EVALUATION OF THE STABILITY AND POTENTIAL USE OF HUMAN ORAL MICROBIOME DNA SIGNATURES FOR INDIVIDULIZATION IN FORENSICS

[1]Iroanya, Onyekachi Ogbonnaya, [2]Iromenu, Nkiru Susan, [3]Egwuatu, Tochukwu Frank, &[4]Folorunso, Stephania Semiire

[1,2,3,]Department of Cell Biology and Genetics, University of Lagos, Akoka Yaba, Nigeria

[4]Department of Nursing,University College, Birmingham

Corresponding Author:
Dr. Onyekachi Ogbonnaya Iroanya
Department of Cell Biology and Genetics, University of Lagos, Akoka Yaba, Nigeria
oiroanya@unilag.edu.ng or onyiiog@yahoo.com
+234 802 954 3243
Scopus Author ID: 35749924100
https://orcid.org/0000-0002-5122-1466
https://scholar.google.com/citations?hl=en&user=u86ISmMAAAAJ

ABSTRACT

In forensic science, human identification is a critical aspect that seeks to match or exclude individuals from crime scenes. Current identification methods focus heavily on DNA analysis. However, where DNA is heavily degraded or biological samples are not readily available, especially relating to sexual crimes involving kissing, licking or spitting then characterization of the oral micrbiome to aid identification might become useful. This study aimed at evaluating the possibility of identifying humans using the DNA fingerprints of their oral microbiome. Saliva samples were collected from the ten participants on days 0 and 30 respectively. Total bacterial DNA was isolated using ZymoBIOMICS™ DNA Miniprep Kitfor microbiome or metagenome analyses and was further subjected to metagenomics assay.

In all the samples, *Streptococcus* species were the most stable. Neisseriawas stable in samples 1, 4, 6 and 9. In samples 1, 2, 4, 5 and 8, *Haemophilus parainfluenzae* was one of the predominant species identified over time. *Prevotella melaninogenica, Streptococcus alactolyticus* and *Porphyromonas* species were identified and stable in samples 1, 4 and 8, samples 2, 4, 8 and 9 and samples 4, 7 and 9 respectively.This study identifies *Streptococcus* as the most abundant microbial specie common to individual oral micrbiome irrespective of nutrition, environment and social status. However, there was no distinct bacterial identifier for each individual, although further studies will be required to establish this. This study aimed at evaluating the possibility of identifying humans using the DNA fingerprints of their oral microbiome.

Keywords: Oral micrbiome, DNA Fingerprints, Saliva, Metagenomics Assay, *Streptococcus* species

1.0 INTRODUCTION

Metagenomics is a technology, or a group of procedures, whose primary goal is to identify the microbial population that may be found in a given environment, as well as the community in which it exists (Garrido-Cardens *et al.,* 2017). Metagenomics has the potential to reveal the genomes of the majority of microbes that are difficult to obtain in pure culture (**Hugenholt, 2002**). The National Research Council (US) Committee on Metagenomics identified the utility of metagenomics in the field of forensic science in 2007 to precisely identify and characterize microbes that have played a role in war, acts of terrorism, and crime events, thus assisting in the discovery of the microbes' source and the party responsible for their use (N.R.C., 2011). Over the last five years, sequencing techniques for non-human deoxyribonucleic acid (DNA) analysis have evolved dramatically, and the cost of using such tools has become inexpensive (Oliveira and Amorim, 2018). However, proper adoption of metagenomics in forensics necessitates more basic research, ranging from sample selection to data processing, and the process remains plagued with problems and uncertainty (Mason-Buck *et al.,* 2020).

The human microbiome is referred to as the total microorganisms found in and on the human body (**Lee *et al*, 2015)**. Human identification is and will continue to be crucial in forensic research (Leake, 2013). However, trace evidence such as poor quality and a low copy number of Deoxyribonucleic acid (DNA) cannot be discovered with the rigor required for the prosecution of violent crimes. An astute criminal can take steps to contaminate the crime scene with blood, sperm, and fingerprints, making it more difficult to identify the perpetrator (**Lee *et al.,* 2015).** The present forensic DNA intelligence methods rely on estimating information from human DNA, but recent evidence suggests that non-human DNA analysis can considerably improve this. Metagenomic profiles (Bacteria, Archaea, and Eukaryota-fungi, plants, and animal DNA) derived from environmental or casework samples can provide independent and supplementary intelligence to aid an investigation (Arenas *et al., 2017;* Oliveira *et al.,* 2019). The aim of this study was to evaluate the possibility of identifying humans using the DNA fingerprint of their oral micrbiomes.

2.0 MATERIALS AND METHODS

2.1 SAMPLE COLLECTION AND PROCESSING

Based on their willingness to participate voluntarily, a total of ten healthy volunteers who were not on any form of antibiotic therapy were selected to participate in the study. Of the 10 participants in this research were eight males and two females. Buccal swab samples were collected from all the participants. The participants were required to take a buccal swipe with swab sticks first thing in the morning before their teeth were brushed. Samples were taken on the first day (Day 0) and the thirtieth day (Day 30) from each participant. A total of twenty oral swab samples were collected for both Day 1 and Day 30. The samples were labeled appropriately. Each sample (swab stick tip) was stored in 1 mL of DNA Shield contained in an Eppendorf tube and stored at 4°C for further analysis.

Ethical Approval

This study was approved by the Health Research Ethics Committee of the University of Lagos, Nigeria (CMULHREC Number: CMUL/HREC/11/21/973).All the study processes were explained to the participants and important information was collected from the participants. The participants also signed a written consent form.

2.2 TOTAL BACTERIAL GENOMIC DNA EXTRACTION PROTOCOL

Approximately 750 µL of each sample preserved and lysed in DNA/RNA ShieldTM was transferred to ZR BashingBead™ Lysis Tubes (0.1 and 0.5 mm) and secured in a bead beater equipped with a 2 mL tube holder assembly. This was processed at 9000 rpm for 1 minute using optimum beat beating conditions. After the bead-bashing procedure, the mixture in the ZR BashingBead™ Lysis Tubes (0.1 and 0.5 mm) was centrifuged at 10,000 x g for 1 minute in a microcentrifuge. After that, 400 µL of supernatant were transferred to a Collection Tube with a Zymo-Spin™ III-F Filter and centrifuged for 1 minute at 8,000 x g. The Zymo-Spin™ III-F Filter was removed from the mix. The filtrate in the Collection Tube was mixed well with approximately 1,200 µL of ZymoBIOMICS™ DNA Binding Buffer. Approximately 800 µL of the mixture was centrifuged at 10,000 x g for 1 minute in a Zymo-Spin™ IICR Column in a Collection Tube. The collection tube's flow through was discarded. In a fresh Collection Tube, 400 µL ZymoBIOMICS™ DNA Wash Buffer 1 was introduced to the ZymoSpinTM IICR Column and centrifuged for 1 minute at 10,000 x g. The flow-through was discarded. Approximately 700 µL of ZymoBIOMICS™ DNA Wash Buffer 2 was added to the ZymoSpin™ IICR Column in a Collection Tube and centrifuged at 10,000 x g for 1 minute. The flow-through was discarded. Approximately 200 µL of ZymoBIOMICS™ DNA Wash Buffer 2 to the ZymoSpin™ IICR Column in a Collection Tube and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IICR Column was transferred to a clean 1.5 mL microcentrifuge tube and 100 µL of ZymoBIOMICS™ DNase/RNase Free Water was directly added to the column matrix and incubated for 1 minute. Afterward, the mixture was centrifuged at 10,000 x g for 1 minute to elute the DNA. Each Zymo-Spin™ III-HRC Filter was placed in a new Collection Tube and 600 µL of ZymoBIOMICS™ HRC Prep Solution was added. The tube was centrifuged at 8,000 x g for 3 minutes. The eluted DNA was transferred to already prepared Zymo-Spin™ III-HRC Filter in a clean 1.5 mL microcentrifuge tube and centrifuged at exactly 16,000 x g for 3 minutes. The filtered DNA was stored at 4°C for further downstream applications.

