Characterization of Dentin Carious Lesions and Supragingival Plaque from Children that Suffer from Severe Early Childhood Caries (S-ECC)

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Abstract

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Purpose

In order to better understand disease progression of Severe-Early Childhood Caries (S-ECC), we applied cutting edge metagenomic sequencing in order to evaluate the microbial diversity and taxonomic profiles of supragingival and dentin plaque from S-ECC lesions.

Methods

Children diagnosed with S-ECC, treatment planned for extractions, and scheduled for dental rehabilitation under general anesthesia were recruited. Supragingival plaque samples from matching sextants of planned extraction sites and deep dentinal plaque from extracted teeth were collected using sterile technique and placed in anaerobic transfer media (ATM). The DNA of each sample was isolated and sequenced on a Illumina MiSeq using paired-end chemistry. A subset of samples were sequenced on an Illumina NovaSeq for WGS analysis using paired end chemistry. Microbiome analysis includes Alpha and Beta diversity, beta dissimilarity, and taxonomic investigation at the strain level.

Results

Supragingival plaque displayed more diversity than deep caries dentin (Weighted Bray Curtis Distance: PC1, $P=1.9x10^{-5}$). Lactobacillus genus was significantly enriched in the deep carious dentin samples, with *L. casei, L. gasseri and L. paracasei* being prominent species. *Streptococcus mutans* was elevated in both the supragingival and deep carious dentin samples.

Conclusions

There is significant difference in the alpha and beta diversity between supragingival plaque and deep carious dentin in children with S-ECC. This data provides the most in depth metagenomic analysis of S-ECC samples to date.

DEDICATION

To my parents, Maggie and Jerry, who lovingly raised us to be curious leaders.

To my sisters, Hannah and Gretchen, who challenge and inspire me.

To my husband, Peter, who is my joy and my jet fuel.

And especially to my brother, Sam, who has been and always will be my reason.

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TABLE OF CONTENCE

INTRODUCTION:

Dental caries is the most common chronic disease of childhood.[1] Severe-Early Childhood Caries (S-ECC), previously known as 'baby bottle tooth decay', is a severe and specific presentation of dental caries that affects infants and young children.[2] S-ECC is defined as any sign of smooth-surface caries in a child younger than three years of age, and from ages three through five, one or more cavitated, missing (due to caries), or filled smooth surfaces in primary maxillary anterior teeth or a decayed, missing, or filled score of greater than or equal to four (age 3), greater than or equal to five (age 4), or greater than or equal to six (age 5).[2,3] S-ECC has reached epidemic proportions in the United States.[4,5] Prevalence of dental caries in children under 6 years old in the U.S. was last reported at 23%, according to the Center for Disease Control.[5]

S-ECC remains a significant public health concern that is seen at higher prevalence among low-socioeconomic status preschool children in the United States[6] and has been linked to increased risk of dental caries in the permanent dentition.[18] Untreated tooth decay in children can lead to poor performance in school,[9] missed days in school,[10] increased number of emergency room visits,[11] and high treatment costs that have an impact on society in the local, regional, and global level.[12,13] In addition to pain and discomfort, dental caries in young children can greatly affect their development and behavior,[14] is associated with lower oral health related quality of life, [6,7] and in extreme cases can lead to serious disability and even death.[14]

The study of dental caries presents challenges due to its multifactorial etiology.[7] Dental caries is a chronic disease that results from a persistent imbalance of risk factors and protective factors.[15] S-ECC is the result of the interaction between the tooth structure, cariogenic bacteria, fermentable carbohydrates, and a variety of host-factors.[15,16] There are many risk-factors that

may modulate the severity of the disease, such as sociodemographic factors, diet, oral hygiene, breastfeeding/bottle feeding, enamel defects, and oral bacterial flora.[16] However, despite complicated etiology and public health implications, many outstanding questions remain. One promising avenue to better understand this complex disease is though the perspective of the oral flora.

For decades the study of oral bacterial flora was looked at as a whole, mostly through saliva collection, in which oral bacteria have been identified by their DNA signatures. These DNA based studies of human saliva have discovered between 1000 to 2000 species present in the mouth.[19] But stimulated saliva may encounter many niches within the mouth, and more recent investigations have come to understand that there are the separate microenvironments and microorganism profiles within the oral cavity.[20] Recent DNA based studies of site-specific plaque samples have discovered that species level phylotypes within oral niches decrease from the 1000 to 2000 found in general saliva samples to values between 100 to 200.[19] These separate microecosystems are astonishingly diverse from each other. The taxonomic profiles of these ecosystems have even been found to fluctuate depending on the progression of carious lesions from initial enamel surface to the deep carious dentin.[21] Therefore, while saliva collection is relatively non-invasive and easy to acquire, its composition is derived from bacteria from all over the oral cavity, primarily from the tongue, which makes it hard to use as a diagnostic marker for studying niche specific microenvironments associated with various types of dental caries, including S-ECC.[22, 23] Similarly, the niche environment of supragingival plaque may not be representative of the ecology of the bacteria responsible for the localized S-ECC caries disease processes.

To date, studies investigating the possible microbial etiology of S-ECC have mostly focused on saliva and supragingival plaque analysis. Although it has been well established that *Streptococcus mutans* and *Streptococcus sobrinus* are key etiological agents of S-ECC,[24, 25] oral bacteria belonging to other genera such as Granulicatella, Actinomyces, Actinobaculum, Scardovia, Atopobium, Aggregatibacter, Slackia, Bifidobacteria, and Prevotella have also been associated with S-ECC through salivary and supragingival plaque analysis.[26, 27] Using methods such as 16S rRNA sequencing, other studies have suggested that *Veillonella atypical, Veillonella dispar, Veillonella parvula,* and Preveotella species may be the major drivers of S-ECC.[28] *Streptococcus parasanguinis, Streptococcus oralis, Streptococcus intermedius, Streptococcus vestibularis,* and *Streptococcus anginosus* as well as *Veillonella parvulla* and *Lactobacillus fermentum* have also been implicated as key species in the S-ECC disease process that seem to thrive in an increasingly acidic environment in the presence of fermentable carbohydrates.[29]

Temporal studies are also indicating complexities in oral flora at different ages. A recent study by Dashper *et al* (2019) demonstrated an ordered temporal development of the oral microbiome in children who suffer from S-ECC, displaying a distinct shift in composition as disease progressed.[30] This shift was seen in children as early as age 19.7 months of age, with a significant increase of salivary *S. mutans* in children who developed S-ECC by 39 months. Another study by Holgerson *et al* (2015) reported that elevated salivary levels of Lactobacilli in 3-month-old children were more prevalent in children who developed caries by 3 years of age.[27]

These recent findings not only suggest that children who suffer from S-ECC may have unique microbial environments, but that there may be a successional difference in the microbial taxonomic profiles of children who develop S-ECC. This study aims to investigate microbial drivers of the S-ECC disease process by comparing the taxonomic diversity of "healthy

supragingival plaque" and "diseased" deep carious dentin plaque in children diagnosed with S-ECC, in order to explore these taxonomic differences in those who developed S-ECC at an earlier versus late age. Early detection in the change in composition of the supragingival plaque to the disease driven microbial environment may help identify S-ECC risk prior to clinical manifestation of caries and aid in the earliest possible detection and prevention of this devastating and widespread disease in children.

OBJECTIVES:

In order to better understand the etiology of dentin caries within Severe-Early Childhood Caries (S-ECC), we applied cutting edge strain level analysis using 16s rRNA gene sequencing to investigate the microbial diversity and taxonomic profiles of newly deposited 'healthy' supragingival plaque in comparison to deep carious dentin plaque from S-ECC lesions.

Primary Objective

- Use high-temporal 16S rRNA gene sequencing to determine microbial taxonomic profiles of supragingival and dentin plaque obtained from the same severe early childhood caries patients and compare results to those reported in the literature.

