Isolating oral bacterial species from a single donor through a multi-step detection method

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Isolating Oral Bacterial Species from A Single Donor Through A Multi-Step Detection Method

by

Khalid Jabir Al-Lakhen

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Master of Science

College of Arts & Sciences
Department of Biological Sciences
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ABSTRACT

The human oral microbiome is one of the most complex microbiome communities in the human body and its role in maintaining health is not fully understood. Previous research shows that the transition from health to disease is accompanied by changes in community abundance and structure. To fully understand the symbiotic relationship formed between bacterial species that leads to the formation of a functional healthy biofilm structure, we must study the various structural relationships and subsequent molecular interactions among individual species within the biofilm. In this thesis, I have isolated a pure bacteria species from a single donor's dental plaque and identified it as *Fusobacterium nucleatum* subsp. animalis. This was done by optimizing media (Valm media), culturing, utilizing several microscopic techniques including FISH, performing PCR, and sequencing the 16S rRNA gene to finally identify the species.
ACKNOWLEDGMENTS

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I would like to extend my appreciation of my committee members, Dr. Prashanth Rangan, and Dr. Cheryl Andam, for their support, and feedback. In addition, I would like to thank my colleagues in the Valm lab for their constant motivation and input.
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Chapter I

Introduction

The human oral microbiome is one of the most complex microbiome communities in the human body, and its role in maintaining health is not fully understood. The complexity is due to the range of substrates in the mouth, such as teeth, tongue, cheeks, and gums, whose different chemistry, topography, and stability provide different habitats for microbial communities to form a biofilm (Mark Welch et al., 2020). A biofilm is defined as a matrix-embedded microbial population, in which microbial cells are adherent to each other and to surfaces at interfaces (Marsh, 2005). Through DNA sequencing, researchers have been able to determine that about 700 different species of bacteria are located in the oral microbiome with 32% being uncultivable (Zhao et al., 2017). Shifts in community structure have been shown to correlate with different diseases of the mouth such as periodontitis and dental caries. Periodontitis is defined as an oral disease caused by the accumulation of bacteria within gingival pockets resulting in inflammation and damage of connective tissue attached to the tooth, while dental caries results in tooth decay due to overabundance of damaging bacteria producing acids (Abusleme et al., 2013). In this thesis, I will demonstrate that the composition of oral microbial biofilms isolated from human donors is extraordinarily complex. By developing a pipeline to isolate and cultivate oral bacterial species from single donors it will be possible to investigate the relations between species in vitro and test specific hypotheses regarding the structure and function of these communities when these isolates are co-cultured together in the future.

Culturing techniques were some of the earliest approaches to studying the human oral microbiome and examining the composition of the various bacterial species present in dental
plaque. The use of culturing to study the oral microbiome provides many opportunities to test specific hypotheses which will be mentioned later in this thesis. To this day, in order to definitively assign the causation of a specific disease to a microbe, it must be isolated in culture as stipulated in Koch's postulates (Grimes, 2006). Koch started getting the idea for his four postulates when he discovered *Bacillus anthracis* as the causative agent of anthrax disease. Together with his work on tuberculosis for which he received The Nobel prize in medicine in 1905, he formed his famous postulates. The postulates act as a guideline to provide evidence that a microbe is the sole cause of a particular disease. First, the microbe should be present in all organisms with the particular disease. Second, the microbe must be isolated from the diseased organism and cultured. Third, introducing the same isolate into a healthy organism causes the disease. Finally, the same microbe must be once again isolated from the newly diseased organism (Grimes, 2006).

**Early Culturing**

Culturing dental plaque in the lab can be a valuable tool to understand the molecular basis and symbiotic relationships of coexisting microbes effecting oral health in addition to providing a basis to develop models to study the spatial structure of the oral microbiome. However, culturing does have its challenges. The oral microbiome has the same species present in healthy and periodontal disease subjects; the difference is the shift in abundance of specific species which is the reason for disregarding Koch's third postulate in this field. Early culturing experiments involved collecting dental plaque from donors, growing the associated bacteria on non-selective media, and then isolating pure colonies based on the cellular morphology. These isolates were then categorized based on morphology, gram reaction, and whether each thrives in aerobic or anaerobic conditions. This work utilized light microscopy, as well as various phenotypic tests,
plate tests, and broth tests (Gibbons et al., 1964). Culturing played a vital role in demonstrating that periodontal disease is not caused by pathogens foreign to the oral microbiome in healthy individuals rather than a change of abundance in the existing community. Socransky, et al. demonstrated this through microscopic viable counting of several organisms extracted from gingival debris collected from the gingival coercive area in healthy and periodontal diseased subjects (Socransky et al., 1963). Spirochetes showed a significant increase in the periodontal disease group when compared to the healthy group as shown in (Table 1.1). This hypothesis was strengthened by demonstrating the inability of pure culture spirochetes isolated from the human mouth to cause infection in animal models (Rosebury, 1950).

<table>
<thead>
<tr>
<th>Organisms</th>
<th>No. of patients examined in each group</th>
<th>Mean counts/g x 10^7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal group</td>
</tr>
<tr>
<td>Total streptococci</td>
<td>10</td>
<td>1400 ± 360*</td>
</tr>
<tr>
<td>Facultative streptococci</td>
<td>10</td>
<td>490 ± 240</td>
</tr>
<tr>
<td>B. melaninogenicus</td>
<td>11</td>
<td>82 ± 55</td>
</tr>
<tr>
<td>Fusobacteria</td>
<td>6</td>
<td>1.2 ± 0.35</td>
</tr>
<tr>
<td>Spirochetes</td>
<td>20</td>
<td>56 ± 9</td>
</tr>
</tbody>
</table>

**Table 1.1. Count of Bacterial groups in gingival crevice.** The Counts is of of specific groups of microorganisms in gingival crevice material from normal and periodontally-involved individuals. (Socransky et al., 1963, P.278)

The difference in abundance of oral microbes seems to be the leading factor in Periodontal disease. However, the different anatomical regions in the mouth must also be taken into consideration. The application of isolating and cultivating bacterial organisms to study the difference in abundance of oral microbes at different anatomical regions in health and periodontal disease subjects was done. A previous study showed a difference in the composition of cultivable bacterial species in gingival epithelial cells and unattached microbiota recovered from the same site in healthy or periodontal disease subjects. The reasoning behind examining
gingival epithelial cell, and unattached microbiota was to explore the difference between surface properties of healthy or periodontal disease individuals. If a difference is observed this could possibly affect the types of bacteria which are able to attach and colonize to these surfaces. A notable difference in proportion of species between the epithelial cell zone than that of the unattached bacterial zone was observed. *Peptostreptococcus micros* and species of black-pigmenting Bacteroides have shown to be higher in the epithelial cell zone. While species of Streptococci, *Capnocytophaga ochracea & Veillonella parvula* showed a higher abundance in loosely adherent plaque as shown in (Figure 1.1). (Dzink et al., 1989).

**Figure 1.1. Comparison of Bacteroides in epithelial and unattached bacterial cells.** Bar chart showing the mean percent of black-pigmenting Bacteroides found in epithelial cell and unattached bacterial cell layers after Percoll density gradient centrifugation. (Dzink et al., 1989, P.3) (Permission granted by John Wiley & Sons, Inc.)

Another study used culturing and isolating bacterial species based on morphology to examine the subgingival microbial changes in relation with development of gingivitis during puberty years at
different stages. Papillary bleeding index (PBI) was used to measure the severity of gingivitis in subjects between the age of 11-14 years old. Subgingival samples were extracted and transported in reduced transport fluid then grown anaerobically and plated on enriched trypticase soy agar. Isolates were then collected and examined via darkfield microscopy then identified based on morphology, gram staining, and aerotolerance. It was shown that *Capnocytophaga* was highest in samples taken before a rise of PBI, while *Bacteroides* was found to be significantly elevated a high PBI correlating to significant gingivitis. This proved that microbial colonization followed a specific sequence for gingivitis to increase, for example, *Capnocytophaga* precedes the appearance of gingivitis followed by *Bacteroides* after initiation and during disease progression (Mombelli A, Lang NP, 1990).

