM2 16S rRNA gene, p. sequence | 48.2 | 48.2 | 10% | 0.12 | 75% |
| 27 | Uncultured Parvimonas sp. partial 16S rRNA gene, isolate 78N_17281 | 46.4 | 46.4 | 15% | 0.40 | 70% |
| 28 | Pelosinus sp. UFO1, complete genome | 46.4 | 46.4 | 4% | 0.40 | 87% |
| 29 | Uncultured Bifidobacterium sp. clone 24B-39 16S rRNA gene, p. sequence | 46.4 | 46.4 | 11% | 0.40 | 74% |
| 30 | Uncultured Myobacterium sp. isolate DGGE gel band my6 16S rRNA gene, partial sequence | 46.4 | 46.4 | 9% | 0.40 | 93% |
| 31 | Uncultured alpha proteobacterium clone WT172B01F 16S rRNA gene, p. sequence | 46.4 | 46.4 | 8% | 0.40 | 74% |
| 32 | Uncultured Microlunatus sp. gene for 16S rRNA, partial sequence, clone, Up30 | 44.6 | 44.6 | 11% | 1.4 | 72% |
| 33 | Actinomyces sp. ICM58a 16S rRNA gene, partial sequence | 44.6 | 44.6 | 11% | 1.4 | 72% |
| 34 | Arcanobacterium haemolyticum strain WH01 16S rRNA gene, partial sequence | 42.8 | 42.8 | 9% | 4.9 | 75% |
| 35 | Arcanobacterium haemolyticum strain WH01 16S rRNA gene, partial sequence | 42.8 | 42.8 | 9% | 4.9 | 75% |

*Continued on next page*

*Table 1 continued*

| S.No | Description | Max Score | Total Score | Query Cover | E value | Ident |
|---|---|---|---|---|---|---|
| 36 | Propionibacterium acnes subsp. acnes strain HKG 284 16S rRNA gene, p. sequence | 42.8 | 42.8 | 9% | 4.9 | 75% |
| 37 | Streptococcus gallolyticus strain VTM1R38 16S rRNA gene, pa. sequence | 354 | 354 | 98% | 4e-94 | 78% |
| 38 | Streptococcus macedonicus strain PON54 16S ribosomal RNA gene, partial sequence | 354 | 354 | 98% | 4e-94 | 78% |
| 39 | Streptococcus pneumoniae SPN994039 draft genome | 345 | 1355 | 98% | 2e-91 | 77% |
| 40 | Uncultured Firmicutes bacterium clone F14_5B_FL 16S ribosomal RNA gene, partial sequence | 345 | 345 | 93% | 2e-91 | 78% |
| 41 | Streptococcus mitis strain CIP 104997 16S rRNA gene, partial sequence | 345 | 345 | 93% | 2e-91 | 78% |
| 42 | Streptococcus cuniculi partial 16S rRNA gene, strain NED 12-00041-7C | 343 | 343 | 92% | 7e-91 | 79% |
| 43 | Streptococcus bovis strain FMA766 16S rRNA gene, partial sequence | 342 | 342 | 96% | 3e-90 | 78% |
| 44 | Uncultured Serratia sp. clone C137 16S rRNA gene, partial sequence | 342 | 342 | 93% | 3e-90 | 78% |
| 45 | Streptococcus sp. oral taxon 431 strain 5-91 16S rRNA | 340 | 340 | 93% | 9e-90 | 78% |
| 46 | Streptococcus equinus strain CCUG 4214 16S rRNA gene, partial sequence | 340 | 340 | 93% | 9e-90 | 78% |
| 47 | Rumen bacterium IVRI-RM-1006 16S rRNA gene, partial sequence | 340 | 340 | 89% | 9e-90 | 79% |
| 48 | Streptococcus macedonicus strain EQ29 16S rRNA gene, partial sequence | 338 | 338 | 99% | 3e-89 | 77% |
| 49 | Bacterium NLAE-zl-C272 16S rRNA gene, p. sequence | 338 | 338 | 96% | 3e-89 | 78% |