Secondary Objectives

- Determine if there are taxonomic differences in children who suffer from "early" severe early-childhood caries (E-SECC) compared to "late" severe early-childhood caries (L-SECC). In this study, we defined E-SECC as children with SECC ages 0-36 months and L-SECC as children with SECC ages 37-71 months.
- Identify differential relative abundance of various taxonomic species that have cariogenic capacity, i.e. are both aciduric and acidogenic, and may facilitate the key etiological

processes associated with dentin caries, in either coordination or in the absence of *Streptococcus mutans*.

HYPOTHESES:

Primary Hypothesis

- Dentin plaque, which has a very different micro-environment in comparison to supragingival plaque, will result in a different microbial profile identified by 16S rRNA sequencing in comparison to supragingival samples derived from the same patient that suffers severe early childhood caries.

Secondary Hypothesis

- There are taxonomic differences in children who suffer from "early" severe early-childhood caries (E-SECC) compared to "late" severe early-childhood caries (L-SECC). In this study, we defined E-SECC as children with SECC ages 0-36 months and L-SECC as children with SECC ages 37-71 months.
- There is differential relative abundance of various taxonomic species involved in the etiological processes associated with dentin caries, in either coordination or in the absence of *Streptococcus mutans*. Suspected bacteria include species of the Lactobacillus and Veillonella genera.

METHODS:

Study Population:

The study population for this investigation was patients of record at The Center for Pediatric Dentistry (CPD). The CPD is an educational facility for pediatric dental training

located in Seattle, Washington. The CPD is affiliated with the University of Washington School of Dentistry (UWSoD) and Seattle Children's Hospital. It is a training site for pediatric dental residents and a rotation for UWSoD pre-doctoral dental students. The clinic provides comprehensive dental care, including preventative, restorative and surgical care is provided to children 18 years and younger. The CPD maintains a Dental Surgery Center (DSC). In the DSC, patients may be treated for comprehensive dental rehabilitation under general anesthesia. The study population consisted of children diagnosed with S-ECC scheduled for extraction and dental rehabilitation under general anesthesia at the CPD DSC between November 18th 2019 through October 28th 2020.

Sample Collection (See Figure 1):

Children diagnosed with S-ECC scheduled for extraction and dental rehabilitation under general anesthesia were recruited. Written informed consent was obtained from all caregivers. Supragingival plaque samples from matching sextants of planned extraction sites and deep dentinal plaque from extracted teeth were collected using sterile technique and placed in anaerobic transfer media (ATM) with glycerol (80%) and then flash frozen in liquid nitrogen (-196˚C) until transferred to UWSOD McLean Lab in which they were stored at -80˚C. DNA for each sample was extracted, quantified, and assessed for quality. Quality samples were sequenced on an Illumina MiSeq platform using paired-end chemistry (300 bp). This study was approved by the University of Washington's Institutional Review Board (STUDY00007221).

16S rRNA Amplicon Sequencing:

DNA and RNA were extracted with the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, CAT#80224) following the protocol for purification from tissues and with Lysing

Matrix E beads (MPBio CAT# 6914050) for cell lysis. DNA samples were further purified and concentrated using DNA Clean and Concentrator Kit-5 (Zymo Research, CAT# D401).

16S rRNA gene sequencing was performed for all DNA samples on an Illumina MiSeq platform. The run included a negative kit control, a no template library preparation control, and a Zymogenetics bacterial community standard (Zymo Research, CAT# D6310) positive control. Library preparation was performed as follows: The V3-V4 variable region of the 16S rRNA gene was amplified using gene-specific primers with Illumina adapter overhang sequences (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′). Each reaction mixture contained 2.5 µl of template, 5 µl of each 1 µM primer, and 12.5 µl of HiFi HotStart ReadyMix (KAPA, CAT# KK2602). Amplicon PCR was carried out with the following parameters: 95°C for 3 min, 35 cycles at [95°C for 30 s, 55°C for 30 s, 72°C for 30 s], 72°C for 5 min. PCR products were verified by gel electrophoresis (1% agarose gel) and cleaned with AMPure XP beads (Beckman Coulter, A63881). Amplicons were indexed using the Nextera XT Index Kit V2 set A (Illumina) and purified again with AMPure XP beads. SequalPrep Normalization Kit (Invitrogen, A10510-01) was used to normalize samples to a concentration of 1–2 ng/µL. Samples were pooled into a single library and checked for quality and quantity using a TapeStation 4200 High Sensitivity D1000 assay (Agilent Technologies) and Qubit High Sensitivity dsDNA assay (Thermo Fisher Scientific), respectively. The final pooled library was loaded on to an Illumina MiSeq with 15% PhiX spike, was run for 301 cycles (2 × 301 bp). A total of 4,821,199 sequencing reads were generated, with an average number of 66,961 reads per sample.

Data Analysis:

Demultiplexed paired-end sequences were downloaded from the Illumina MiSeq platform, imported to QIIME2 (v2019.10), and trimmed and denoised using the DADA2 package. [31] Forward reads were trimmed by 15 nucleotides (nt) from the 5′ end and truncated to 290 nt on the 3′ end; reverse reads were trimmed by 10 nt from the 5′ end and truncated to 250 nt from the 3′ end. Samples had an average of 73,000 reads with a minimum sampling depth of 22,639 reads. Taxonomic assignment was assigned to amplicon sequence variants (ASVs), which represent strain level diversity, using the feature-classifier suite trained on the Human Oral Microbiome Database (HOMD v15.1) using Qiime2. [32] The .biom file generated in QIIME2 was imported to RStudio v1.2.1335 for further processing.

Data were then merged with metadata, tree, and analyzed using Phyloseq v1.30.0. [33] Kit contaminants and unassigned taxonomy were discarded. Analyses, including Alpha and Beta diversity, were performed in RStudio with the Phyloseq package and visualized using ggplot2 v3.3.0 [34]. Alpha diversity metrics (Shannon, Chao1, and Observed) and Beta Diversity (Weighted Unifrac) was generated using Phyloseq. Taxonomic diversity was analyzed using agglomerated data at the phylum, genus, and species levels respectively and compared by plaque source (supra v dentin) as well as stratified by early and late S-ECC designation. Statistical analysis was performed using the non-parametric Wilcoxon test adjusted by Bonferroni FDR in which $P < 0.05$ was considered statistically significant using the rstatix and ggpubr packages (Kassambara, 2020).

Chi-squared test of association was utilized to examine the descriptive statistics of the participants and sample demographic information. Level of significance was predetermined at $p<0.05$.

RESULTS:

A total of 15 patients were included in the study. Two patients were excluded due to inability to extract viable DNA from collected samples, leaving a total of 13 patient samples included in analysis (N=13). Of these patients, 5 patients met the criteria for Early Severe Early Childhood Caries (E-SECC) and 8 met the criteria for Late Severe Early Childhood Caries (L-SECC). We defined E-SECC as children with SECC ages 0-36 months and L-SECC as children with SECC ages 37-71 months.

Descriptive Statistics: Patient Population, See Table 1 and Table 2

Overall patient age ranged from 22-71 months, with the mean age of patients 46.9 months. 46% were male and 54% were female. Ethnicity demographics were as follows: White (30.77%), Hispanic (7.69%), Asian or Pacific Islander (30.77%), Middle Eastern or North African (7.69%), Black or African American (15.38%), and Other (7.69%). The majority of patients were ASA class I, and patients that were classified as ASA class II were due BMI >95% (84.62% and 15.38% respectively, P=0.012).

The mean age of patients within the E-SECC stratification was 29.4 months, 60% were male and 40% were female. Ethnicity demographics were as follows: White (60%), Hispanic (20%) and Asian or Pacific Islander (20%). Eighty percent of the E-SECC patients were ASA class I, and patient's that were classified as ASA class II were due to Body Mass Index (BMI) being greater than 95%.