2.3 METAGENOMICS ANALYSIS PROTOCOL

Targeted *16S rRNA* gene PCR was carried out using the ultra pure DNA isolated (first PCR).The *16S rRNA* gene was amplified with the following primers that are tagged with PacBio M13 adaptors,

Universal 27-F (Forward 5'-AGAGTTTGATCMTGGCTCAG-3', Reverse 5'-TAATACGACTCACTATAGGG-3) and

1492-R (Forward-5'-TACGGYTACCTTGTTACGACTT, Reverse 5'-GGTCAACAAATCATAAAGATATTGG-3').

Afterward barcoding PCR was performed (second PCR). During this process, each sample was assigned a barcode in a limited cycle PCR. This was immediately followed by a quality control (QC) and Pooling process. All samples were pooled equimolar and SMRTbell library was prepared according to PacBio M13 protocol.

2.4 PREPARING SMRTBELL® LIBRARIES USING PACBIO BARCODED M13 PRIMERS FOR MULTIPLEX SMRT® SEQUENCING

The 16S rRNA gene was amplified using target-specific PCR primers that were followed by M13 forward and reverse primers. The PCR product was then employed as an input into a second-round PCR reaction with barcoded M13 primers, yielding an asymmetrically barcoded PCR product for SMRTbell library creation. Multiple samples were processed simultaneously using this procedure, and the barcoded PCR products were pooled for SMRTbell library creation and sequenced (in multiplex) using the PacBio® Sequel® System or the Sequel II and Sequel IIe Systems (Sequel Systems). Different barcode sequences are utilized on the forward (F) and reverse (R) PCR primers in asymmetric barcoding. Each of the M13 forward PCR primer's barcodes can be used with any or all of the M13 reverse PCR primer's barcodes, and vice versa. A set of 32 forward primer sequences and 32 reverse primer sequences recommended by PacBio can be used to barcode up to 1,024 samples.

3.0 RESULTS

For sample one and on day 0, the phylum Firmicutes was the most predominant, followed by Proteobacteria, while least phylum was Verrucomicrobia. Streptococcus (47.17%), *Haemophilus parainfluenzae* (11.31%), *Neisseria* (6.82%) and*Prevotella melaninogenica* (3.24%) were the more predominant species on day 0. However, on day 30 the most common phylum was Firmicutes, which was followed by Actinobacteria, while least phyla were Verrucomicrobia, Tenericutes and Nitrospirae. On day 30, *Veillonella dispar* (31.68%), *Actinomyces* (20.81%), *Streptococcus* (12.96%), *Neisseria* (9.74%), *Haemophilus parainfluenzae* (4.05%), *Prevotella melaninogenica* (2.99%), *Streptococcus alactolyticus* (2.33%) and *Veillonella parvula* (2.21%) were more predominant. Figure 1a and 1b shows the bacteria which were present in sample one on days 0 and 30. *Streptococcus*, *Neisseria*, *Haemophilus parainfluenzae* and *Prevotella melaninogenicas*species were the most stable over time.

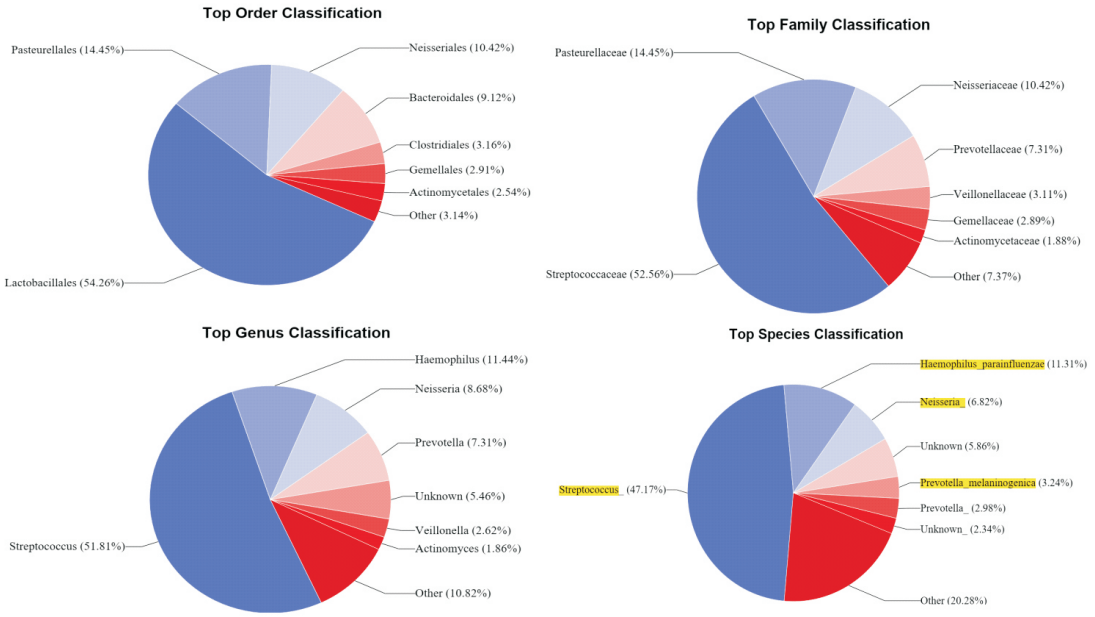

Figure 1a: Order, Family, Genus and Species classification 0f Bacteria in Sample 1 on day 0

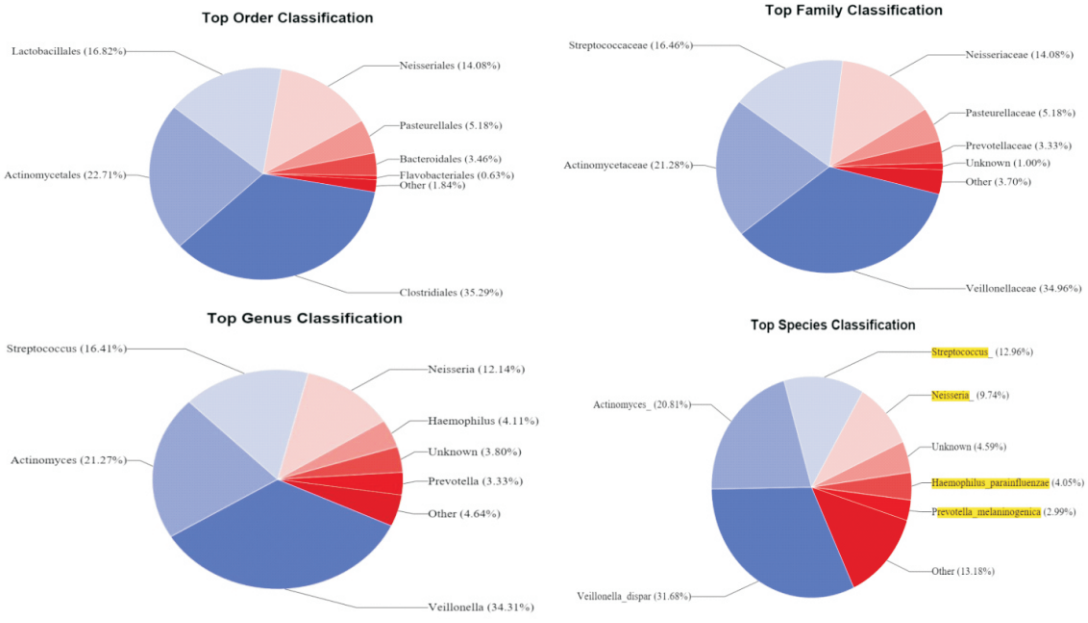

Figure 1b: Order, Family, Genus and Species classification 0f Bacteria in Sample 1 on day 30