These findings exemplify the utility of culturing and isolating oral microbiome species from dental plaque as an approach to study periodontal health and the sequential events of change in abundance of microbial species associated with disease. In addition, when combining microscopy with culturing techniques it is possible to examine the location and spatial structure of microbial species at specific location of the oral cavity.

The use of culturing techniques to isolate and speciate microbes based on morphology, gram stain and other phenotypic identification, led to the hypothesis that an ecological succession of different taxa among early colonizers occurs during biofilm development. The hypothesis was initiated by (Nyvad & Fejerskov, 1987) in a study that they identified microbial isolates from four subjects that carried artificially made enamel and root surface in the oral cavity for periods of 4, 8, 12, and 24 hours. The samples were then grown anaerobically on tryptone yeas extract blood agar plates resulting in a total of 1742 isolates that were characterized in three groups based on gram staining reaction, cell morphology, and catalase reaction. Within each group
known isolates species were identified via various biochemical reaction tests such the capability of fermenting specific sugars, and production of specific biochemicals from sugars. The various Streptococci species were examined at the different time intervals. *Streptococcus mitis* was the most abundant species at all time intervals with a mean of 30-52%, while *Streptococcus oralis* showed an increase with time in the enamel, and root surfaces. Furthermore, *Streptococcus sanguis* showed to significant change with time while *Streptococcus salivarius* exhibited a dramatic decrease as time progressed. In both enamel, and root surfaces *S. mitis* & *S. oralis* are the predominant *Streptococcal* species as shown in (Figure 1.2). There was also a relative decrease in proportion of all gram-positive rods between 4- and 8-hour mark, followed by an increase from the 8 to 12-hour mark. The increase in gram positive rods was mostly due to an increase in *Actinomyces* suggesting it to be the prime taxa at this time period (*Nywad & Fejerskov, 1987*). The major findings of this study as well as decades of subsequent work have been assembled into the Ecological Plaque Hypothesis. According to this hypothesis, oral disease is driven by dysbiosis of the microbiome, dysbiosis is the imbalance between the microorganisms that are present in a healthy microbiome. Dysbiosis can be triggered by environmental changes, ultimately effecting the ecology of the ecosystem through changes in spatial relation, host-microbe, and microbe-microbe interaction within the biofilm, which effects competitiveness and prevalence of certain bacterial species of the microbial community within the oral cavity resulting in increased risk of disease (*Marsh & Zaura, 2017*).
Figure 1.2. Percentage distribution of different streptococci after 4, 8, 12, and 24 h on enamel (E), and root surfaces (C). (Nyvad & Fejerskov, 1987, P.375) (Permission granted by John Wiley & Sons, Inc.)

_Fusobacterium nucleatum_, is an obligate anaerobic, elongated, gram-negative rod, that is a prevalent bacterial species with great importance to the oral microbiome. According to (Kolenbrander et al., 2010) spatiotemporal model of oral bacterial colonization, it’s shown that _F. nucleatum_ had the most partnership among the bacterial community. In addition, it acts as an essential bridging intermediate organism between, initial, early, and late colonizers (Figure 1.3). This paints a broad picture of _F. nucleatum_’s role in the formation of the oral biofilm structure, to dig deeper its critical to understand how the various fusobacterium species and subspecies effect the biofilm structure differently. A recent study by (Thurnheer et al., 2019) utilized fluorescent in situ hybridization (FISH) to test the effects of _F. nucleatum_ as a control and 6 various _Fusobacterium_ species or subspecies on in vitro biofilm formation and structure in three
different oral biofilm models. Although all six tested *Fusobacterium* variants established well in biofilms, *F. nucleatum ssp. nucleatum* and *Fusobacterium periodonticum* exhibited constantly higher numbers and evenly scatter positions in the three biofilm models. In the subgingival model, the count of *S. oralis, Prevotella intermedia, Porphyromonas gingivalis*, among other species significantly decreased in the presence of any of the six *Fusobacterium* species compared to the control. This shows that different *Fusobacterium* species and subspecies effect the growth of the surrounding bacteria, as a result will also affect the overall biofilm structure. Due to essential role of *F. nucleatum* in the formation of a multispecies biofilm, isolating it in the future will help us understand the spatial structure of the oral microbiome. It is important to note that isolating various *Fusobacterium* species and subspecies from a single donor is critical to understanding the bridging hypothesis.
**Figure 1.3. Spatiotemporal model of oral bacterial colonization.** Showing receptors on salivary pellicle coaggregation with initial colonizers, early colonizers, Fusobacteria, and finally late colonizers. *(Kolenbrander et al., 2010, P.472)* (Permission granted by Springer Nature)

The isolation of individual microbial species and growth in pure culture have been instrumental in uncovering the basic principles behind dysbiosis. However, it does not give an accurate quantitative representation of community structure due to 35% of the oral microbiome being uncultivable and not having media that is able to grow the 65% of previously cultivated microbes in the same experiment. Furthermore, culture-based approaches are limited to studying abundant and fast-growing organisms with lower abundance and slower-growing species not being accurately represented. The use of culturing cultivable bacterial to study the oral microbiome was done early on before many advancements in molecular techniques such as sequencing. In addition, microscopy was not as advanced as it is today which limited the methods in which the
isolates can be identified to its morphology and gram stain. Modern fluorescence microscopy provides an added arsenal with more depth in genus level microbial detection.

**Microscopy**

Microscopy allows identification of the morphology of individual bacterial species, as well as the in situ spatial relationships within communities. Bacterial plaque biofilms have been studied with optical and electron microscopy to observe their structure. Images of plaque biofilms demonstrated the highly non-random structure of the community and further contributed to the hypothesis that there is an ecological succession in dental plaque. However, optical and electron microscopy provide limited information on taxonomic identity.

Electron microscopy and optical microscopy were used in one of the earliest studies to identify the sequential development of bacterial plaque on a given surface. This was done by observing the growth of dental plaque on constructed epoxy resin dental crowns worn by volunteers over a period of two months. At different time intervals, plaque formation was observed using electron microscopy. The growth of plaque at day one consisted mostly of coccoid shaped cells with both gram positive, and gram-negative cell walls. While at week one, a mixed plaque flora containing rods, filaments, and coccoid shaped cells formed. Finally, at the two month mark, the bulk of the plaque was composed of densely packed filamentous microorganisms oriented perpendicular to the crown surface (Listgarten et al., 1975).

These findings provided the field with a glimpse of the sequence in which bacterial species establish in oral communities. As shown by (Listgarten et al., 1975) imaging provided the capability to identify early colonizers demonstrating coccoid shaped morphologies which is believed to be Streptococcus species. A change in cellular morphology was observed when the
sequential formation of different microbial species occurred. The final stages of formation of subgingival plaque was demonstrated as a filamentous microorganism which is believed to be caused by the abundance of *F. nucleatum*, this was a steppingstone to the hypothesis mentioned earlier of *F. nucleatum* acting a bridging organism in the oral biofilm.

Microscopy is still used to aid in identification of microbial species isolated from the oral microbiome. A recent study was done to culture *Treponema* species using filtration and selective media, followed by Scanning Electron Microscopy (SEM) to verify the isolation of treponemes with their characteristic spirochete morphology. The isolated spirochetes were then identified using a combination of matrix assisted laser desorption ionizing time of flight mass spectroscopy (MALDI-TOF MS) followed by sequencing ([Belkacemi et al., 2019](#)). Another imaged the ultrastructure of subgingival dental plaque via Field emission SEM (FE-SEM), which produces a smaller electron beam spot size that offers greater resolution and image contrast than SEM. ([Holliday et al., 2015](#)) managed to provide clear images of a range of morphological distinct cells adhered to one another with visible extracellular components present. The use of electron microscopy and optical microscopy is a useful, quantitative, tool to examine the morphological shape of bacteria species and type of cell wall whether in dental plaque or isolated species in culture. The downside is that it only distinguishes bacteria based on morphology, with different types of bacteria having the same morphology this can be problematic.