The mean age of patients within the L-SECC stratification was 57.8 months. 37.5% were male and 62.5% were female (P=0.039). Ethnicity demographics were as follows: White (12.5%), Asian or Pacific Islander (37.5%), Middle Eastern or North African (12.5%), Black or

African American (25.0%), and Other (12.5%). The majority of patients were ASA class I, and patients that were classified as ASA class II were due BMI >95% (87.5% and 12.5% respectively, P=0.034).

Descriptive Statistics: Sample Population, See Table 3 and Table 4

There was a total of 13 supragingival plaque samples and 30 dentin lesion plaque samples due to multiple carious lesions within an individual. Of the supragingival plaque samples, 69.23% were collected from anterior teeth and 30.77% were collected from posterior teeth. Of the dentin lesion plaque samples, 86.67% were collected from anterior teeth and 13.33% were collected from posterior teeth ($P=1.158 \times 10^{-5}$). There were 10 dentin lesion samples (33.33%) that had been treated with silver diamine fluoride (SDF) prior to collection, and of these 8 were anterior tooth samples and 2 were posterior tooth samples.

In the E-SECC sample group, all samples were collected from anterior tooth locations – 5 anterior supragingival samples and 18 dentin lesion plaque samples ($P= 2.21 \times 10^{-5}$). 11.11% of the E-SECC dentin samples had been previously exposed to SDF and all were from anterior teeth ($P= 9.67 \times 10^{\wedge -4}$).

In the L-SECC sample group, 8 samples were collected from supragingival plaque, 50% from anterior tooth locations and 50% from posterior tooth locations. There were 12 L-SECC dentin lesion plaque samples, 66.67% from anterior tooth locations and 33.33 from posterior tooth locations. Of the dentin plaque L-SECC samples, 16.67% had been previously treated with SDF, 8.33% from anterior tooth locations and 8.33% from posterior tooth locations (P= 0.021).

Microbiome Characterization

There was a significant difference in the alpha diversity – the evaluation of biodiversity within a sample – between the supragingival plaque and the deep carious dentin plaque. Dentin plaque exhibited significantly less alpha diversity in the dentin plaque when compared to the healthy supragingival plaque of the same subjects (Observed P=0.035, Chao1 P=0.034, Shannon P=0.01, Figure 2).

The principle coordinate analysis (PCoA) showed genetic variation separating the microbial flora extracted from different plaque sources. This indicates there was separation in the beta diversity – the magnitude of diversity of the microbial communities when compared to each other – between the supragingival plaque microbiome and the deep carious dentin microbiome in the S-ECC samples (Weighted Bray Curtis Distance: PC1, $P=1.9x10^{-5}$, Figure 3).

On the phylum level (Figure 4), relative abundance of Firmicutes was significantly higher in dentin plaque samples compared to supragingival samples (P=0.0042, Figure 5). Conversely, relative abundance of Saccharibacteria (P=0.016, Figure 6), Fusobacteria (P=0.0019, Figure 7) and Proteobacteria (P=0.00079, Figure 8) was significantly elevated in the supragingival plaque compared to dentin plaque.

At the genus level within the Firmicutes phyla (Figure 9), relative abundance of Lactobacillus was significantly increased in the dentin lesion plaque compared to supragingival plaque ($P=7.2x10^{-5}$, Figure 10). There was no significant difference in the observed relative abundance of Streptococcus between the supragingival and dentin plaque samples (P=0.80, Figure 11).

At the species level, *Lactobacillus casei* (P=0.0012, Figure 12), *Lactobacillus gasseri* (P=0.039, Figure 13), and *Lactobacillus paracasei* (P=0.039, Figure 14) reported significantly higher relative abundance in the dentin plaque samples compared to the supragingival plaque.

Microbiome Characterization of Early-SECC versus Late-SECC Stratification

There was no significant difference in alpha diversity between supragingival plaque and dentin plaque lesions in the E-SECC stratification (Observed P=0.4, Chao1 P=0.42, Shannon P=0.15, Figure 15) which was unexpected. However, there was a significant difference in the alpha diversity between the supragingival plaque and dentin plaque samples in the L-SECC stratification (Observed P=0.05, Chao1 P= 0.05, Shannon P=0.038, Figure 15).

Analysis of the beta diversity of the supragingival versus dentin plaque environments when the data was stratified into the E-SECC and L-SECC groups displayed separation in community and site (Figure 16).

At the phylum level (Figure 17), there was a significantly increased relative abundance of Firmicutes species in dentin lesion plaque samples compared to the supragingival plaque samples in the L-SECC group, but there was no significant difference in abundance between the plaque sources in the E-SECC group (P=0.015 and P=0.22 respectively, Figure 18). There was a significantly elevated relative abundance of Proteobacteria in the supragingival plaque compared to the dentin lesion plaque samples in the L-SECC group, as well as elevated in the supragingival plaque in the E-SECC group but not significantly (P= 0.00016, P= 0.31, Figure 19). There was also elevated relative abundance of Fusobacteria in the supragingival plaque compared to the dentin lesion plaque samples in the L-SECC group but not the E-SECC group (P=0.021, P=0.056, Figure 20).

At the genus level within the Firmicutes phyla (Figure 21), the Lactobacillus abundance was significantly elevated in the deep carious dentin plaque compared to the healthy supragingival plaque within the L-SECC stratification ($P=6.2x10^{-4}$, Figure 22). When comparing the relative abundance of Lactobacillus in the dentin plaque of the E-SECC group compared to the L-SECC group, there was no significant difference in abundance (P=0.44, Figure 22). Streptococcus abundance was elevated in both dentin and supragingival plaque in the E-SECC vs L-SECC stratification, but no significant difference between the two environments nor stratification subgroups (Figure 23).

At the species level *Lactobacillus paracasei* (P=0.021), *Lactobacillus casei* (P=0.007), *Lactobacillus gasseri* (P=0.028), *Lactobacillus pentos* (P=0.05) *Streptococcus mutans* (0.015), and *Streptococcus anginosus* (P=0.038) were all significantly elevated in the L-SECC dentin plaque compared to the L-SECC supragingival plaque (Figure 24). *Streptococcus mutans* was significantly elevated in the supragingival plaque when comparing the E-SECC versus L-SECC groups (P=0.011), but no difference in abundance in the dentin plaque when comparing the E-SECC to the L-SECC group (P=0.13, Figure 25). When comparing the dentin plaque communities of the E-SECC versus the L-SECC groups, *Streptococcus anginosus* was the only species within the Firmicutes phyla that was significantly elevated in the L-SECC group relative to E-SECC (P=0.045, Figure 26), while *Streptococcus vestibularis* was the only species elevated in the E-SECC group relative to the L-SECC group (0.045, Figure27).

DISCUSSION:

The sugar fermenting acidogenic species *Streptococcus mutans* has been considered to be the bacterial etiologic agent of dental caries and the cornerstone of cariology microbial investigation for the past 50 years. But through advances in technology, it has been revealed that

oral ecosystem is inhabited by hundreds of bacterial species in environmental niches that are subject to change and fluctuation.[21, 35] In this study, 16s rRNA amplicon sequencing was utilized to explore the differences in microbial diversity between the 'healthy' supragingival plaque and deep carious dentin plaque in children who suffer from S-ECC to further our understanding of microbial drivers of this disease. Additionally, we sought to investigate the proposed temporal differences between children who presented with S-ECC early in life (designated E-SECC, children ages 0-36 months) verses later in life (designated L-SECC, children ages 37-71 months).