For sample two on day 0, Firmicutes was the most predominant phylum followed by Bacteroidetes while least phylum was Verrucomicrobia.*Streptococcus* (36.59%), *Prevotella melaninogenica* (10.59%), *Haemophilus parainfluenzae* (10.42%), *Veillonella dispar* (9.46%), *Actinomyces* (5.07%), *Prevotella* (4.58%), *Streptococcus alactolyticus* (3.13%) were the predominant species on day 0. However, on day 30 the most common phylum was Proteobacteria, which was followed by Firmicutes, while least phylum was Spirochaetes. The predominant species on day 30 were *Neisseria* (46.84%), *Streptococcus* (19.13%), *Veillonella dispar* (7.72%), *Haemophilus parainfluenzae* (3.245) and *Streptococcus alactolyticus* (2.16%). Figure 2a and 2b shows the bacteria which were present in sample two on days 0 and 30. *Streptococcus*, *Haemophilus parainfluenzae*, *Veillonella dispar* and *Streptococcus alactolyticus* species were the most stable among all the bacteria identified over time.

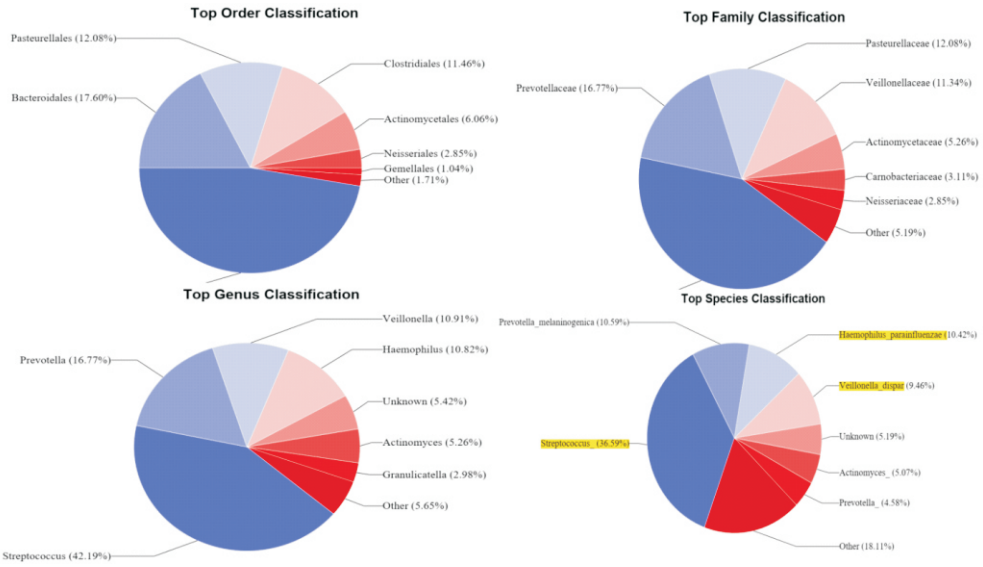

Figure 2a: Top Order, Family, Genus and Species classification 0f Bacteria in Sample 2 on day 0

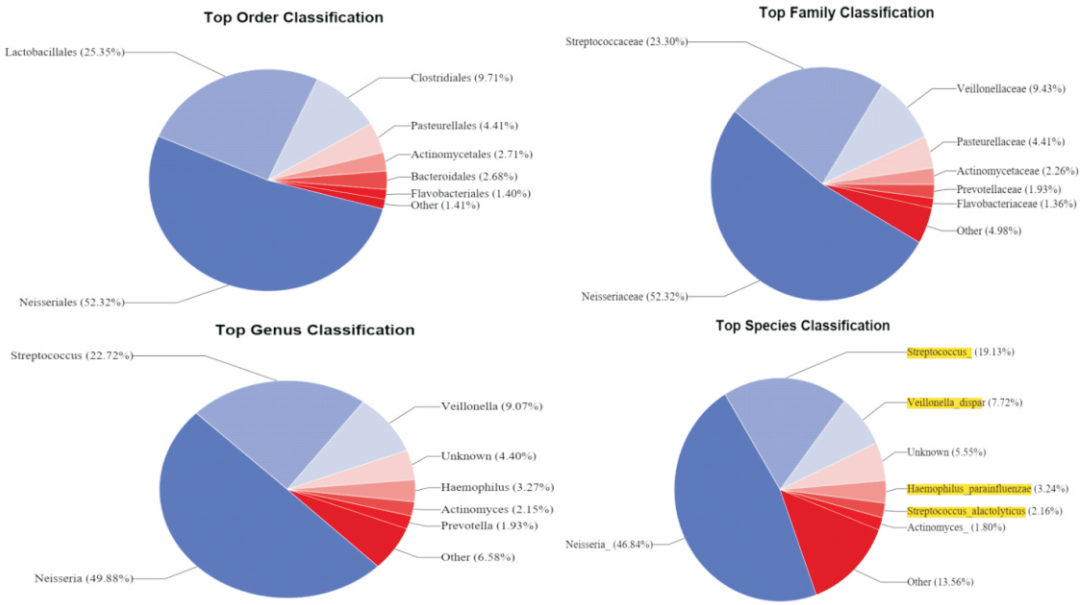

Figure 2b: Order, Family, Genus and Species classification 0f Bacteria in Sample 2 on day 30

For sample three, the sample for day 0 was lost in transit, hence, a comparison cannot be made regarding the bacteria obtained on both days.

For sample four and on day 0, the most common phylum was Proteobacteria, followed by Firmicutes and Bacteriodetes, while least phyla were Verrucomicrobia and Nitrospirae.*Streptococcus* (11.60%), *Neisseria* (11.60%), *Veillonella parvula* (7.61%), *Haemophilus parainfluenzae* (5.82%), *Porphyromonas* (5.76%), *Prevotella nanceiensis* (5.53%), *Prevotella melaninogenica* (4.52%), *Neisseria subflava* (3.27%), *Streptococcus alactolyticus* (2.86%) are some of the species identified on day 0. However, on day 30 the most common phylum was Firmicutes, followed by Bacteriodetes while the least phylum was Cyanobacteria. Streptococcus (29.39%), *Neisseria* (26.81%),*Porphyromonas* (5.74%),*Neisseria subflava* (3.62%), *Prevotella melaninogenica* (3.47%), *Streptococcus alactolyticus* (2.95%), *Prevotella nanceiensis* (2.25%), *Haemophilus parainfluenzae* (2.14%) were identified on the 30th day. *Streptococcus, Neisseria, Haemophilus parainfluenzae,Porphyromonas, Prevotella melaninogenica, Neisseria subflava, Streptococcus alactolyticus* species were the most stable among all the bacteria which were identified over time. Figure 3 shows the bacteria present in sample four on days 0 and 30.