## RESULTS AND DISCUSSION

### Sequence Analysis

To determine the bacterial species present in the buccal swab samples of patients with and without Type II diabetes, the DNA extracted from the buccal swabs of five uncontrolled diabetic patients and five non-diabetic controls were first amplified by PCR with universal bacterial primers that are capable of amplifying the 16S rRNA gene of all bacterial species in any given sample. The PCR amplicons thus obtained were subjected to sanger sequencing. Of the above samples, all diabetic samples showed interpretable sequence outputs, whereas only one from the control group showed interpretable sequence output.

This could be because the amounts of bacterial DNA in these controls were either absent or were present below detectable limits of the present amplification protocol. Sequence analysis by comparison of the identified sequence with those in the oral microbiome database (http://www.homd.org/) indicated the presence of Granulicatella in most of the uncontrolled diabetic samples. One sample showed Veillonella as the top hit, followed by Granulicatella during data analysis. The one control sample showed none of the above two bacteria but had mostly streptococcus species.

### Real-Time PCR Analysis

To determine the relative copies of Veillonella and Granulicatella in the buccal samples, the samples were amplified in the presence of species-specific primers as described in the methods section. Veillonella and Granulicatella specific signals were identified by running a melt curve analysis cycle (Graph 1, Graph 2, Graph 3, Graph 4,Graph 5, Graph 6, Graph 7, Graph 8, Graph 9, Graph 10, Graph 11, Graph 12, Graph 13, Graph 14, Graph 15, Graph 16). This involved a temperature ramp step that ranged between 50°C to 99°C with an initial hold for 90 seconds followed by a rise of 1°C at each step with a 5-second hold to enable the melting process. Analysis of the melt curve showed a sharp peak for Veillonella at 82.5°C and Granulicatella at 86°C. Those samples that showed similar peaks were included for analysis, while those that deviated from these control peaks were excluded from further analysis as these indicated primer-dimers or non-specific amplification. The data analysis was done after taking the average of indicated copy numbers for each of the bacteria. This showed Veillonella to be present 3.5 fold more in the diabetes samples than the controls, while Granulicatella was present 1.5 fold more in the diabetes samples than controls.

Arctaeus of Cappadocia coined the term Diabetes, literally meaning "siphon," characteristic of polyuria in uncontrolled disease. In the recent times, Diabetes has taken over the title, " Captain of the men of death," from tuberculosis and syphilis so much so, that the diabetologist is considered the last of internists, a physician who needs to have intimate knowledge of the function of each organ of the body in order to effectively treat his patient.

Recent works of literature displayed a bidirectional relationship between diabetes and periodontitis in which subjects with controlled glycemic metabolic level as measured by HbA1c demonstrated a slower attachment loss than uncontrolled subjects, in contrast, few studies showed an improvement in glycemic control following periodontal treatment (Chang and Lim, 2012).

Po-Chun Chang and Lum Peng Lim, in their study, suggested an epidemiological correlation between diabetes and periodontal health wherein, diabetes is said to affect the periodontia due to hyperglycemic levels and indirectly regulated by advanced glycation end-products(AGEs).

The main virulence factor of the microorganisms involved in periodontitis is lipo, 125 Polysaccharides (LPSs) an endotoxin, the pathogenesis is activated by identification of pathogen-associated molecular structures from Toll-like receptors (TLRs), release of ROS from defending cells by correspondingly inducing oxidative stress, proinflammatory cytokines, and immunoregulatory complexes through the NF-kB pathway (Chang and Lim, 2012).

J. Michael Janda and Sharon L. Abbott, in their literature, showed that the exposed taxa are directly in debt to ease the role of the 16S rRNA gene sequence disputed to the more incommodious administration of DNA-DNA hybridization investigations. DNA-DNA hybridization is the emphatically gold standard for recommended unfamiliar species and for the precise stint of a strain with obscure properties to correct the taxonomic unit (Janda and Abbott, 2007).