This study found significant differences in microbial diversity between the supragingival plaque and the deep carious dentin plaque, and these differences were maintained when the data was stratified by the proposed E-SECC and L-SECC designations. Diversity in this study was measured by established metrics to evaluate alpha (Shannon, Chao1, and Observed) and beta (Weighted UniFrac distance) diversity. The significant difference in alpha diversity in this study reflects that strain level diversity represented by amplicon sequence variants (ASVs) were found to be higher in supragingival plaque compared to dentin plaque. Beta diversity evaluates how similar or different are the samples are to each other. Figure 16 shows clearly that there was a distinct separation in communities from the site where the samples were taken and the early versus late S-ECC proposed designations. This confirms our primary hypothesis that dentin plaque, which has a very different micro-environment in comparison to supragingival plaque, will result in a different microbial profile identified by 16S rRNA sequencing in comparison to supragingival samples derived from the same patient that suffers S-ECC.

The results of this study corroborates results from previous studies using 16S rRNA sequencing reported in the literature, confirming that *Streptococcus mutans* is one of the key

etiological agents of S-ECC.[25] *Streptococcus vestibularis* and *Streptococcus anginosus* had previously been associated as possible etiological agents of S-ECC due to their high activity at low pH in work published by Edlund et al.[29] However, this study suggests that there may be a temporal difference between establishment of the two species. *Streptococcus vestibularis* was more elevated in the dentin plaque in the E-SECC lesions compared to the L-SECC dentin plaque, which suggests it may aid in establishment of the severe disease progression. While *Streptococcus anginosus* was found elevated in the L-SECC dentin plaque when compared to the E-SECC dentin plaque and may suggest that it is associated as a microbial driver of disease within more established carious lesions.

While the *Veillonella* genus had previously been reported as drivers of disease in S-ECC lesions, [36, 37] results of this study found *Lactobacillus* and *Streprococcus* genus to be significantly more prevalent. The results of our study found lactobacilli such as *L. casei, L paracasei, L. gasseri,* and *L. pentos* to be more prevalent dentin plaque compared to the healthy supragingival plaque, particularly in the proposed Late S-ECC group. *L. casei, L paracasei* have previously been implicated in S-ECC dentin lesions.[24, 39] Caufield et al (2015)[38] proposed the notion that lactobacilli may be opportunistic invaders of precarious lesions. Caufield proposes that species like *S. mutans* begin colonization of teeth and establish an increasingly acidic environment in the presence of a fermentable carbohydrates. Enamel demineralization creates a "retentive niche" with low pH and anaerobic milieu that the primarily planktonic lactobacilli can colonize. With this in mind, the results of this study suggest that *S. mutans, S. anginosus* and *S. vestibularis* may play a critical role in colonization and initiation of the S-ECC lesion, but lactobacilli such as *L. casei, L paracasei, L. gasseri,* and *L. pentos* may contribute to the rapid and severe progression that we see in S-ECC carious lesions.

Of clinical significance, lactobacilli species have been found to be up to 10-fold more tolerant of fluoride than *S. mutans*.[38, 40] Additionally, lactobacilli have been found to be tolerant of alternative sugar alcohol sweeteners: 36%, 50% and 52% of *Lactobacillus* strains isolated from high caries risk adults could metabolize xylitol, mannitol and sorbitol respectively. [41]

Limitations

Contamination is a potential limitation with any sequencing study. Though great measures were taken to ensure sterile technique, contamination from other microbial niches of the mouth and saliva could have influenced the number and abundance of species found in the samples.

Additionally, the method of collection – scooping out the deep carious dentin with a sterile excavator – does not allow for analysis of any possible microbial architecture to the biome. It is possible that there is an enrichment of a species at the surface of the lesion – for example a facultative anaerobe such *S. mutans* – that is separate microbial profile than the enrichment of species at the deepest aspect of an active S-ECC lesion. Additionally, 16s rRNA only evaluates what species are present within the sample, not what species are metabolically active. It is possible that while some species found in the S-ECC dentin in this study had low abundance or non-significant differences between the healthy supragingival plaque, there may be significant differences in metabolic activity and gene expression and should be the focus of future studies.

There was also a number of subjects who had received antibiotics within 12 months of the collection date and who had teeth that had history of SDF application. These possible

confounding variables were investigated, but the power was not sufficient enough to report results. Further investigation into these variables is required with an increased sample size.

Directions for Further Research

The 16S rRNA amplicon sequencing of the collected samples yielded a vast amount of data that has yet to be evaluated. Over 400 species were found in this dataset and analysis was focused mainly on evaluating those taxa that have been previously reported in the literature. Investigation of bacteria in other phyla such as Actinobacteria and Proteobacteria, which was elevated in both the E-SECC and L-SECC subgroups has yet to be explored. Additionally, further investigation of the dataset with the addition of more samples (i.e. more dentin and supragingival plaque collection) could increase the power and validity of the data set. For example, while *Streptococcus anginosus* was elevated in the L-SECC dentin, there were four samples that were extremely enriched. Similarly, while *Streptococcus vestibularis* was elevated in the E-SECC dentin, there were two samples that were exceptionally enriched. Further investigation with increased sample size could help determine if these species are drivers of disease or outliers within this sample population.

Deeper understanding of the S-ECC disease process can further be accomplished through whole genome sequencing (WGS) and mRNA metatranscriptomic sequencing (RNAseq). While the 16S rRNA sequencing utilized in this study is very cost efficient and uses a ubiquitous marker that is often rich within samples, it often results in shallow insight or even misrepresentation of the true taxonomic diversity within studies due to the inability to distinguish live from dead cells or account for copy number variation across species.[42, 43]This approach does not provide any insight into what active expressing bacteria are doing, which is essential when trying to understand temporal changes in relation to progression of a disease etiology. WGS and

RNAseq may provide a more accurate representation of species present and active within the communities. RNSseq has the ability to offer deeper insight into microbial samples by investigating global mRNA expression. When applied to microbial communities, this metatranscriptomics approach not only provides the ability to identify active species within samples, but as well as the genes and pathways that are actively being transcribed by those species within a given sample.

Lastly, the National Health and Nutrition Examination Survey (NHANES) Food Frequency Questionnaire was given to parents of each study participant. Previous studies have found that there are nutritional differences and changes in eating habits in children who suffer from S-ECC.[17] Based on this study, there could be certain foods that may increase S-ECC caries risk as well. The lactobacilli found prevalent in patients in this study such as *L. casei* and *L. paracasei* have also been found prevent in probiotic medicines, foods such as yogurt and cheese, and are commonly isolated from Asian fermented fruits and vegetables.[44] Using this survey, comparison of S-ECC microbial diversity and dietary habits such as snacking frequency and breastfeeding duration can be explored and evaluated in future studies.

CONCLUSION:

There is significant difference in the alpha and beta diversity between supragingival plaque and deep carious dentin plaque within children who suffer from S-ECC, and these differences were maintained when the data was stratified between the proposed early and late SECC designations. Lactobacilli species such as *L. casei, L paracasei, L. gasseri*, and *L. pentos* were found to be more prevalent in dentin plaque compared to the supragingival plaque, particularly in the proposed Late S-ECC group. *Streptococcus vestibularis* was more elevated in the dentin plaque in the E-SECC dentin samples compared to L-SECC dentin samples, which

suggests it may aid in establishment of the severe disease progression earlier rather than later. Results from this study provide clarity of taxonomic diversity of S-ECC and fill current gaps in caries etiological research. This new foundational knowledge will hopefully aid in the development of a microbial-based profile for caries risk assessment as well as the development of novel therapeutics that reduce the burden and impact of this disease.