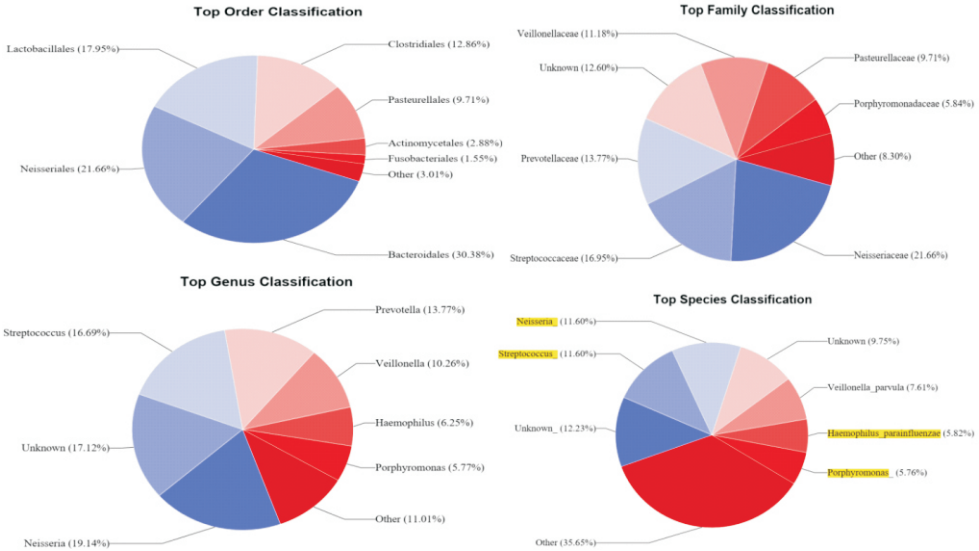

Figure 3a: Order, Family, Genus and Species classification 0f Bacteria in Sample 4 on day 0



Figure 3b: Order, Family, Genus and Species classification 0f Bacteria in Sample 4 on day 30

Figure 4 shows the bacteria that were present in sample five on days 0 and 30. For sample five and on day 0, the most common phylum was Firmicutes (71.98%), Bacteroidetes (14.16%), Proteobacteria (7.05%) and Actinobacteria (4.74%) while Synergistetes, Cyanobacteria and Spirochaetes were the least phyla.*Streptococcus* (44.24%), *Veillonella dispar* (12.62%), *Prevotella* (6.29%), *Streptococcus alactolyticus* (3.16%), *Actinomyces* (2.96%), *Prevotella melaninogenica* (2.95%), *Haemophilus parainfluenzae* (2.94%) were predominant on day 0. Firmicutes (68.45%) Proteobacteria (21.40%) and Bacteroidetes (8.67%) phyla were predominant while Lentisphaerae and Tenericutes were the least on day 30 *Streptococcus* (50.92%), *Haemophilus* (7.35%), *Granulicatella* (6.74%), *Haemophilus parainfluenzae* (6.04%) *Porphyromonas* (5.63%), *Gemella* (3.27%) species were more predominant on day 30. However, *Streptococcus* and *Haemophilus parainfluenzae* species were the most abundant in this sample on days 0 and 30.

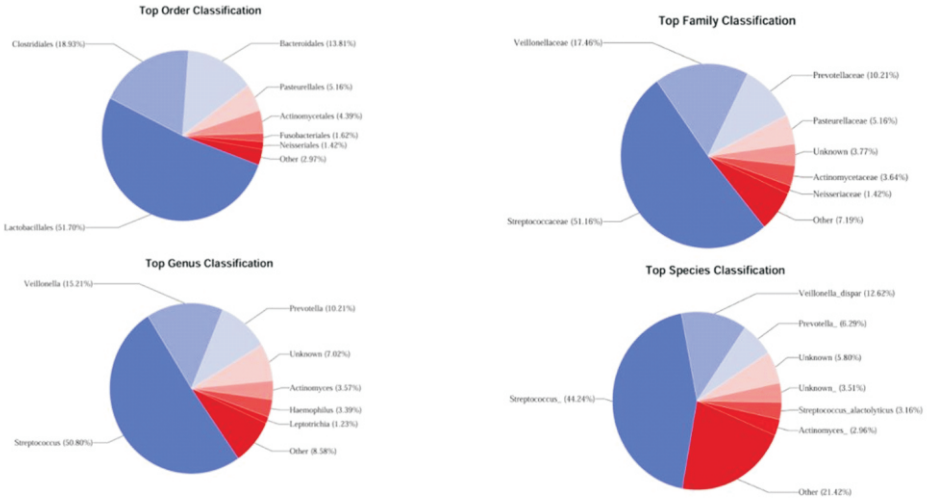

Figure 4a: Order, Family, Genus and Species classification 0f Bacteria in Sample 5 on day 0

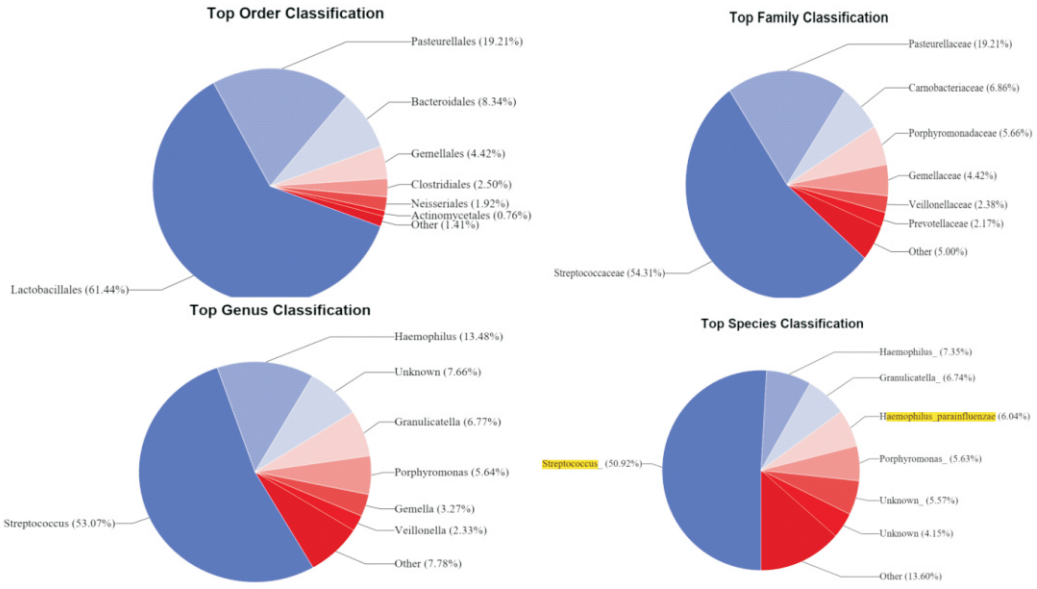

Figure 4b: Order, Family, Genus and Species classification 0f Bacteria in Sample 5 on day 30

Figure 5 shows the bacteria which were present in sample six on days 0 and 30. For sample six and on day 0, Proteobacteria was the most predominant phylum while least phyla were Verrucomicrobia and Nitrospirae.*Neisseria* (23.79%), *Streptococcus* (15.30%), *Actinobacillus parahaemolyticus* (4.60%), *Neisseria subflava* (3.83%), *Haemophilus parainfluenzae* (3.11%) and *Porphyromonas* (3.00%) species were identified on day 0. However, on day 30 Proteobacteria was the most predominant phylum while least phyla were Cyanobacteria, Planctomycetes, Chloroflexi and Verrucomicrobia. *Pseudomonas* (36.41%), *Streptococcus* (13.87%), *Neisseria* (7.54%), *Neisseria subflava* (3.22%), *Granulicatella* (2.36%) and *Streptococcus infantis* (2.32%) werepredominant on day 30. *Neisseria* and *Streptococcus* species were the most stable among all the bacteria identified over time.