Carl Woese and many other researchers commenced to analyze and sequence the 16S rDNA genes of diverse bacterial phylotypes using DNA sequencing, a prompt technology used three decades before, which is still under process with many advancements in technology till date (Woo *et al.*, 2008).

Ramya Srinivasan et al. proposed a genus-level concordance rate of 96% and species-level concordance rate of 87.5%, in their study, to distinguish the difference between 16S rRNA based on conventional culture-based identification in a wide range of

**Table 2: Bacterial Quantification of Veillonella in Type II Diabetic and Non Diabetic Subjects**

| Sample No. | Veillonella | | | | | |
|---|---|---|---|---|---|---|
| | Diabetes 1 to 12 | | | Control 1 to 12 | | |
| | Individual id | Ct | Calc Conc (copies/ul) | Individual id | Ct | Calc Conc (copies/ul) |
| 1 | 1v | 6.54 | 4,423,798,058 | 1v | 19.27 | 776,075 |
| 2 | 2v | 8.68 | 1,153,702,723 | 2v | 26.21 | 9,937 |
| 3 | 3v | 18.91 | 3,477,366,073 | 3v | 5.88 | 1,868,724 |
| 4 | 4v | 27.79 | 7,084 | 4v | 20.95 | 270,201 |
| 5 | 5v | 14.25 | 34,856,671 | 5v | 16.22 | 5,257,039 |
| 6 | 6v | 15.58 | 15,140,325 | 6v | 16 | 6,045,280 |
| 7 | 7v | 10.08 | 480,195,179 | 7v | 13.86 | 23,148,047 |
| 8 | 8v | 5.13 | 10,730,874,309 | 8v | 18.99 | 920,269 |
| 9 | 9v | 11.61 | 85,890,380 | 9v | 17.66 | 1,922,050 |
| 10 | 10v | 10.29 | 197,383,466 | 10v | 17.59 | 2,010,276 |
| 11 | 11v | 6.73 | 1,844,990,568 | 11v | 14.34 | 15,516,216 |
| 12 | 12v | 6.04 | 4,188,128,590 | 12v | 5.42 | 2,839,090,722 |
| | | Average = | 1,817,316,517 | | Average = | 643,447,504 |

**Table 3: Bacterial Quantification of Granulicatella in Type II Diabetic and Non Diabetic Subjects**

| Sample No. | Granulicatella | | | | | |
|---|---|---|---|---|---|---|
| | Diabetes 1 to 12 | | | Control 1 to 12 | | |
| | Individual id | Ct | Calc Conc (copies/ul) | Individual id | Ct | Calc Conc (copies/ul) |
| 1 | 1g | 9.25 | 805,183,962 | 1g | 17.61 | 2,192,350 |
| 2 | 2g | 8.04 | 1,726,794,022 | 2g | 24.83 | 23,585 |
| 3 | 3g | 10.24 | 1,470,358,588 | 3g | 7.25 | 432,372,988 |
| 4 | 4g | 19.63 | 1,191,166 | 4g | 14.41 | 16,423,520 |
| 5 | 5g | 12.31 | 118,198,939 | 5g | 16.16 | 5,468,252 |
| 6 | 6g | 17.91 | 3,498,443 | 6g | 8.91 | 517,898,421 |
| 7 | 7g | 8.01 | 1,759,612,598 | 7g | 15.27 | 9,537,470 |
| 8 | 8g | 8.27 | 1,496,015,219 | 8g | 7.66 | 1,136,519,350 |
| 9 | 9g | 15.84 | 6,021,388 | 9g | 12.26 | 57,189,921 |
| 10 | 10g | 15.37 | 8,085,091 | 10g | 9.86 | 258,863,518 |
| 11 | 11g | 7.02 | 1,532,943,937 | 11g | 12.34 | 54,301,449 |
| 12 | 12g | 8.75 | 518,618,518 | 12g | 6.42 | 2,239,925,060 |
| | | Average = | 700,711,356 | | Average = | 480,725,124 |