APPENDIX:

Table 1: Study Patient Population Demographics, Total

Subject Demographics		Subjects Total (N=13)	(% of Subjects Total)	Chi Squared Total (Adj P value)
Age				
	Range = 22 - 71 Months	Average age = 46.9 months		
Sex				
	Male	6	46.15%	0.163
	Female	7	53.85%	
Ethnicity				
	White	4	30.77%	0.413
	Hispanic	1	7.69%	
	Asian or Pacific Islander	4	30.77%	
	Middle Eastern or North African	1	7.69%	
	Black or African American	2	15.38%	
	Other	1	7.69%	
ASA Class				
	I	11	84.62%	0.012
	II: BMI>95%	2	15.38%	

Table 2: Study Patient Population Demographics, Early-SECC versus Late-SECC Stratification

Subject Demographics - Early vs. Late SECC		Early SECC, 0-36 months (N = 5)	Chi Squared (Adjusted P value)	Late SECC, 37-71 months (N = 8)	Chi Squared (Adjusted P value)
Age					
	Range = 22 - 71 Months	mean = 29.4		mean = 57.8	
Sex					
	Male	3 (60.0%)	0.107	3 (37.5%)	0.039
	Female	2 (40.0%)		5 (62.5%)	
Ethnicity					
	White	3 (60.0%)	0.145	1 (12.5%)	0.548
	Hispanic	1 (20.0%)		0 (0%)	
	Asian or Pacific Islander	1 (20.0%)		3 (37.5%)	
	Middle Eastern or North African	0 (0%)		1 (12.5%)	
	Black or African American	0 (0%)		2 (25.0%)	
	Other	0 (0%)		1 (12.5)	
ASA Class					
	I	4 (80.0%)	0.180	7 (87.5%)	0.034
	II: BMI>95%	1 (20.0%)		1 (12.5%)	

Table 3: Sample Demographics, Total

Sample Demographics		Supragingival Plaque (N = 13)	(% of Subjects)	Chi Squared (Adjusted P value)	Dentin Lesion Plaque (N = 30)	(% of Subjects)	Chi Squared (Adjusted P value)
Tooth Location							
	Anterior tooth	9	69.23%	0.166	26	86.67%	1.158 x 10^-5
	Posterior tooth	4	30.77%		4	13.33%	
Antibiotics Exposure in last 12 Months							
	Yes	3	23.08%	0.052	10	33.33%	0.068
	No	10	76.92%		20	66.67%	
SDF Exposure							
	Yes	4	30.77%	0.165	10	33.33%	0.068
	Anterior tooth	2	15.38%		8	26.67%	
	Posterior tooth	2	15.38%		2	6.67%	
	No	9	69.23%		20	66.67%	

Table 4: Sample Demographics, Early-SECC versus Late-SECC Stratification

Early vs. Late S-ECC Stratification Sample Demographics		E-SECC Supragingival Plaque	E-SECC Dentin Lesion Plaque	Chi Squared (Adjusted P value)	L-SECC Supragingival Plaque	L-SECC Dentin Lesion Plaque	Chi Squared (Adjusted P value)
		(N = 5)	(N = 18)		(N = 8)	(N = 12)	
Tooth Location							
	Anterior tooth	5 (100%)	18 (100%)	2.21 x 10^-5	4 (50.0%)	8 (66.67%)	0.248
	Posterior tooth	0 (0%)	0 (0%)		4 (50.0%)	4 (33.33%)	
Antibiotics Exposure in last 12 Months							
	Yes	1 (20.0%)	8 (44.4%)	0.637	2 (25.0%)	2 (16.67%)	0.021
	No	4 (80.0%)	10 (55.56%)		6 (75.0%)	10 (88.33%)	
SDF Exposure							
	Yes	2 (40.0%)	2 (11.11%)	9.67 x 10^-4	2 (25.0%)	2 (16.67%)	0.021
	Anterior tooth	2 (40.0%)	2 (11.11%)		1 (12.50%)	1 (8.33%)	
	Posterior tooth	0 (0%)	00 (0%)		1 (12.50%)	1 (8.33%)	
	No	3 (60.0%)	16 (88.89%)		6 (75.0%)	10 (88.83%)	

Figure 1: Sample Collection Methods

Figure 2: Alpha Diversity of S-ECC Microbiome, Dentin Plaque vs. Supragingival Plaque, DNA

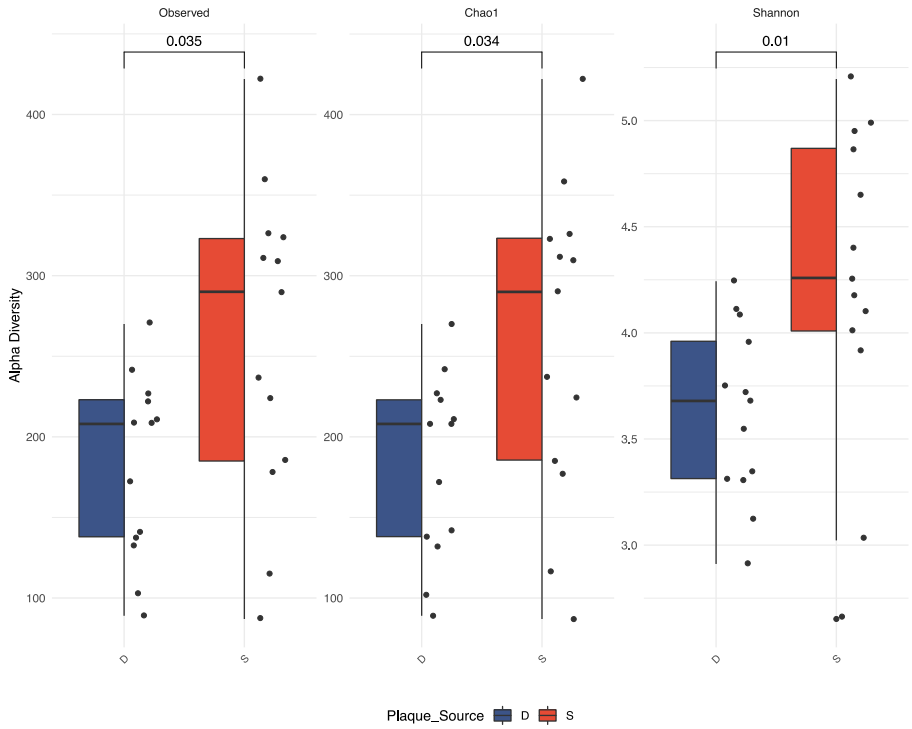


Figure 3: Beta Diversity of S-ECC Microbiome, Principle Coordinate Analysis Using Weighted UniFrac Distance