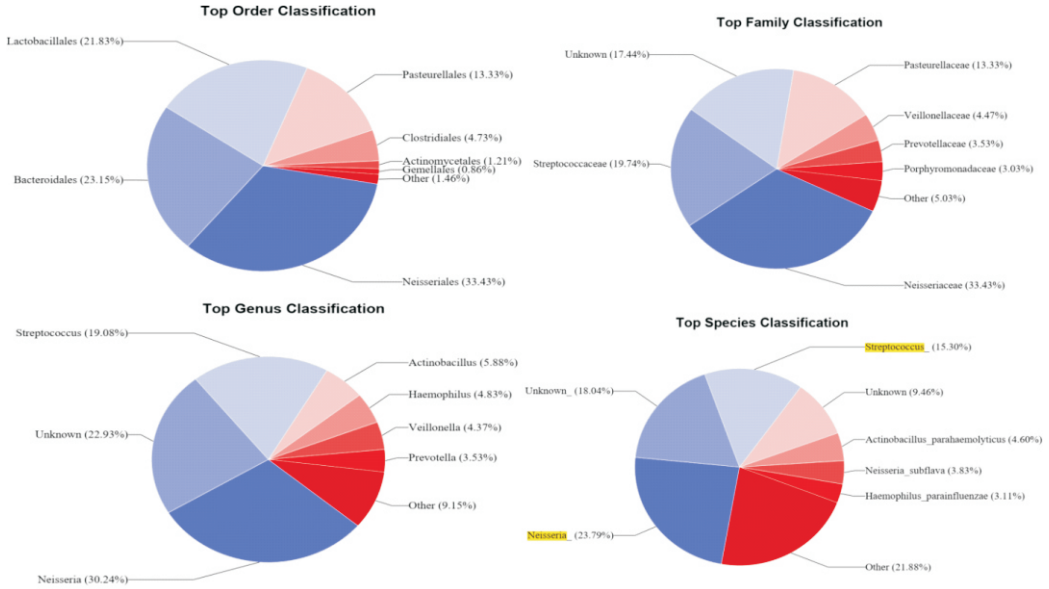

Figure 5a: Order, Family, Genus and Species classification 0f Bacteria in Sample 6 on day 0

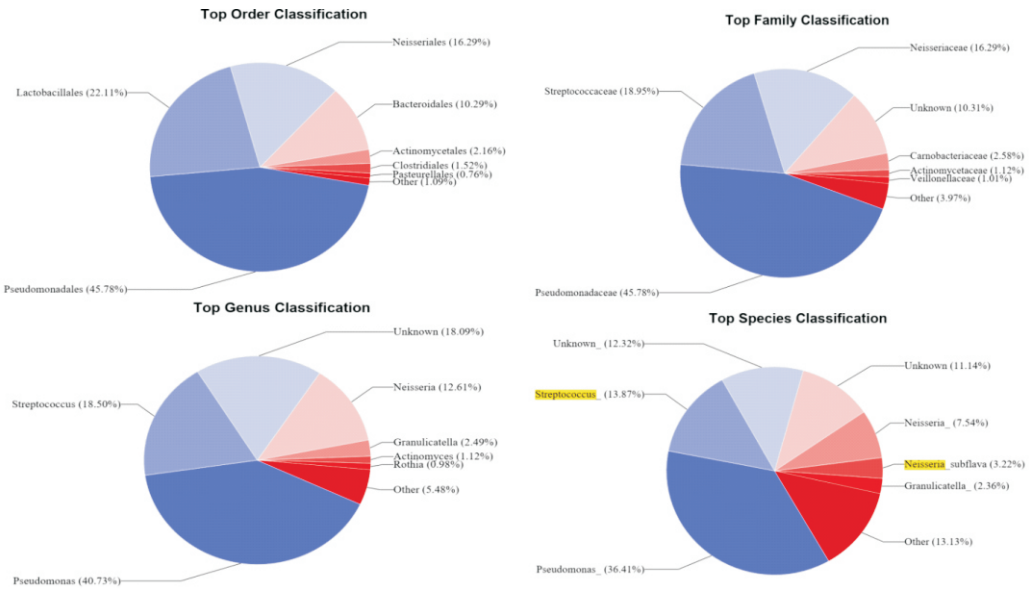

Figure 5b: Order, Family, Genus and Species classification 0f Bacteria in Sample 6 on day 30

For sample seven and on day 0, Firmicutes was the most predominant phylum while least phylum was Spirochaetes.*Streptococcus* (47.10%), *Porphyromonas* (8.68%), *Veillonella* (7.36%), *Granulicatella* (5.54%) and *Haemophilus* (2.89%) species were identified on day 0. However, on day 30 Firmicutes was the most abundant phylum while least phylum was Planctomycetes.*Streptococcus* (54.98%), *Porphyromonas* (12.20%), *Granulicatella* (3.63%), *Veillonella* (2.89%), *Haemophilus* (2.56%) and *Actinobacillus parahaemolyticus* (2.30%) species were predominant on day 30. Figure 6 shows the bacteria which were present in sample seven on days 0 and 30. *Streptococcus, Porphyromonas, Veillonella, Granulicatella*and Haemophilus species were the most stable among all the identified bacteria over time.

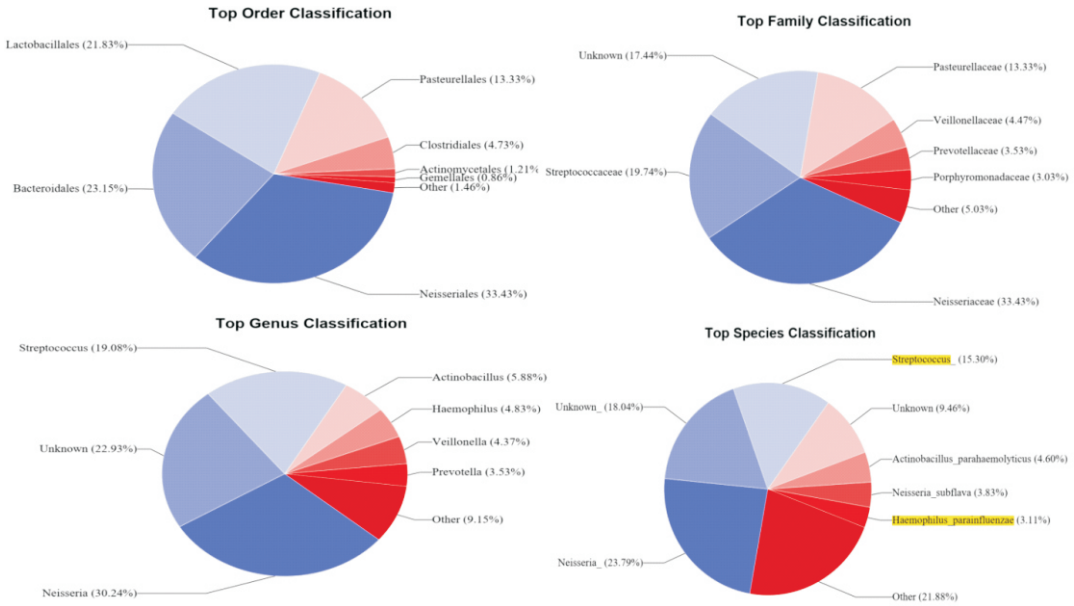

Figure 6a: Order, Family, Genus and Species classification 0f Bacteria in Sample 7 on day 0