A New staining method was introduced by ([Delong et al., 1987](#)) called FISH, which used fluorescently labeled oligodeoxynucleotides complementary to phylogenetic group-specific 16S ribosomal RNA (16S rRNA) on intact cells. The use of multiple phylogenetic probes labeling with different fluorescent dyes can provide information on the taxonomy of the organisms within a single field of view. By using FISH in combination with confocal or fluorescent microscopy
we can solve the issues of electron & optical microscopy mentioned previously. However, due to technical difficulties and overlapping of wavelengths it has not been possible to detect more than three species at a given time, which has hindered examining the spatial structure of a full biofilm.

The limitation imposed by overlapping wavelength has been overcome by using Combinatorial Labeling and Spectral Imaging Fluorescence In Situ Hybridization (CLASI-FISH) as shown by (Mark Welch et al., 2019). This method uses multiple fluorophores, imaged on spectral detectors, after which computational algorithms are applied to deconvolve different fluorophores with overlapping spectra (Valm et al., 2011). The result is shown in (Figure 1.4) which gives a possibility of 28 different colors to each label type in proof of principle with Escherichia coli. By using this method, it is possible to detect as many as 120 different microbes at a given time in a single field of view (Valm et al., 2017). CLASI-FISH was used to detect 10 different taxa in intact plaque biofilms by using different probes for genus-level detection and the EUB338 probe which is the universal probe to most bacteria. A plaque complex with an assortment of microbes with Corynebacterium filaments and streptococcus at the periphery was detected; this structure was referred to as a hedgehog. 10 taxa have been identified in this hedgehog structure by using different fluorophore channels detecting the specific probe of interest and assigning pseudo colors to each as shown in (Figure 1.5). Utilizing this approach provides spatial information on the formation of the oral biofilm that could not be achieved previously with other microscopy techniques (Mark Welch et al., 2016).
Figure 1.4. CLASI-FISH proof of principle with *E. coli*. (C) raw spectral images in which the color at each pixel corresponds to a merge of all eight fluorophore channels after unmixing. (D) segmented images of the field of view in (C), the color of each segmented cell corresponds to one of 28 different label types; each label type is assigned an arbitrary color. *(Valm et al., 2011)*, P.4154) (Permission granted by National Academy of Sciences)
Figure 1.5. *A hedgehog structure in plaque showing spatial organization of the plaque microbiome.* Plaque was hybridized with a set of 10 probes each labeled with a different fluorophore. Each panel shows the superposition of several of these individual fluorophore channels. A–D and F–H show a single focal plane near the center of the structure, with two to three fluorophore channels shown in each of A–C and all nine specific probes superimposed in D.(E) Maximum intensity projection of three planes, representing a total of ~2 μm of thickness, to visualize the continuity of *Corynebacterium* filaments from the center toward the edge of the structure. F is a detailed view of corncob structures. G is a detailed view of mixed filaments. H shows the fluorophore channel corresponding to the universal bacterial probe, showing that the specific probes (D) identify most of the cells that hybridize to the universal probe. I–L show a second focal plane near the periphery of the structure. Fluorophore channels shown correspond to the following genera in the figure: (A, E, and I) *Corynebacterium* and *Streptococcus*; (B and J) *Capnocytophaga, Porphyromonas,* and *Haemophilus/Aggregatibacter;* (C and K) *Fusobacterium, Leptotrichia,* and *Neisseriaceae;* (D and L) all nine specific probes; (F) *Corynebacterium, Streptococcus, Porphyromonas,* and *Haemophilus/Aggregatibacter;* (G) *Corynebacterium,*
Although the use of microscopy a powerful tool in understanding spatial relations in the oral microbiome, it lacks comparison measurements in abundance changes of specific organisms, which is an important factor in the comparison between health and diseased states in the oral cavity. In addition, it does not narrow our identification microbes down to the species level that is achieved through modern molecular techniques.

**Early Molecular Techniques**

As molecular techniques have advanced, DNA sequence analysis is routinely applied to studying the human oral microbiome. Checkerboard DNA-DNA hybridization was one of the first molecular techniques used to determine the clustering and abundance of oral bacteria. Checkerboard DNA-DNA hybridization utilizes the use of up to 40 chromosomal fluorescence DNA probes at once that target specific taxa known to exist in the oral cavity. Dental plaque samples collected from donors are placed on a rectangular shaped membrane with each probe located at a specific position within the membrane, computer software analysis is then able to measure the abundance on a scale of 1-5 based on the intensity of the hybridization. This technique was used to perform the first cluster analysis of dental plaque communities.

*(Socransky et al., 1998)* utilized this technique by using 40 probes of prevalent subgingival taxa, they managed to identify five different co-occurring complexes of bacterial species present in subgingival plaque (red complex, orange complex, green complex, yellow complex, purple complex). Each complex was identified by examining 32 of the highest detected species and the association they have with one another based on an analysis of intensity relationship between the species. Several complexes exhibited consequential relationship to one another. For example, the
orange complex consisting of species such as *F. nucleatum*, and *P. intermedia* was a required predecessor to the red complex which consisted of species such as *P. gingivalis*, and *Treponema denticola*. This suggested that the growth of certain complexes must proceed others. In addition, correlation between different complexes and pocket depth of subjects with periodontal disease was examined. The red complex was associated with the most pocket depth indicating severe periodontal disease, while the green complex correlated with the lowest pocket depth indicating periodontal health (*Socransky et al., 1998*). Importantly, this reinforced the idea that no single organism is responsible for periodontal disease--the failure of Koch's postulates.

In addition to checkerboard DNA-DNA hybridization 16S rRNA-based Microarray method was later used. The Oral Microbe Identification Microarray (HOMIM) is based on the use of 16S rRNA-based oligonucleotide probes that have been synthesized, with a total of 400 fluorescence probes printed on each microarray targeting over 300 bacterial taxa previously identified in the oral microbiome. A universal probe that hybridizes with all species is used as a positive control, while a negative control is used to detect the array background levels. The intensity is then measured on a scale of 0-5 as previously for DNA-DNA hybridization. A study compared the subgingival microbial profiles of three different groups, healthy periodontal subjects, subject with sever periodontitis, and subjects with refractory periodontitis which is identified as destructive periodontal disease that respond poorly to conventional therapy. Subjects with any form of periodontitis exhibited a greater diversity in subgingival microbiota than that of healthy subjects. All 300 taxa were detected in most periodontitis patients while healthy subjects lacked 28% of the 300 taxa. Some of the more prevalent species in the periodontal healthy subjects are *Actinomyces sp.*, *Capnocytophaga sputigena*, and *S. sanguinis*. Importantly, this study
strengthened the understanding that periodontal disease is caused by an increase in diversity of the oral microbiome (Colombo, 2009).

Although checkerboard DNA-DNA hybridization and 16S rRNA-based microarrays are powerful tools in identifying different taxa present at different conditions in the oral microbiome these approaches are limited in their ability to provide quantitative information. This is due to measurements being restricted to a dynamic range determined by biological factors, probe specificity, and detector sensitivity unlike the freedom that is available when utilizing advanced microscopy techniques such as SEM, and FISH. In addition, these molecular techniques are limited to the probes available and cannot identify species not represented by these probes.

**Sequencing:**

As molecular technology advanced sequencing was introduced which has taken the place of many of the methods mentioned earlier due to the higher flexibility of identifying bacterial species directly from the environment. Although Sanger sequencing was invented in the 1970s, sequencing was not used as a frequent detection tool until recent years due to the affordable cost and advancements in efficiency of sequencing which led to the creation of The human oral microbiome database (HOMD). HOMD is a 16S rDNA database that provides us with the information of around 700 predominant bacterial species which is present in the human oral microbiome. This database was created in 2010 and provides a way to identify unnamed species, and phylotypes (Dewhirst et al., 2010). With such a database available it is easy to identify bacterial species from the oral cavity by using 16S rRNA sequencing, whether by older sequencing methods such as sanger sequencing or next-generation sequencing (NGS) such as, pyrosequencing, and Illumina sequencing.
Pure bacteria cultures or samples from the oral cavity can be identified by via the use of its 16S rRNA and utilizing either Sanger sequencing or NGS, such as 454 Pyrosequencing, and Illumina sequencing (Krishnan et al., 2017). Sanger sequencing has a higher accuracy then NGS due to it producing one large fragment of a forward and reverse, however NGS has a higher throughput capability by producing millions of fragments at a time, and faster turnaround time making it the better option when investigating a high number of different genes at a time. 16S rRNA sequencing is currently utilized in the human oral microbiome field to identify and compare the abundance of the bacterial species present at a given time. Unlike other molecular methods presented earlier such as checkerboard DNA-DNA hybridization, and Human Oral Microbiome Identification Method (HOMIM), sequencing is not restricted by a set measuring scale allowing for quantifiable results relative to the abundance of the community. Furthermore, since probes are not required it is not limited to that amount and instead is determined based on the similarity a sequence to an already known taxa or species. With the current advancements and cost-effective use of sequencing it is a viable tool to measure the abundance of species in a microbiome at a given time.