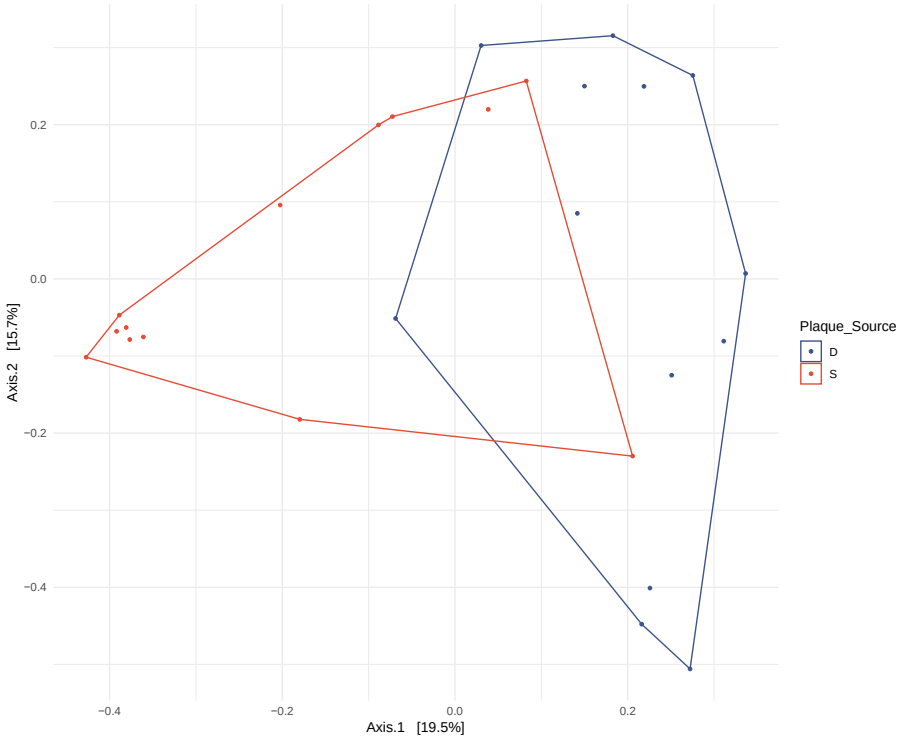


Figure 4: Phylum Relative Abundance in the S-ECC Microbiome

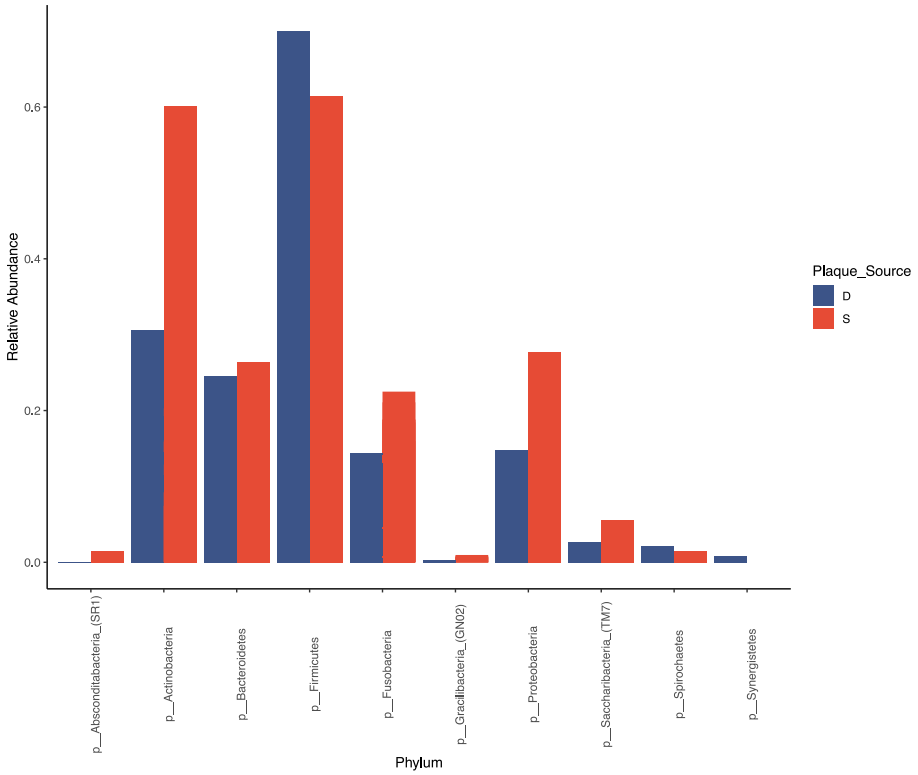


Figure 5: Phylum of Firmicutes Relative Abundance Supragingival Plaque vs. Dentin Plaque, DNA

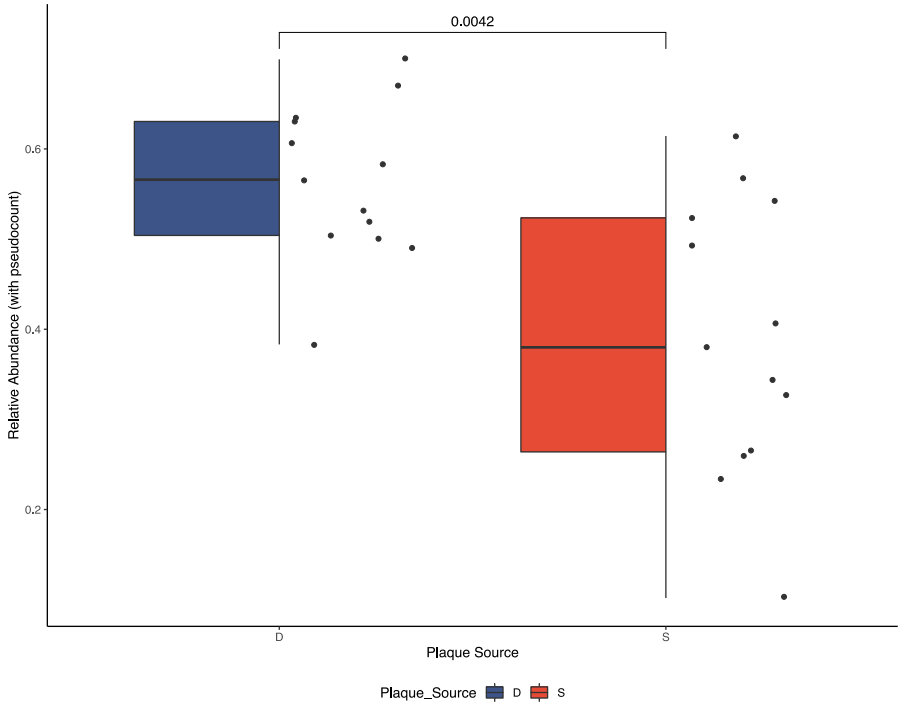


Figure 6: Phylum of Saccharibacteria Relative Abundance Supragingival Plaque vs. Dentin Plaque, DNA

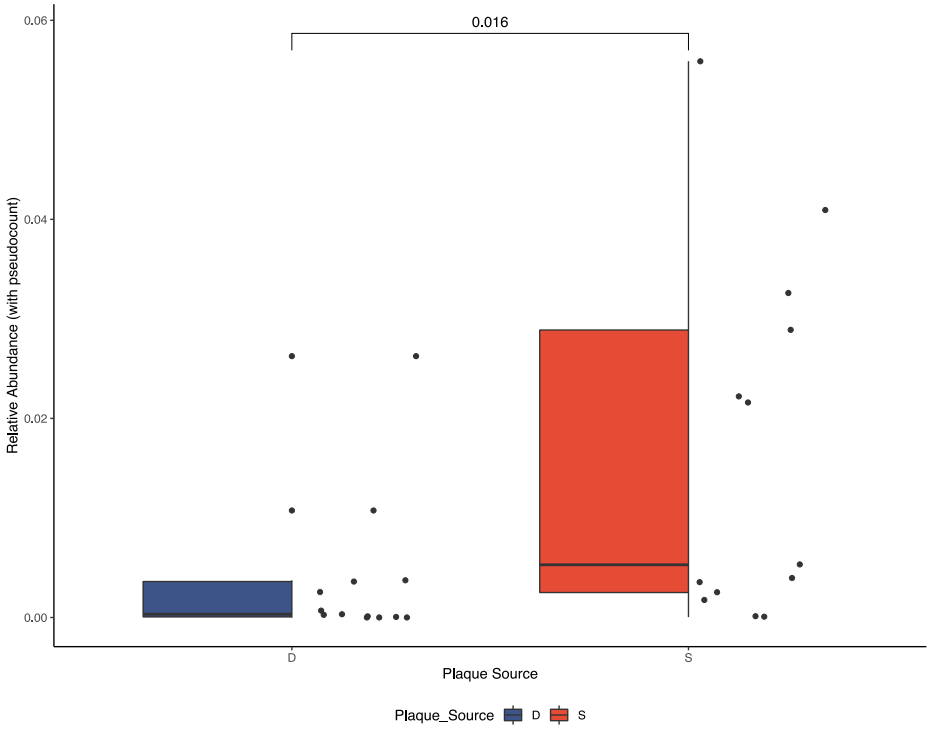


Figure 7: Phylum of Fusobacteria Relative Abundance Supragingival Plaque vs. Dentin Plaque, DNA