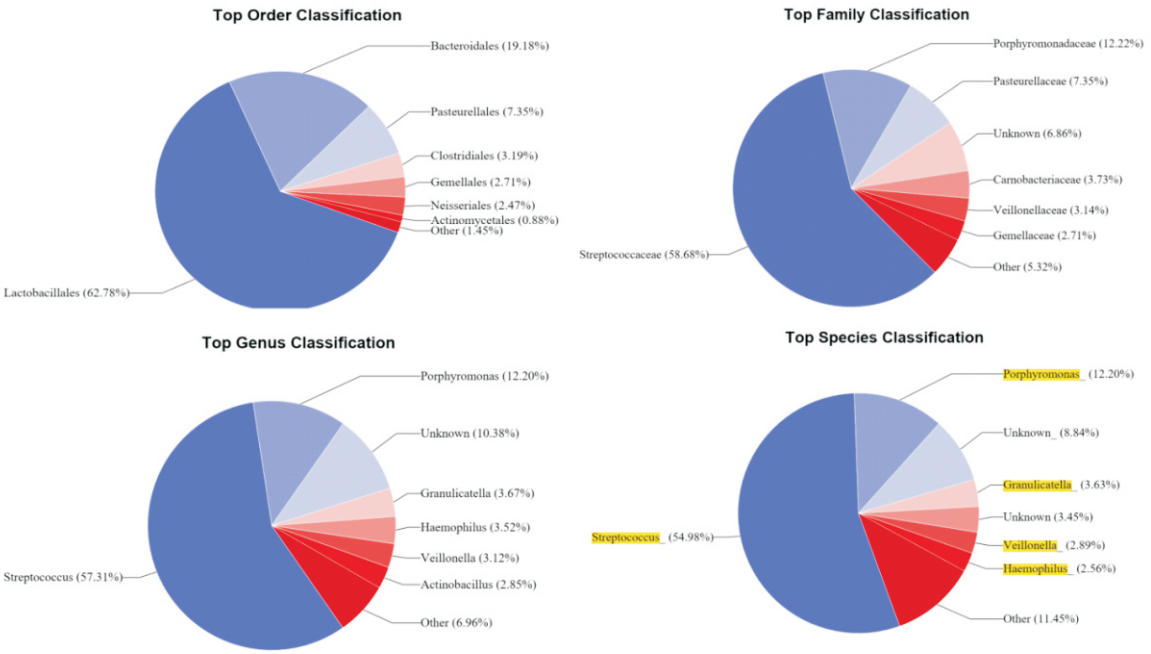

Figure 6b: Order, Family, Genus and Species classification 0f Bacteria in Sample 7 on day 30

For sample eight and on day 0, the phylum Firmicutes was the most common, followed by Bacteriodetes while least phyla were Tenericutes and Synergistetes.*Streptococcus* (34.62%), *Prevotella melaninogenica* (29.86%), *Haemophilus parainfluenzae* (5.02%), *Veillonella parvula* (3.84%), *Granulicatella* (2.44%), *Streptococcus alactolyticus* (2.35%) and *Prevotella nanceiensis* (2.23%) species were identified on day 0. However, on day 30 Firmicutes was the most abundant phylum while least phylum was Spirochetes. Streptococcus (36.97%), *Haemophilus parainfluenzae* (19.20%), *Veillonella parvula* (10.68%), *Prevotella melaninogenica* (4.27%), *Rothia mucilaginosa* (3.10%), *Streptococcus alactolyticus* (2.50%), Granulicatella (2.49%), *Actinomyces* (2.31%) and *Neisseria* (2.22%) species were predominant on day 30. Figure 7 shows the bacteria which were present in sample eight on days 0 and 30. Streptococcus, *Haemophilus parainfluenzae*, *Veillonella parvula*, *Prevotella melaninogenica*, *Streptococcus alactolyticus* and *Granulicatella*species were more stable among all the bacteria identified over time.

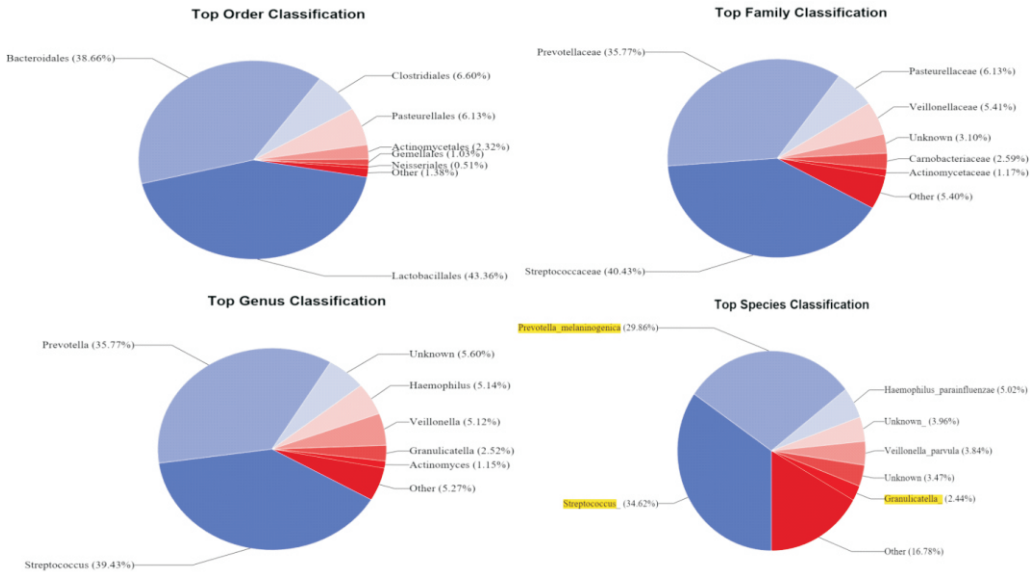

Figure 7a: Order, Family, Genus and Species classification 0f Bacteria in Sample 8 on day 0

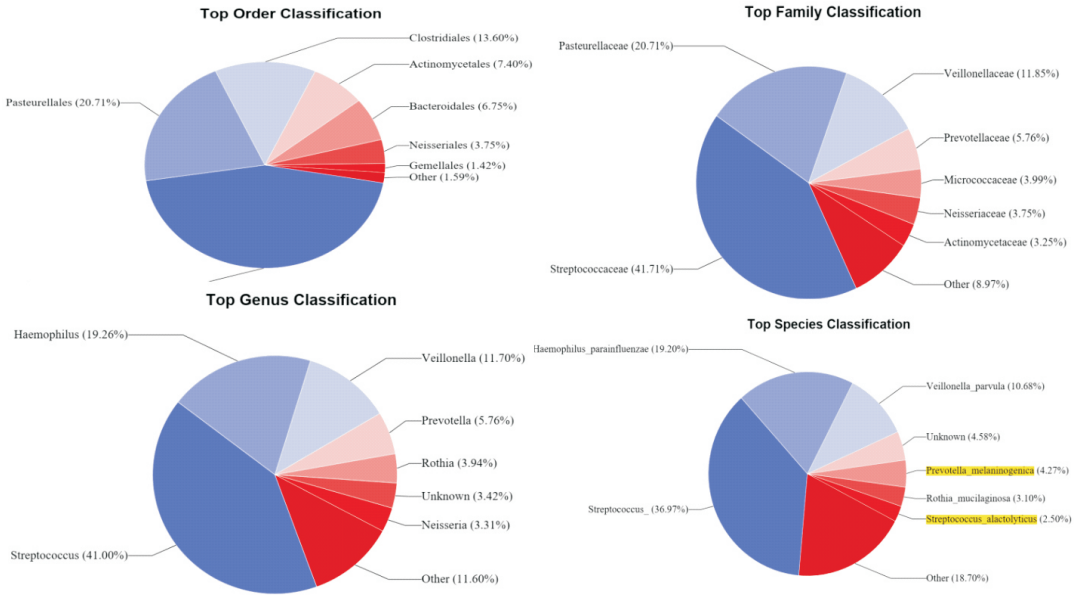

Figure 7b: Order, Family, Genus and Species classification 0f Bacteria in Sample 8 on day 30

For sample nine and on day 0, Firmicutes was the most predominant phylum followed by Bacteriodetes while least phylum was Spirochaetes. Among the species identified on day 0 were*Streptococcus* (28.84%), *Porphyromonas* (13.70%), *Neisseria* (9.49%), *Prevotella nanceiensis* (6.45%), *Haemophilus parainfluenzae* (4.24%), *Granulicatella* (3.18%), *Streptococcus alactolyticus* (2.71%).However, on day 30 the most common phylum was Firmicutes, which was followed by Proteobacteria and Bacteriodetes while least phylum was Spirochaetes.*Streptococcus* (23.53%), Neisseria (20.21%), *Porphyromonas* (8.67%), *Veillonella parvula* (3.78%), *Prevotella pallens* (2.86%), *Neisseria subflava* (2.60%), *Streptococcus alactolyticus* (2.60%), *Prevotella nanceiensis* (2.38%) were identified on the 30th day. Figure 8 shows the bacteria which were present in sample nine on days 0 and 30.*Streptococcus*, *Porphyromonas*, *Neisseria*, *Streptococcus alactolyticus* and *Prevotella nanceiensis* species were the most stable among all the bacteria identified over time.