The concept of a core healthy microbiome was strengthened by (Zaura et al., 2009), who were one of the first to utilize NGS in the field. This was done by sequencing the V5-V6 region of the 16S rRNA in a few locations in the oral cavity in several different healthy subjects. within the mouth, samples were collected from the cheek, hard palate, tongue, and saliva of the oral cavity, with the aim of detecting the similarity in abundance of taxa, and phylotypes in different healthy individuals. Overlapping of phylotypes between the individuals was compared. The clustering of unique sequences into operational taxonomic units (OTUs) was done at a 97% similarity, which is a standard metric in community microbiology. OTUs are used to cluster organisms based on
similarity in the DNA sequence. The results showed about 100 species-level phylotypes that belonged to abundant OTUS in an indivial microbiome where abundance is referred to the phylotype contributing to at least 0.1% of the microbiome community. The predominant taxa that were identified in all samples of healthy individuals were Firmicutes (genus Streptococcus, family Veillonellaceae, genus Granulicatella), Proteobacteria, Actinobacteria, Bacteroidetes and Fusobacteria (genus Fusobacterium) (Zaura et al., 2009).

The development of the HOMD provided direct information to understand the organisms sequencing from the oral microbiome. As shown by (Abusleme et al., 2013) in which they combined 454-pyrosequencing and quantitative PCR to better understand the subgingival microbiome and its relationship with community biomass and inflammation in health and periodontitis. Sequencing data collected from subgingival plaque in healthy and periodontal subjects was analyzed using the HOMD, indicating a shift in community abundance. For example, Rothia and Actinomyces were identified as health-associated genera with higher relative abundance in healthy individuals, while Treponema and P. gingivalis had a higher relative abundance in periodontal individuals. The observed shift of community abundance between health and periodontitis subjects is demonstrated in (Figure 1.6). In addition, qPCR showed an increase in overall community bacterial load at inflamed locations in periodontitis compared to health, this increase of bacterial load was not observed in in individual health-associated microbes such as Actinomyces. This suggests periodontitis is caused by the emergence of newly dominant community members such as P. gingivalis, resulting in an ecological shift of community structure and not community membership (Abusleme et al., 2013).
Figure 1.6. The core subgingival microbiome in health and periodontitis. OTUs that are part of the core microbiome were first defined as those present in at least 50% of subjects in both the healthy and periodontitis groups (gray), in at least 50% of healthy subjects (green) or in at least 50% of subjects with periodontitis (red). Prevalence and relative abundance data were used for further filtering. OTUs in gray represent the core subgingival OTUs, present at equal prevalence and relative abundance in health and periodontitis. OTUs in green represent the core health-associated species, appearing at increased prevalence and relative abundance in health compared to disease. OTUs in red represent the core periodontitis-associated species, present at increased prevalence and relative abundance in periodontitis compared to health. Inner circles labeled with 1, contain highly prevalent and highly abundant OTUs, that is, present in at least 2/3 of samples from each group (core, health, or periodontitis) and numerically dominant with a mean relative abundance of ≥2% of total sequences. Middle circles labeled with 2 contain OTUs highly prevalent but present in low abundance, that is present in at least 2/3 of samples from the specific group but with a mean relative abundance of <2% of total sequences. Outer circles labeled with 3 contain OTUs moderately prevalent and present in low abundance, that is OTUs present in 1/2 to 2/3 of samples from each group and with a mean relative abundance of <2% of total sequences. (Abusleme et al., 2013, P.1021) (Permission granted by Springer Nature)
The hypothesis that a community shift of already present bacterial taxa leads to periodontitis is supported by (Griffen et al., 2012). They sequenced amplicons from the V1-2 and V4 regions of 16S rRNA genes of subgingival bacterial communities in subjects with chronic periodontitis. The data identified 123 species that are more abundant in individuals with chronic periodontitis, among them *P. gingivalis, T. denticola, and Filifactor alocis*. This shows that sequencing can provide us with the abundance and makeup of bacterial communities and can be used as an accurate method to identify a single bacterial species. However, understanding the structure of the oral microbiome is important in understanding the relations between various species in forming the biofilm therefore sequencing only provides us with one part of the story.

**Modern Culturing**

Modern molecular techniques such as shotgun metagenomic sequencing can provide us with a culture-independent approach to obtain information on complex bacterial communities and their association with their environment; however, it is important to not neglect the value of culturing and isolating pure species from such communities which is necessary in understand their physiological and pathological properties. As mentioned earlier, not one introduced bacterial species acts as a pathogen that leads to oral disease such as periodontal disease. Instead, it is the change in abundance and symbiotic relationships between species in the large oral biofilm. Although isolating pure cultures is not important in understanding the pathological causation of oral disease as mentioned earlier, it is important to help us gain knowledge on the molecular basis that occurs between species specific microbe-microbe interaction when grown together that otherwise is involved in a complex biofilm with hundreds of various species. In addition to studying the unique ecological progression that the oral microbiome follows. This was shown by (Eren et al., 2014) through analyzing data from the Human Microbiome Project (HMP) and
comparing the variations between different anatomical regions of the mouth and different individuals. There was a variation of strains at both, different anatomical locations, and different individuals at the same anatomical location. This provides important evidence that studying oral microbes extracted and isolated from the same donor might be more beneficial than using commercial strains.

Approximately 99% of microorganisms found in the environment cannot be cultivated in the lab. This was originally addressed in a review by (Staley, 1985) in which a trend was observed in research showing a dramatic difference by a number of magnitudes in viable plate counting of grown bacteria compared to total direct microscopic count of the bacteria from the environment. This phenomenon was referred to as the “The Great Plate Count Anomaly”. This rapid decrease in bacteria when growing in vitro is due to several reasons, one being the difficulty of optimizing media for growth of all organisms with being selective to specific species. Bacteria can be unculturable due to the exceptionally low chance of isolating a slow growing organism from a community that has a majority population of other organisms. In addition, some bacteria are resistant to isolation in media due to the nature of their fastidious growth requirements, including nutrients, pH levels, oxygen levels, and in some cases its dependency on another organism for these ideal conditions (Sonia R. Vartoukian et al., 2010).