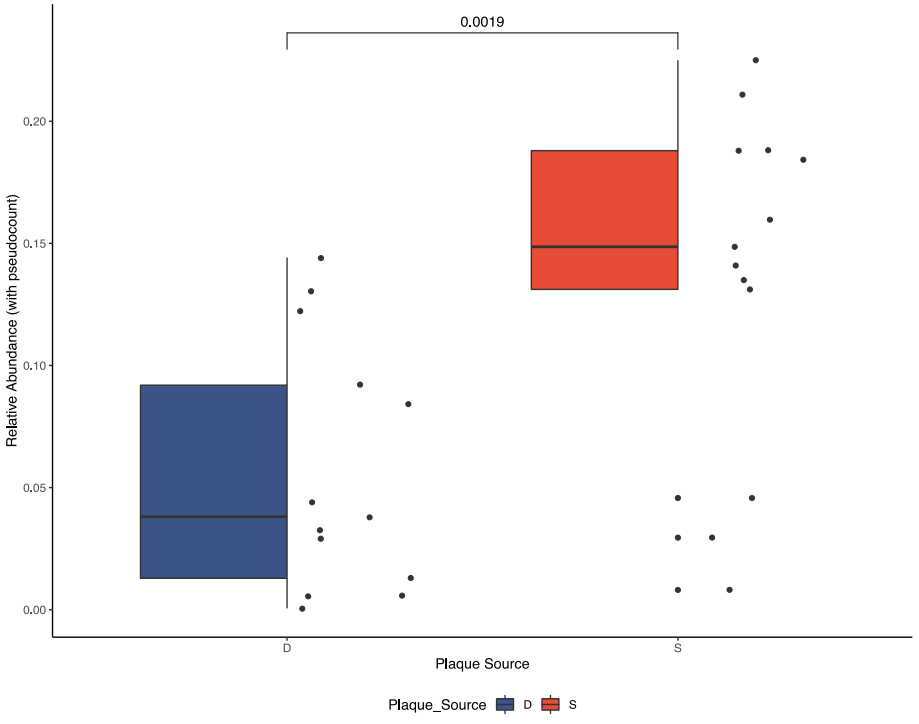


Figure 8: Phylum of Proteobacteria Relative Abundance Supragingival Plaque vs. Dentin Plaque, DNA

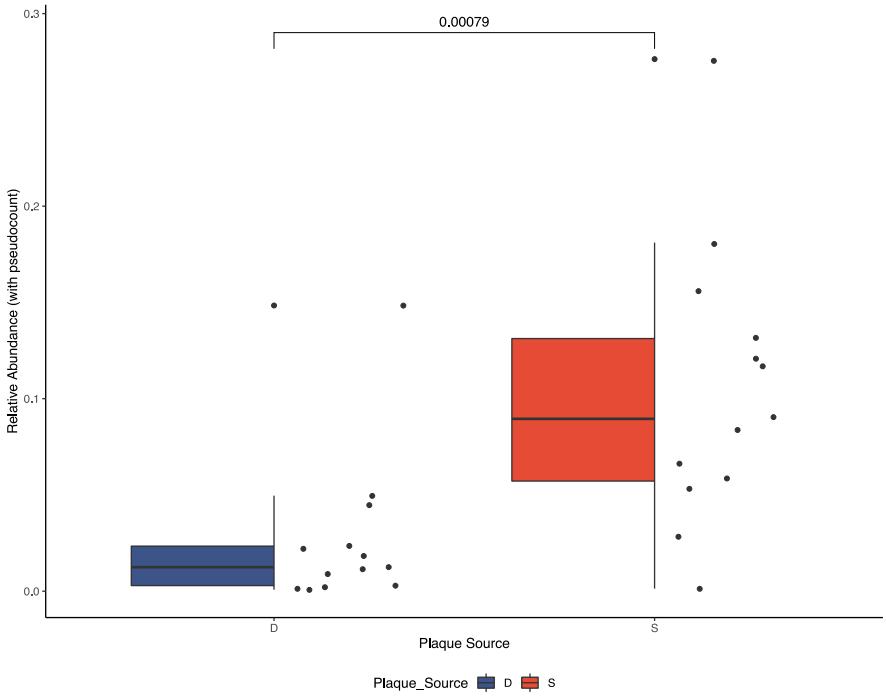


Figure 9: Genera Within the Firmicutes Phyla, Relative Abundance in the S-ECC Microbiome