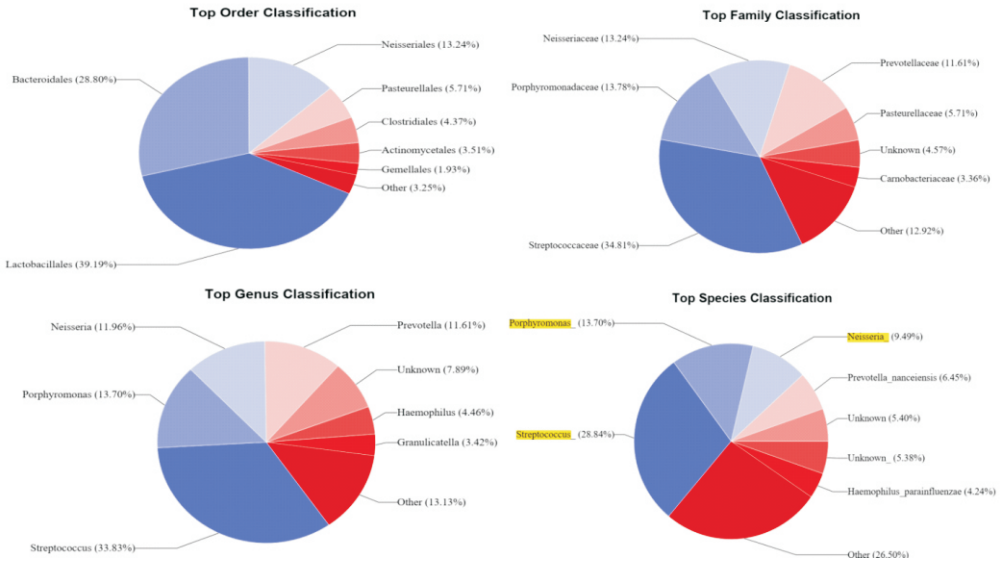

Figure 8a: Order, Family, Genus and Species classification 0f Bacteria in Sample 9 on day 0

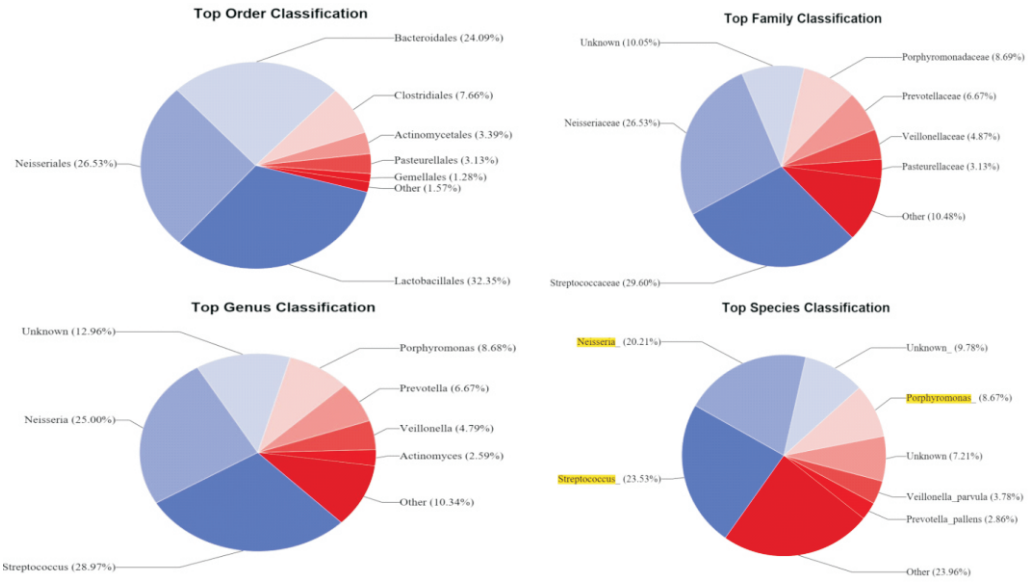

Figure 8b: Order, Family, Genus and Species classification 0f Bacteria in Sample 9 on day 30

For sample ten, on day 0 Proteobacteria was the most predominant phylum while least phylum was Verrucomicrobia.*Neisseria* (26.93%), *Streptococcus* (14.10%), *Porphyromonas* (6.76%), *Haemophilus parainfluenzae* (5.38%), *Haemophilus* (5.03%), *Neisseria subflava* (3.73%), *Prevotella melaninogenica* (1.93%) and *Prevotella nanceiensis* (1.22%) species were among the organisms identified on day 1. However, on day 30 the most common phylum was Firmicutes, which was followed by Actinobacteria while least phyla were Nitrospirae and Spirochaetes.*Streptococcus* (37.31%), *Prevotella melaninogenica* (20.76%), *Veillonella dispar* (6.18%), *Actinomyces* (4.87%), *Streptococcus alactolyticus* (4.07%), Neisseria (3.38%) and *Actinomyces hyovaginalis* (2.67%) species were identified on the 30th day. Figure 9 shows the bacteria which were present in sample ten on days 0 and 30. *Streptococcus* species were the most stable among all the bacteria which were identified over time.

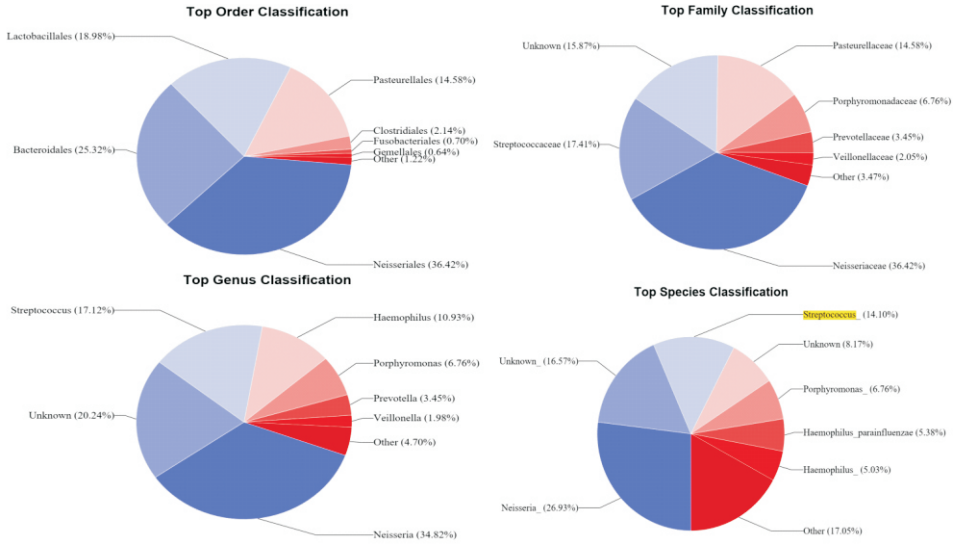

Figure 9a: Order, Family, Genus and Species classification 0f Bacteria in Sample 10 on day 0