Finding the appropriate media is the first step in culturing a more diverse community representing the oral microbiome, which allows the possibility of further isolating pure species from in vitro cultures. (Tian, 2010) was able to develop an optimized media (SHI media) that far succeeded previous media used such as brain heart infusion (BHI), Lysogeny broth (LB), basal medium mucin (BMM), and others. By utilizing 454 pyrosequencing, and PCR of the 16S rRNA genes, then denaturing gradient gel electrophoresis (PCR-DGGE) they were able to observe that
SHI media exhibited the least selectivity when cultivating saliva. This was proven by the high similarity of the microbial community grown in SHI media in vitro compared to that of the saliva derived community. In addition, SHI media cultures showed an increase from other media types to 40 different genera of bacterial species detected (Tian, 2010). SHI media was further optimized in a study by (Edlund et al., 2013), They included the optimized SHI media in the development of an in vitro biofilm model that closely represents the oral microbiome obtained. Ideal sucrose levels for SHI media was calculated by sequencing the 16S rRNA of the community in culture using different levels of sucrose and comparing it to sequencing data obtained from saliva samples. Using 0.5% sucrose for the in vitro model, obtained the highest similarity from extracted dental plaque in terms of genera observed. In addition, the reproducibility of the 16S rRNA profile outcome was measured for different batches as shown in (Figure 1.7).
Figure 1.7. Correspondence analysis showing reproducibility and 16S profile similarities within biofilm and saliva samples. Axis 1 explains 47% of the variation in the dataset; axis 2 explains 19% of the variation. Replicate biofilm samples representing batches 1 and 3 cluster closely together whereas batch-2 biofilms that derive from an SIP experiment cluster more distantly along the first ordination axis. Saliva-derived replicates (1 to 3) also show similar 16S diversity. (Edlund et al., 2013, P7) (Permission granted by Springer Nature)

Although SHI media has shown to be a great nonselective media that can cultivate a diverse community of bacterial species extracted from dental plaque. It is important to note that subgingival plaque included a high proportion of uncultivated oral species, this might be due to the different requirements and environment required when comparing it to the supragingival region that is easier to access. A novel culture media was developed to mimic the nutritionally environment of periodontal pockets that are located in the subgingival, this included Trypticase peptone .3%, pig gastric mucin .25%, and grown in anaerobically. Furthermore, The Calgary Biofilm Device (CBD) was used which is a 96 well plate with pegs extended from the slide where the dental plaque is grown. This makes inoculation of media over a long period feasible.
The result was a diverse oral bacterial community with several oral bacterial taxa that was previously uncultivatable. Through further culturing the previously uncultivated species *Lachnospiraceae bacterium* HOT 500 was successfully isolated (*Thompson et al., 2015*).

Another challenge faced when cultivating some bacterial species *in vitro* is the inability to mimic the natural environment which provides specific conditions and chemicals that stimulates growth. Therefore, providing microbes in culture with the chemicals in their natural environment should aid in their growth. (*Kaeberlein et al., 2002*) designed a diffusion chamber with membranes that allow the exchange of chemicals while restricting the cells from leaving or entering the chamber. This device was submerged in an aquarium of natural water which the microbial communities were originally extracted from, this allowed for the exchange of chemicals from the natural environment. Colonies exhibited growth and were then further attempted to isolate on petri dishes, the isolates were checked for purity by using SEM or by performing PCR on the 16S rRNA full length gene. This led to the isolation of the organisms MSC1 and MSC2, which was previously uncultivatable. It is important to add that both isolates exhibited difficulty growing on petri dishes outside of the chamber. However, when cocultured with other organisms from the natural community on plates outside of the chamber they were able to grow. The results suggest’s there is a form of codependency between some organisms to grow in specific environments (*Kaeberlein et al., 2002*).

An important codependent relationship that requires more research is the role that *Veillonella* has in protecting the strict anaerobe, *F. nucleatum* under microaerophilic conditions. In addition it prevents *Streptococcus gordonii*’s production of H2O2 from killing and inhibiting *F. nucleatum* growth (*Zhou et al., 2017*). This shows the importance of isolating bacterial species from a single donor, once isolated we may seed them *in vitro* under controlled conditions in the lab, by
doing this we will further understand the microbe-microbe interactions on a molecular level and how they might influence the formation of the biofilm. due to the crucial role that *Fusobacterium* seems to have in the structural formation of the oral biofilm, it is the initial target for isolation in this thesis. Once various *Fusobacterium* species and subspecies are isolated from a single donor, we will aim to isolate other organisms such as *Veillonella*. By co-culturing *F. nucleatum* with various other species isolated from the same donor, we will be able to gain information on the structural formation of the oral biofilm at the molecular level by utilizing molecular techniques such as RNA sequencing.

**Techniques in Modern Culturing**

Due to the reliance of some microbes on beneficial bacterial interaction within the source environment, the use of helper strains has shown successes in growing some bacteria under lab conditions which otherwise are uncultivable. *(Nichols et al., 2008)* show that factors released from helper strains such as growth-promoting signals allow for the growth of uncultivable bacteria. They show that an uncultivable marine microbe MSC33 (*Psychrobacter* spp.) was able to grow on Agar media plates when cocultured with MSC33c, in addition the MSC33c condition-media without MSC33c promoted growth of MSC33 on agar media plates. by combining bioassay-guided fractionation, High Performance Liquid Chromatography, and mass spectrophotometry, they managed to identify the growth promoting-signal, a 5-amindo-acid peptide LQPEV. This provides evidence that the conditioning of media with spent culture supernatant or cell-free extracts derived from helper strains can promote growth of some uncultivable microbes. *(Nichols et al., 2008).*
These observations are critical in studying the oral microbiome by culturing the community. The environment in the oral cavity is ever changing with a significant amount of constant change occurring. The mouth contains the following selective forces influencing the interactions occurring between oral microbes: first, the flow of saliva and adhesion of initial colonizers onto the salivary pellicle, and coaggregation with one another. Second, the constant shedding and colonization of microbes on an ever-changing biofilm. Third, the host microbial interaction including the immune system and biochemical molecules (Mark Welch et al., 2020). To optimize the environment of oral communities grown in the lab we have been using the IBIDI pump system, which provides the cultured dental plaque with a constant controlled flow of SHI media which will mimic the flow of saliva in the mouth. This has been shown as successful model in various studies, including microfluidic organ-on-a chip design that allows the interaction of endothelial cells with pericytes, and the extracellular matrix in 3D vessel structures. The IBIDI pump system ultimately provided a tool for live in vitro imaging of human vasculature with circulating cells to mimic diseased environment (van Dijk et al., 2020), which can transfer to a powerful tool in understanding oral biofilm structure formation throughout time.

Advancements in modern molecular and imaging techniques are constantly used and applied to help detect and culture difficult organisms. An example was the use of PCR cloning, and then FISH to detect unculturable oral taxon Synergistetes and investigate its diversity and morphology in periodontitis subjects. (S. R. Vartoukian et al., 2009) Were able to establish a strong association of Synergistetes abundance and development of periodontitis, in addition the morphology was identified as a large, curved bacilli. By gaining such information such as the morphology and having a FISH probe that can detect this unculturable bacteria, it should help in future isolation or association studies that Synergistetes has on structure formation of the oral
biofilm. However, using FISH can be difficulty when identifying a bacterial taxon within a community without having a pure culture and prior knowledge on the metabolic properties of said taxon. This is because FISH targets the rRNA, therefore the hybridization is affected by metabolism and state of the cells such as cell size, growth rate, and cellular ribosome content. Catalyzed reporter deposition (CARD)-FISH has been as a technique utilizing horseradish peroxidase to enhance bacterial cell detection when using FISH on soil bacterial communities that are slow growing and not at their peak metabolic state (Ferrari et al., 2006).

Another way to gain genomic information on unculturable bacteria is via Flow cytometry and cell sorting (FACS). (Zengler et al., 2007) Were able to use gel microdroplets to encapsulate single bacterial cells from mixed environmental samples with specific oligonucleotide probes that makes it possible to detect and sort microcolonies from single cells. The microcolonies were grown in situ and then sorted microdroplets containing microcolonies from single cells via FACs. Although it is not possible to subsequently cultivate sorted cells due to the probes effecting cell viability, they were able to gain information on their genome sequence by amplifying the whole genome. By using this method it can provide critical information on the species requirement for in vitro cultivation (Zengler et al., 2007).
Chapter II
Materials & Methods