**Table 4: Mean and Standard Deviation for Veillonella**

| Paired Samples Statistics | | Mean | N | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Pair 1 | Veillonella | 1230382010.9167 | 24 | 2476820103.60452 | 505578786.54154 |
| | Group | .5000 | 24 | .51075 | .10426 |

**Table 5: Mean and Standard Deviation for Granulicatella**

| Paired Samples Statistics | | Mean | N | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| Pair 1 | Granulicatella | 590718239.7917 | 24 | 728975981.57345 | 148801599.13329 |
| | Group | .5000 | 24 | .51075 | .10426 |

**Table 6: Paired T-test for Veillonella**

| Paired Samples Test | | | Paired Differences | T | Df | Sig. (2-tailed) |
|---|---|---|---|---|---|---|
| | | | 95% Confidence Interval of the Difference Upper | | | |
| Pair 1 | Veillonella Group | – | 2276251414.94015 | 2.434 | 23 | .023 |

**Table 7: Paired T-test for Granulicatella**

| Paired Samples Test | | | Paired Differences | T | Df | Sig. (2-tailed) |
|---|---|---|---|---|---|---|
| | | | 95% Confidence Interval of the Difference Upper | | | |
| Pair 1 | Granulicatella Group | – | 898537799.86157 | 3.970 | 23 | .001 |

gram-negative rods and gram-positive cocci as well as common gram-negative cocci (Srinivasan *et al.*, 2015).

Jørn A. Aas et al., in their study on defining the normal oral microflora conducted in nine different sites of the oral cavity, suggested that the predominant bacterial species found were; Gemella, Granulicatella, Streptococcus, and Veillonella evident involving most of the sites (Aas *et al.*, 2005).

Izumi Mashima et al. reported the distribution and frequency of Veillonella species showcasing the prime species in both periodontal pockets and gingival sulcus is V.parvula also with other species such as V.atypica, V.dispar, V.tobetsuensis with V.rogasae the least found in the oral cavity (Mashima *et al.*, 2018).

Supathep Tansirichaiya et al. determined the genes causing the reduction in antimicrobial susceptibility by oral microbes. In their study, the gene fabl enclosing enoyl-acyl carrier protein reductase(ENR), an essential protein for fatty acid synthesis and the gene gale, was evidenced in E.coli and Veillonella parvula species which inhibits the binding of antiseptics to bacteria thus conferring antimicrobial resistance (Yungun-Dong and Jongno-Gu, 2007).

Peng Zhou et al. considered that Veillonella species as early colonizers and which can aggregate with initial colonizer and periodontal pathogen. Veillonella species provide various nutrients for the growth of the pathogen. They also provide a bridging effect in the ecology of the oral cavity, they modify the local environment and produce hemin, which helps the growth of lateral colonizers. Thus the knowledge on them will help in disease prevention. These species are more tolerant of O2. Veillonella is not only to aggregate with various bacteria in the oral cavity but can also optimize the microbes for periodontopathogen (Zhou *et al.*, 2017).

As in this study, the chief microbial organisms involved in periodontitis like Porphyromonas gingivalis, Tannerella forsythia, and Treponema denticola were not identified. Inconsistent with the previous pieces of literature, this study proposed a significant predominance of Veillonella and Granulicatella species in Type II diabetic subjects projecting as a causative factor for periodontitis in comparison to non-diabetic subjects.

## CONCLUSIONS

This study demonstrates the efficacy of metagenomic analysis of 16S rRNA for defining the bacterial flora and also adding on to the previous kinds of literature, projects the abundance of bacterial quan-

tity in Type II Diabetic subjects to non-diabetic subjects with statistical correlation. It is fundamental to have a thorough knowledge on the bacterial diversity, the impact of diabetes on periodontia and vice-versa, for executing appropriate management of oral infections in diabetic patients with specific antibiotic therapy to avert antibiotic resistance, an upcoming global treat.

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