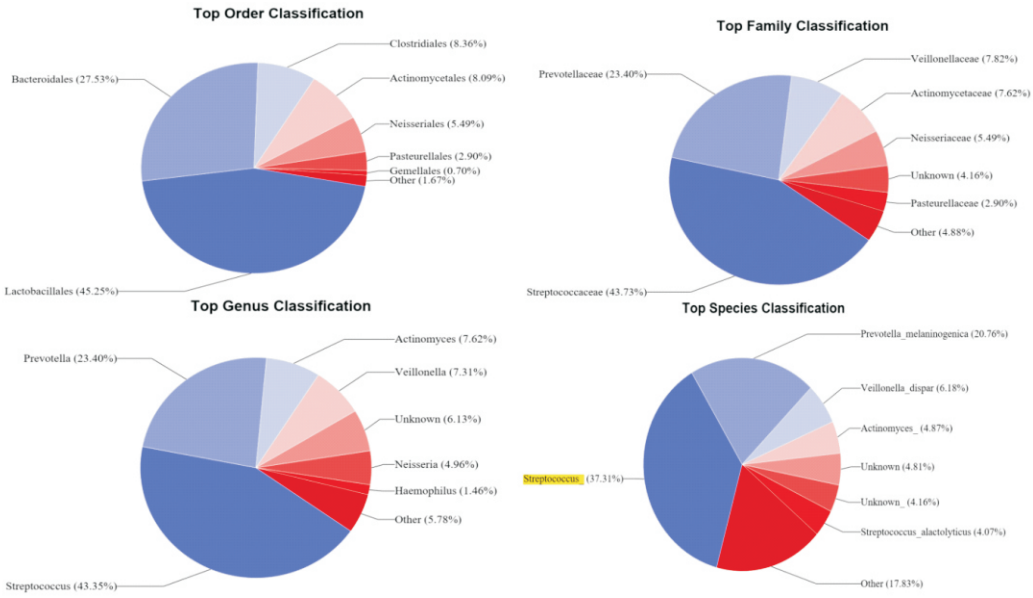

Figure 9b: Order, Family, Genus and Species classification 0f Bacteria in Sample 10 on day 30
In all the samples, *Streptococcus* species were the most stable.

4.0 DISCUSSION

Fierer *et al*. (2010) studied the use of bacteria for human identification, specifically the possibility of examining skin bacterial communities. They proposed that microorganisms left on a surface after a person has touched it may be utilized to identify the person. These findings are encouraging, implying that microbiome analysis could be used for forensic identification. The use of bacteria detected in saliva for human identification, on the other hand, has only been studied in relation to bite-mark analysis, specifically streptococcal DNA analysis (Rahimi et al., 2005; Kennedy et al., 2012).This opens the door to a complete human identification procedure based on bacteria present in saliva (Leake, 2014).

The microbial diversity of the 20 buccal swab samples were analyzed by targeting the *16S rRNA* regions of the gene. The results showed that the most common phyla present in the mouth were Firmicutes, Proteobacteria, Bacteriodetes and Actinobacter. This is in agreement with previous studies carried out by Stahringer *et al., (*2012) and Leake, (2014). However, bacterial abundance appeared to be markedly different for each of the samples. This falls within the variations observed in studies by Stahringer *et al.* (2012) who examined 264 saliva samples and found that bacterial abundances varied substantially.

In the current investigation, there was evidence of microbial species diversity across individuals (inter-individual variation) as well as within the individual (intra-individual variation) because bacteria found in both samples from the same subject varied. Microbial species diversity within an individual (intra-individual variation) was not as large as differences between individuals. This is consistent with the report of Leake 2014). Furthermore, Nasidze *et al*., (2009) found that the level of variation between persons was substantially larger than within the same individual in a study on the worldwide diversity of the human salivary microbiome

From this study, *Streptococcus* species were the most abundant in all samples. *Streptococcus* spp. is the most prevalent genus found in the mouth (Whiley and Beighton, 1998). Previous research has also revealed that humans have several distinct strains of the same *Streptococcus* species, with many strains being unique to individuals (Rudney and Larson, 1994; Wisplinghoff *et al*., 1999). Bik *et al*., (2010) investigated a co-occurrence of bacteria in their study and reported *Streptococcus*, *Neisseria* and *Haemophilus* as the most abundant genera. This study also revealed that Firmicutes phylum had the highest score in all samples. This shows that species segregation and competitive species interactions are conceivable. This is intriguing because Streptococcus is the most common genus found in saliva and belongs to the Firmicutes, implying that due to this interaction, different people may have different Streptococcus species. Furthermore, bacterial abundances will vary, particularly as a result of poor oral hygiene and periodontal disease (Curtis *et al*., 2011; Liu *et al*., 2012; Ge *et al*., 2013; Belstrm *et al*., 2014). Nevertheless, *Streptococcus*, *Neisseria*, *Haemophilus*, *Campylobacter*, *Veillonella*, *Fusobacterium*, *Rothia*, *Actinomyces*, *Prevotella*, *Corynebacterium*, *Capnocytophaga*, *Atopobium*, *Granulicatella*, and *Bergeyella* are the most common genera found in the oral cavity and present in everyone, with *Streptococcus*, *Prevotella*, *Veillonella*, *Neisseria*, *Haemophilus* and *Rothia* as the most abundant (Bik *et al.,* (2010). The oral microbiome is highly dynamic throughout the day (affected by eating, hygiene, sleep). However, individuals often maintain a relatively stable core microbiome over time (Hu *et al*., 2022). In this study, participants followed their usual diet and maintained their regular oral hygiene practices, with samples collected first thing in the morning before tooth brushing. These routines are unlikely to have had a significant impact on the oral microbiome. A review on oral microbiome sampling recommends collecting

saliva in the morning before tooth brushing or between meals to reduce variability due to daily activities (Lu *et al*., 2022). Many studies explicitly instruct participants not to brush or eat before sampling, reinforcing that this standardization is important for consistency (Hallang *et al*., 2021). The loss of sample 3 during transportation represents a minor limitation of the present study. Although this resulted in a slight reduction in sample size, the overall analysis was still considered robust. Nonetheless, this factor should be acknowledged when interpreting the findings.

5.0 CONCLUSION

This study clearly identifies *Streptococcus* as the most abundant specie of microorganisms common to individual oral micrbiome irrespective of nutrition, environment and social strata. However, there was no distinct bacterial identifier for each individual and further studies using larger population sizes will be required to establish this.

Authors' contributions:

All the Authors (**Iroanya**, Onyekachi Ogbonnaya* (IOO); **Iromenu**, Nkiru Susan (INS); **Egwuatu**, Tochukwu Frank (ETF) and **Folorunso**, Stephania Semiire (FSS)) conceptualizedand designed the research and participated in sample collection and processing. INS and IOO funded the research.INS, ETO, ETF and FSSwrote the ***original draft.*** IOO coordinated and supervised the researchand carried out the final editing/ formatting of the manuscript. All authors read and approved the final version of the manuscript.

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Name, address, e-mail, and telephone number of the corresponding author:

Onyekachi Ogbonnaya Iroanya (PhD)

Department of Cell Biology and Genetics, Faculty of Life Science, University of Lagos, Nigeria.

oiroanya@unilag.edu.ng or onyiiog@yahoo.com

+234 802 954 3243

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