This section will provide materials, methodology, and results of the experiments mentioned in this manuscript. With approval from the University at Albany IRB (protocol #18E-068) dental plaque was self collected from a single healthy donor with dental floss. Dental floss was handed over to laboratory personnel, then immediately submerged in a reduced Dental Transport Media (DTM). Dental plaque was removed from the floss with vigorous pipetting up and down of the DTM. The liquid suspension was transported to an anaerobic chamber (Coy Systems) and aliquotted in pre-reduced, anaerobic glycerol solution into anaerobic crimp vials. The dental plaque was then stored at -80 C. Previous lab members have sequenced the community of the dental plaque by sequencing the 16S rRNA, which shows the donor dental plaque to be highly diverse and abundant in prevalent oral bacterial taxa (Figure 2.1) In order to increase the starting material volume due to the scarcity of human dental plaque imposed by COVID-19 restrictions, dental plaque was inoculated in SHI media broth with a microscope slide submerged in the tube which was grown anaerobically for 10 days (See Appendix P.53 for recipe). Two portions were then collected, first the planktonic bacteria in suspension of the broth, second the biofilm that formed on the slide was scraped and collected in the anaerobic chamber. Anaerobic glycerol stocks were prepared in the anaerobic chamber then stored at -80 C. Thus, two kinds of inocula were used in this project hereafter referred to as planktonic or biofilm inocula. For all growth experiments, SHI media broth and plates were prepared and placed in the anaerobic chamber 48 hours prior to use.
Figure 2.1. Sequencing data of the 16S rRNA region of healthy donor. Left 2 bars representing the abundance of bacteria collected from the donor on the genus level, left part is the sequencing data collected from the saliva of the donor while the right part is from the plaque portion. Each color represents the abundance of the given genus corresponding to the matching colors to the right.
Colony picking

Both the planktonic and biofilm inocula were removed from the -80 C, an inoculation loop was used to inoculate each type of starting inoculum in separate tubes containing 5 mL of SHI media, which were grown anaerobically for 10 days. After the 10 days 1 ul was removed from each tube and diluted in 1000 ul of SHI media, the solution was then vortexed. 50 uL was transferred and spread plate on SHI media plates, which was incubated at 37 C for 72 hours. This dilution was picked based off a dilution test I performed, using the material in dilutions ranging from 1:10 to 1:10000 dilution. The optimal dilution was a 1:1000 dilution providing 70-300 colonies when grown on SHI media plates. After 72 hours 24 colonies were picked from each plate based off morphological differences observed by eye, these colonies were then grown in 24 well plates containing 0.5 mL of SHI media for 72 hrs. In the anaerobic chamber, 100 uL from each well was removed and mixed with an equal amount of 1:1 glycerol-SHI media solution, the mixture was transferred to sterile crimp vials, capped, snap frozen, then stored in the -80 C. The wells containing the picked colonies were then imaged using a 40x evos microscope for an initial morphological screening.

Screening by Imaging

In attempt to identify *Fusobacterium* and *Veillonella* samples, candidates based off morphology were further imaged through a higher objective phase contrast microscope. Candidate samples were picked based off the morphology; *Fusobacterium* is a spindle-shaped bacillus with pointy ends also referred to as (filamentous), and *Veillonella* has a coccoid morphology that appear as single cells, in pairs or in short chains. The following samples were picked from the -80 C: biofilm A4, biofilm C1, biofilm C2, planktonic B1, planktonic C6, and planktonic D4. They were then grown anaerobically for 48 hours in 6 well plates with 3 mL of Valm media (see
Appendix P.54 for recipe). Valm media has shown to favor anaerobic organism growth due to the presence of the reductant L-cystine as shown by resazurin, a redox indictor in (see Appendix P.55). Valm media was prepared in the anaerobic chamber 72 hours prior to use. After 48 hours, 1 mL was transferred from each sample to an Eppendorf tube and centrifuged for 10 min at 15,000 RPM. The samples were then resuspended in 100 uL of water and transferred to a hydrophobic region of a microscope slide, a coverslip was then placed on top and sealed with nail polish, the samples were imaged using a phase contrast 100x objective on a widefield microscope.

FISH was then performed on each of the six candidate samples to preliminarily identify the different bacterial taxa present in each sample by using a EUB probe targeting all bacteria as a control, and six genus-level probes targeting some of the most prevalent bacterial taxa in the human oral microbiome. The genus level probes used are Actinomyces AF488, Porphyromonas AF488, Veillonella AF514, Fusobacterium AF555, Streptococcus AF594, Prevotella AF633, and EUB AF647. A reference image was needed before imaging the six samples, this was done by performing FISH on E.coli that was previously fixed at mid log-phase and stored at -80 C. The probes used on E.coli were EUB AF488, EUB AF514, EUB AF555, EUB AF594, EUB AF533, and EUB AF647. Utilizing Zen Black software, linear unmixing was then done to spectrally record data and overcome overlapping spectra. The unmixed data was then applied to each sample with a pseudo color being assigned for each peak as performed by (Valm et al., 2011).
**Isolating *Fusobacterium***

In attempt to isolate a pure culture of *Fusobacterium*, planktonic D4 was chosen and grown anaerobically in 5 mL of Valm media at 37 C for 48 hours. As performed previously, 1 uL of the planktonic D4 in Valm media was diluted in 1000 uL of Valm media and vortexed, 50 uL was then spread plate on Valm media plate and grown at 37 C for 48 hours. After 48 hours, 6 colonies were picked and grown anaerobically in 3 mL of Valm media in one of the wells of a 6 well plate at 37 C for 48 hours. 100 uL from each sample was used for the snap freezing protocol mentioned earlier and stored at -80 C. The remaining volume was used for FISH, utilizing the following probes; *Actinomyces* AF488, *Veillonella* AF514, *Fusobacterium* 555, and EUB AF647, the experiment was replicated two additional times.

**DNA Extraction**

Samples D4.1, D4.2, D3, and D4.4 were grown anaerobically for 48 hours in 5mL of Valm media at 37 C for DNA extraction. In addition, *E.coli* K12 was grown aerobically in 5 mL of LB broth in an incubator shaker at 37 C for a positive control. The samples were then centrifuged at 10,000 rpm for 10 minutes, each sample pellet was then resuspended in 1,000 uL of TE buffer and aliquoted in a sterile Eppendorf tube, another 1,000 uL of TE buffer was aliquoted in an Eppendorf tube as a negative control. 130 uL of freshly made lysis buffer (See Appendix P.56 for recipe) was added to each tube and incubated for 30 min at 37 C in a water bath, DNeasy Blood and Tissue Kit (QIAGEN, catalog number: 69504) protocol was used to perform DNA extraction. The DNA product was eluted in 53 uL of buffer EB that does not contain EDTA that might inhibit PCR reactions, the DNA concentration was measured using a (Nanodrop ND-1000) and stored at -20 C.
**PCR & Gel electrophoresis**

Polymerase Chain Reaction (PCR) was performed to amplify the full sequence of the 16S rRNA region. Each reaction contained 5 ul of 10x High fidelity PCR buffer (Invitrogen), 1 ul of dNTP mix (10 mM Invitrogen), 1 ul platinum taq DNA polymerase high fidelity (Invitrogen), 1 ul of each primer 8F, and 1492R (Table 2.1), 3 ul of 50 mM MgSO4 (Invitrogen), 150 ng of bacterial DNA, and complete the reaction with nuclease free H2O to achieve a final volume of 50 ul. The thermocycles were set to denature at 95 c for 2 min, followed by 30 cycles of; denaturation for 1 min at 95 c, annealing for 1 min at 59 c, and elongation at 72 c for 2 min, ending with single elongation at 72 c for 5 min. PCR purification was done using (QIAquick PCR Purification Kit Catalog no. 28104), product was stored at -20 c.

Gel electrophoresis was performed to identify the amplification of the 16S rRNA region which should represent a heavy band at the 1.5 kb mark. The gel was made with 0.7% agarose and 10 ul of SYBR green loaded in the gel. The Gene ruler 1 kb plus DNA ladder (ThermoFisher, SM1331) was used as a reference and loaded in the first well, the PCR cleanup products, including the positive, and negative controls were loaded in order and the gel was ran for 3 hours. The gel was then transferred to a molecular imager to visualize the gel.

**Sequencing**

The sample D4.3, which exhibited a strong band at the 1.5 kb mark on the gel, was sent out to EtonBioscience (Eton, NJ) for Sanger sequencing of the full 16S region shown in (Figure 2.2). Primers 8F, 515F, and 1492R were used for the sequencing, see (Table 2.1) for the sequence of primers. The sequencing data was analyzed via SnapeGene Viewer 5.24 and the two forward and one reverse sequence fragments were merged manually. Taxonomic identification was
performed with The Basic Local Alignment Search Tool (BLAST) via webserver at the National Center for Biotechnology Information (NCBI).

Figure 2.2. Full 16S region with commonly used primers. Green arrows are Forward primers going from 5’ to 3’. Orange arrows are reverse primers going from 3’ to 5’.

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<tr>
<th>Primer (F=forward, R=reverse)</th>
<th>Sequence</th>
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<tr>
<td>8F</td>
<td>AGAGTTTGATCCTGGCTCACG</td>
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<tr>
<td>515F</td>
<td>GTGCCAGCMGGCGGCTGAA</td>
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<tr>
<td>1492R</td>
<td>GGTACCTTGTACGACTT</td>
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Table 2.1. Sequence of primers used for sequencing of the full 16S region.
Chapter III
Results

**Morphological Detection**

Both biofilm and planktonic colonies initially grown and imaged using an EVOS microscope exhibited predominantly coccoid shaped morphologies, with approximately 80% of the images having an obvious coccoid shape as shown in (Figure 3.1 & 3.2). Another bacterial morphology of importance identified were filamentous shaped cells, this morphology was minimally observed in the samples with only planktonic B1, planktonic C6, and planktonic D4 showing an obvious dominance of filamentous shaped cells. The EVOS images were an efficient method for initial screening, however, to identify the samples morphology with greater confidence, specific samples of interest were imaged using a phase contrast 100x objective.

The samples biofilm A4, biofilm C1, biofilm C2, planktonic B1, planktonic C6, and planktonic D4 were picked and imaged using a phase contrast 100x objective. The images obtained showed a mix of several morphological distinct cells, with samples planktonic B1 and planktonic D4 having an apparent filamentous shape showing in (Figure 3.3).
Figure 3.1. EVOS images of colonies picked from biofilm cells. Colonies in SHI media wells initially picked from SHI media plates containing biofilm materiel. All 24 samples were directly imaged from 24 well plates using an EVOS microscope at 40x magnification. Samples labeled A1 to D6.

Figure 3.2. EVOS images of colonies picked from planktonic cells. Colonies in SHI media wells initially picked from SHI media plates containing Planktonic materiel. All 24 samples were directly imaged from 24 well plates using an EVOS microscope at 40x magnification. Samples labeled A1 to D6.
Figure 3.3. Phase contrast images of six picked samples. Samples are diluted in water, placed on microscope slides and dehydrated. Filamentous morphology apparent in samples planktonic B1 and planktonic D4. Located on the bottom left corner and bottom right corner respectively. Images were taken using a widefield microscope phase contrast 100x objective.

**Genus level Detection**

FISH was performed on *E. coli* aliquots labeled with 6 probes that had the same oligonucleotide sequence, but a different fluorophore attached. These same six fluorophores were used with probes for genus level detection. Linear unmixing was performed on the images using reference spectra extracted from pure populations of each *E. coli* standard as shown in (Figure 3.4). From the FISH images taken of the six-candidate plaque isolate samples, all exhibited a mixed culture of more than one bacterial taxon present at the genus level. *Veillonella* was not detected in any of the six, interestingly *Fusobacterium* was detected with high intensity staining in biofilm A4, planktonic B1, planktonic C6, and planktonic D4 shown in (Figure 3.5).
Sub-cultured samples from planktonic D4 provided evidence of possible samples consisting of pure *Fusobacterium* isolates. FISH images showed consistent reoccurring filamentous cells with only *Fusobacterium* probes being detected. Although some samples such as D4.5 did not contain any *Fusobacterium*, the majority obtained *Fusobacterium*, with a good yield of possible pure cultures due to no other bacteria being detected by the EUB probe (Figure 3.6). Replicates of the experiment were consistent with this outcome (images not provided).

**Figure 3.4. Linear unmixing with *E. coli* as a reference.** Image of *E. coli* labeled with six EUB probes using FISH (Left image). Linear unmixing was performed on the image using Zen black, pseudo colors were arranged for each fluorophore (Right image). Images were taken using a confocal microscope.
Figure 3.5. Genus level detection with FISH. Six candidate samples, each with seven genus level probes including a general EUB probe as a control, targeted genera, and corresponding color (On right). Green filamentous shaped cells are *Fusobacterium*, shown in biofilm A4, planktonic samples B1, C6, and D4.
Figure 3.6. Detection of pure isolates on genus level of sub-cultured samples. FISH images of six samples that were obtained from colonies grown of planktonic D4 on Valm media plates.

Species Detection

Concentration of DNA extracted from the potential *Fusobacterium* isolate samples D4.1, D4.2, D4.3, and D4.4 ranged from 55.7 ng/μL to 246.8 ng/μL (Table 3.1). After amplifying the 16S region through PCR and performing PCR cleanup, an agarose gel was made and each sample was loaded in a well, this included a positive control and negative control. Both the positive control and D4.3 had a strong band correlating to the 1.5 kb region when compared to the ladder in (Figure 3.7). Samples D4.1, D4.2, and D4.4 had no visible band. The DNA concentration of each of the samples after performing the PCR cleanup was measured, all 4 samples showed a significant reduction in concentration compared to the initial concentration observed prior to PCR. All 3 samples D4.1, D4.2, and D4.4 had a concentration below 10 ng/μL which is below
the required concentration for sequencing. Although D4.3 had a significant reduction in DNA concentration it was still above 10 ng/μL, with a concentration of 26 ng/μL (Table 3.2). The data provided gives an explanation on why some of the samples did not appear on the gel.

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Table 3.1. DNA concentration of sub-cultured samples after DNA extraction. Measured using (Nanodrop ND-1000)

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<tr>
<td>(-) Control</td>
<td>4.2</td>
<td>0.085</td>
<td>0.045</td>
<td>1.9</td>
<td>6.35</td>
</tr>
</tbody>
</table>

Table 3.2. DNA concentration of Sub-cultured samples after PCR & PCR cleanup. Measured using (Nanodrop ND-1000)
**Figure 3.7. Gel with expected *Fusobacterium* isolated samples.** Gel was made of 0.7% agarose and ran for ~3 hours. Wells 1, 2, and 3 corresponding to the ladder, the positive control, and D4.3, respectively. Arrow shows band location for 1500 base pairs (length of the full 16S region)

**Sequencing**

The sample D4.3 was sequenced using Sanger sequencing of the full 16S rRNA to confirm the purity and gain information at the species level. Using BLAST, D4.3 identified the highest with *Fusobacterium nucleatum subsp. animalis strain JCM 11025* with a 99.78% identical sequence shown in (Figure 3.8). Other species that produced significant alignments are *Fusobacterium nucleatum subsp. animalis ATCC 51191*, *Fusobacterium simiae strain JCM 17465*, and *Fusobacterium nucleatum subsp. nucleatum ATCC 25586*. 
Figure 3.8. BLAST obtained of top 5 alignment results from D4.3 sequencing data.
Chapter IV
Conclusion

This thesis aimed to develop a reproducible pipeline for isolating and identifying pure oral bacterial species extracted from a single donor. Based on the results of imaging and sequencing data, it can be concluded that this work provided a multi-step detection method based on the identification of taxonomic hierarchy of the bacterial species by media optimization, culturing, brightfield microscopy, phase contrast microscopy, FISH imaging, and sequencing of the 16S rRNA. The first step of the multi-step detection method utilized brightfield and phase contrast microscopy to detect the morphology of each sample from colonies picked and grown from dental plaque. This step aids in detection of mixed cultures by identifying more than one morphology present in a sample. Furthermore, it provides us with a general idea of the species, such as a filamentous morphology indicating a possible *Fusobacterium* sample. The second step examined the sample based on probe detection at the genus level through FISH. This was an essential step prior to sequencing to confirm that the sample is pure at the genera level, which can be shown in the multiple FISH images only being detect by the *Fusobacterium* AF-555 probe. Finally, sequencing of the full 16S region provided the pure species and subspecies *F. nucleatum* subsp. animalis. The results achieved are important in showing our ability of building a library of species isolated from a single donor, which can lead to understanding the unique ecological succession of dental plaque that occurs differently among individuals.

The work presented here shows that FISH is a powerful tool in screening possible isolated species based on genus level detection prior to sequencing, this has not been done previously in
the human oral microbiome field. Incorporating FISH into the pipeline provides substantial benefits by providing a preliminary genus-level assignment of unknown bacteria. Combined with the use of the EUB338 general bacteria probe, FISH allows a robust method to assay the purity of a candidate isolate culture at the genus level. Another benefit is the detailed morphological structure that is presented within a single field of view, this will layout information on the structural relation between two organisms that are codependent and cannot be isolated. Although FISH adds much value for the detection of unknown bacteria, it is dependent upon the amount of rRNA at a given time, which is most abundant at the mid-log phase. The exact time of mid log phase can be difficult to know when working with unknown bacterial species. In addition, the pipeline must be tested more to assess the ability and possible future implications that might come up when targeting other organisms for isolation.

**Future Directions**

The pipeline developed will help obtain more information on the human oral microbiome at the structural and molecular level, by providing an *in vitro* model of the oral biofilm with control of the bacterial species making up the biofilm. In addition, we can analyze the variations in bacterial species exhibited in different individuals and how that may affect the formation of the oral biofilm. The effectiveness of this pipeline is supported by the successful isolation of *F. nucleatum subsp. animalis*, a fastidious strict anaerobe that is challenging to isolate and plays a crucial role in biofilm formation. The current hypothesis of *F. nucleatum* acting as a bridging organism in the structure of the biofilm can be further tested *in vitro*. In addition, the dependency of *Fusobacterium* on other organisms for survival in an aerobic environment can be studied by seeding the isolated fusobacterium with organisms that will be isolated in the future, *in vitro*, under different conditions as illustrated in the proposed model in (Figure 4.1). In the future, I
will attempt to isolate more species from the same donor and study the structural and molecular outcomes of coculturing specific species with one another utilizing this model.

**Figure 4.1. Controlled *In vitro* oral biofilm model.** Individual isolated bacterial species shown at the top of the graph in test tubes, flow system for culturing shown after the first arrow at the bottom of the graph, and a confocal microscope for FISH imaging is shown after the second arrows to the far right of the graph.
References


Gibbons, R. J., Socransky, S. S., de Araujo, W. C., & van Houte, J. (1964). Studies of the


Mombelli A, Lang NP, B. W. et al. (1990). Microbial changes associated with the development of puberty.


Appendix

Shi Media Preparation

1a Preparation of stock solutions
   a. Hemin Solution – Combine in flask and heat to boiling. Store at 20°C
      | Hemin | Sigma, 51280 |
      | 50mg  | Hemin     |
      | 1.74g | K2HPO4    | Fisher, P288=500 |
      | 100mL | ddH2O     | Fisher, BP2470=1 |
   b. NAM Solution – Dissolve NAM in ddH2O and filter sterilize; store at 20°C
      | N=Acetyl Muramic Acid | Sigma, A3008=100MG |
      | 100mg | ddH2O     | Fisher, BP2470=1 |
      | 10mL  | ddH2O     | Fisher, BP2470=1 |
   c. Vitamin K Solution – Combine Vitamin K and ethanol and filter sterilize; store at 20°C
      | Vitamin K | Alpha Aesar, L10575 |
      | 10uL  | ddH2O     | Fisher, BP2470=1 |
      | 10mL  | ddH2O     | Fisher, BP2470=1 |

2. Combine the following in a clean, autoclavable bottle for 1L Shi Media:
   | Proteose Peptone | Fisher, BP1420=500 |
   | 10g              | BD Bacto, 211705 |
   | Trypticase Peptone | BD Bacto, 212750 |
   | 5g               | Yeast Extract    | Fisher, P217=500 |
   | KCl              | 2.5g             | Fisher, P217=500 |
   | 5g               | Hemin Solution   | (see 1a)         |
   | 0.06g            | Urea             | Fisher, U15=3    |
   | 0.17g            | Arginine         | Fisher, BP370=100 |
   | 992.5mL          | ddH2O            | Fisher, BP2470=12|

3. Autoclave at 121°C for 15min, remove from autoclave and let cool to 50°C or place in 50°C water bath and allow temperature to equalize.
4. In the biological safety cabinet Add the following to 1L autoclaved mixture
   | 100 uL | Vitamin K | (see 1c) |
   | 1 mL   | NAM Solution | (see 1b) |

5. Let cool to room temperature to use immediately or store at 4°C.
6. Just before you are ready to use the Shi medium (to prepare a liquid culture tube or to pour plates) In the biological safety cabinet add fresh filter sterilized, heat inactivated FBS (Thermofisher 10082139) so that the final concentration is 10%.
Valm Media Preparation

1a Preparation of stock solutions
   a. Hemin Solution – Combine in flask and heat to boiling. Store at 20°C
      
      |   |   |   |
      | 50 mg | Hemin | Sigma, 51280 |
      | 1.74 g | K2HPO4 | Fisher, P288=500 |
      | 100 mL | ddH2O | Fisher, BP2470=1 |
   b. NAM Solution – Dissolve NAM in ddH2O and filter sterilize, store at 20°C
      
      |   |   |   |
      | 100 mg | N=Acetyl Muramic Acid | Sigma, A3008=100MG |
      | 10 mL | ddH2O | Fisher, BP2470=1 |
   c. Vitamin K Solution – Combine Vitamin K and ethanol and filter sterilize; store at 20°C
      
      |   |   |   |
      | 10 uL | Vitamin K | Alpha Aesar, L10575 |
      | 10 mL | ddH2O | Fisher, BP2470=1 |

2. Combine the following in a clean, autoclavable bottle for 1L Shi Media:

   |   |   |   |
   | 10 g | Proteose Peptone | Fisher, BP1420=500 |
   | 3 g | Trypticase Peptone | BD Bacto, 211705 |
   | 5 g | Yeast Extract | BD Bacto, 212750 |
   | 2.5 g | KCl | Fisher, P217=500 |
   | 10 mL | Hemin Solution | (see 1a) |
   | 0.06 g | Urea | Fisher, U15=3 |
   | 0.87 g | L-Arginine | Fisher, BP370=100 |
   | 0.182 g | L-lysine | Fisher BP386-100 |
   | 0.075 g | Glycine | Fisher BP381-500 |
   | 0.5 g | Cysteine hydrochloride | Fisher BP376-100 |
   | 972.5 mL | ddH2O | Fisher, BP2470=12 |

3. Autoclave at 121°C for 15min, remove from autoclave and place the bottle in a 65°C water bath. Do not let the media get below 60°C until it is in the anaerobic chamber.

4. In the biological safety cabinet add the following to the 1L autoclaved mixture

   |   |   |
   | 100 uL | Vitamin K | (see 1c) |
   | 1 mL | NAM Solution | (see 1b) |

5. Take the media to the anaerobic chamber while its temperature is higher then 60°C and transfer in a 50 mL tube the amount of media required for the week. Add sterilized human serum (Sigma H3667) equivalent to 10% of the final volume of media.

6. For plates, substitute, 20g agar (BP 1423-500) for 20 mL of ddH2O in the base media stock. After adding human serum in the anaerobic chamber, the media should be poured in the plates and allowed to dry for 1 hour.
Appendix 4: Resazurin appears blue at the most oxidized state then pink, then clear as it gets reduced and loses oxygen content. Starting from the top left resazurin containing Cystine HCL is already more reduced the control. As time progresses in the anaerobic chamber to 72 hours to the bottom left the tube containing Cystine HCL has less oxygen than the control due to the clear color compared to pink.
## Lysis Buffer Preparation

<table>
<thead>
<tr>
<th>Volume per mL</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>35.38 mg lysozyme</td>
<td>20 mg/mL</td>
</tr>
<tr>
<td>972 uL H$_2$O</td>
<td></td>
</tr>
<tr>
<td>20 uL 1 M Tris (pH 7.0)</td>
<td>20 mM</td>
</tr>
<tr>
<td>8 uL 0.5 M EDTA</td>
<td>2 mM</td>
</tr>
</tbody>
</table>