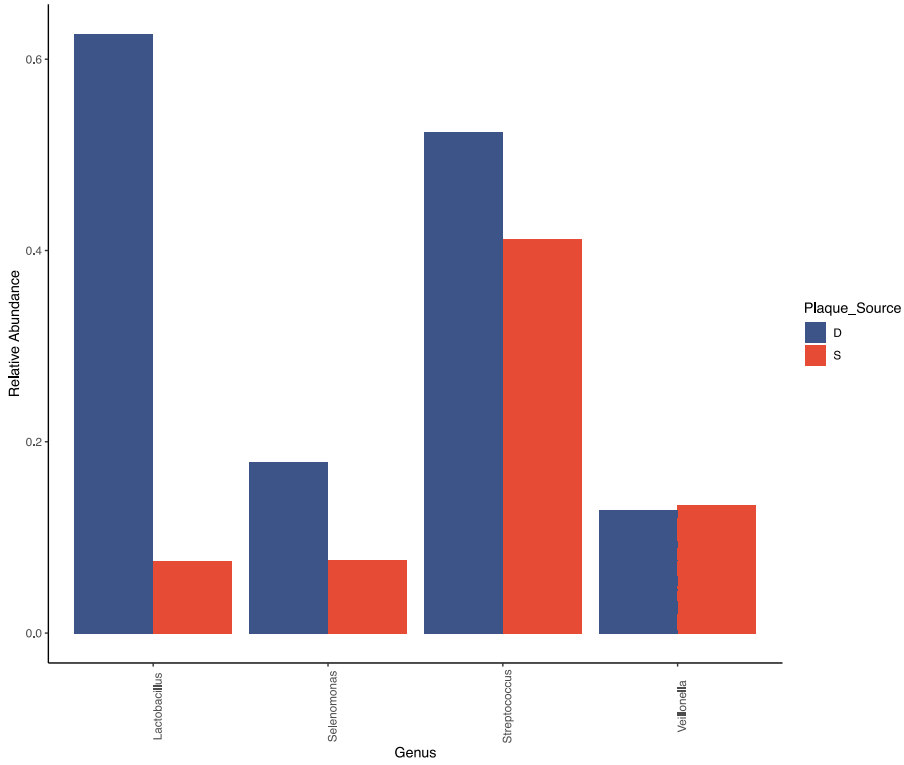


Figure 10: Lactobacillus Genera, Relative Abundance in the S-ECC Microbiome

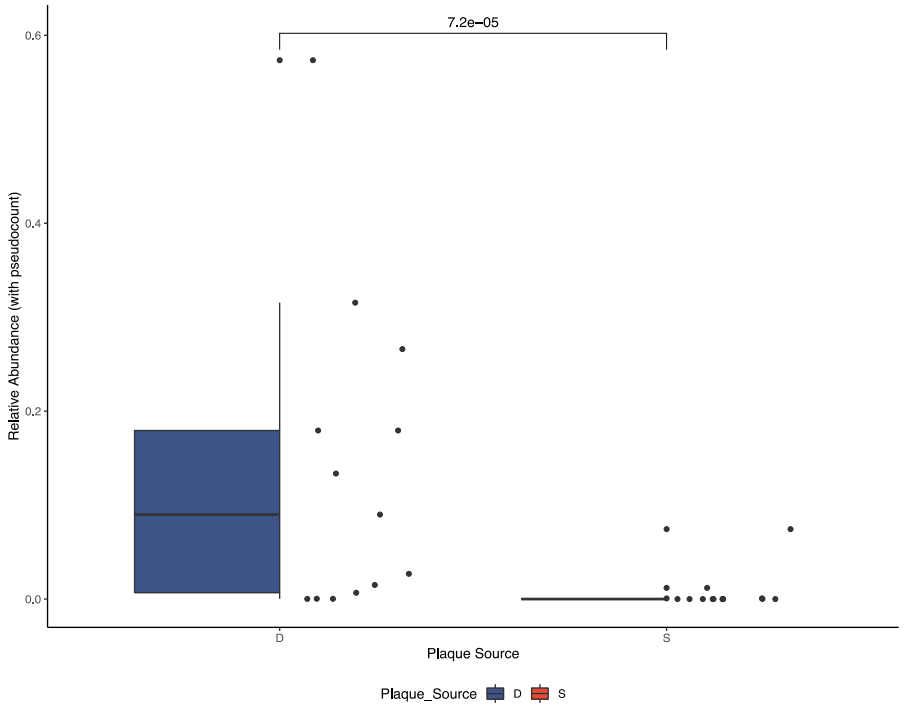


Figure 11: Streptococcus Genera, Relative Abundance in the S-ECC Microbiome

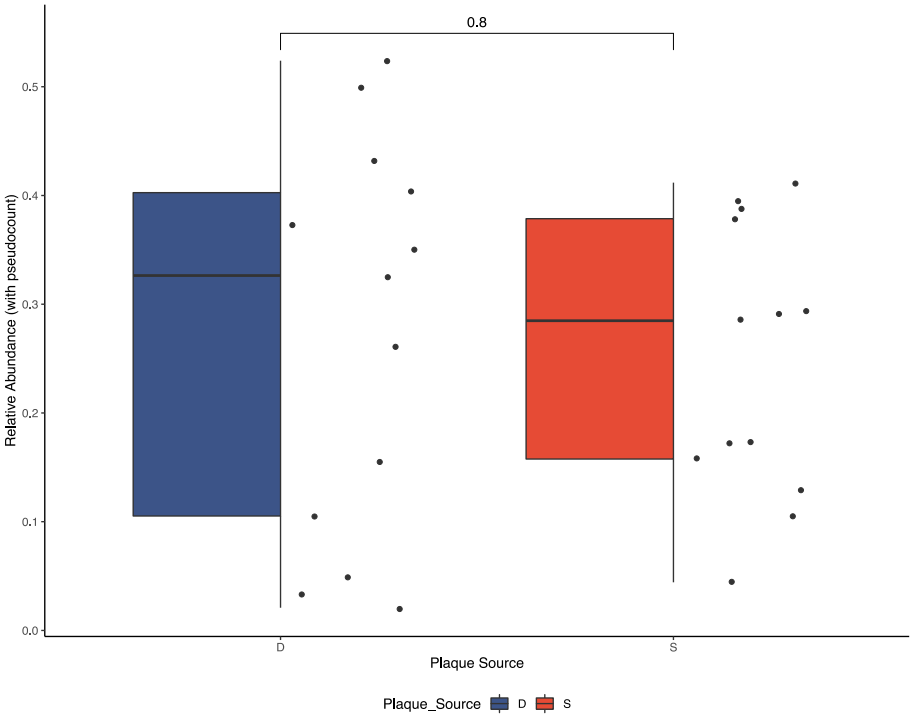


Figure 12: *Lactobacillus casei*, Relative Abundance in the S-ECC Microbiome

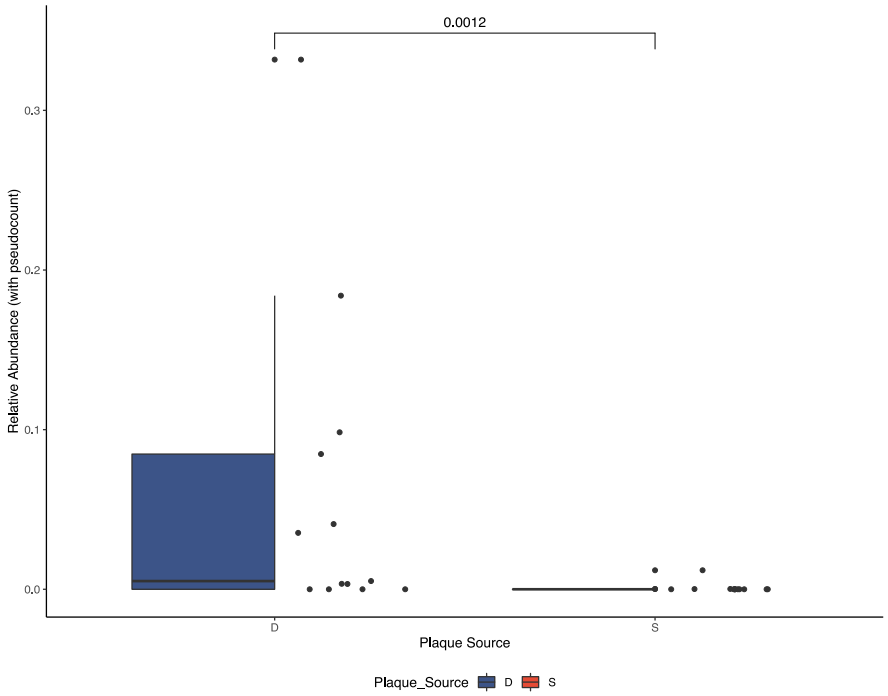


Figure 13: *Lactobacillus gasseri,* Relative Abundance in the S-ECC Microbiome

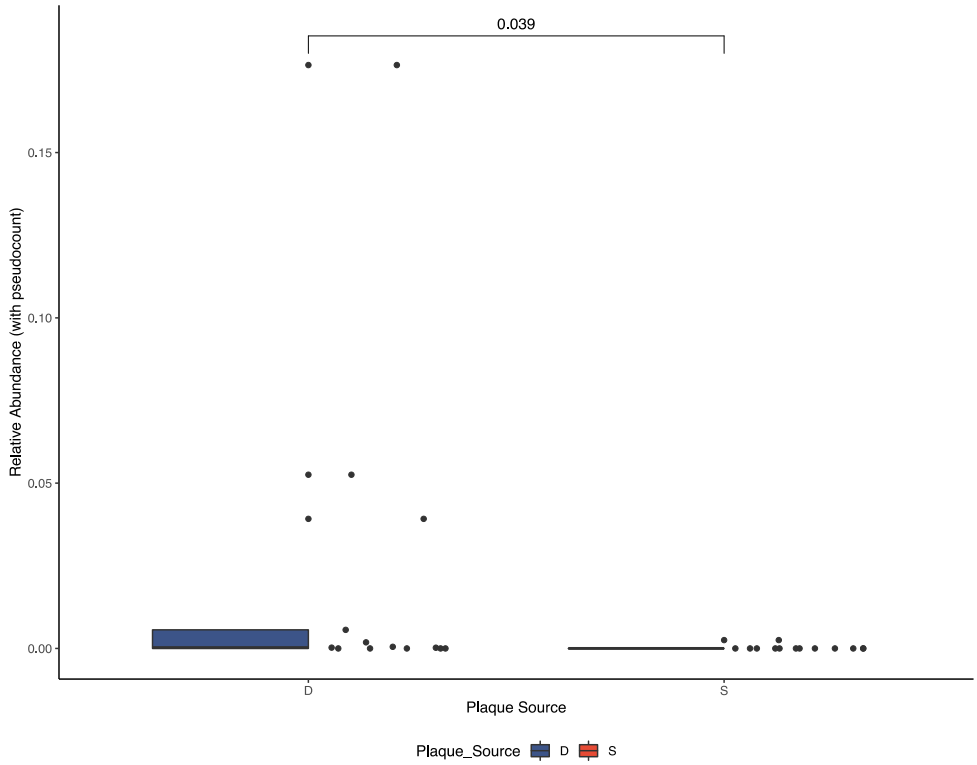


Figure 14: *Lactobacillus paracasei*, Relative Abundance in the S-ECC Microbiome

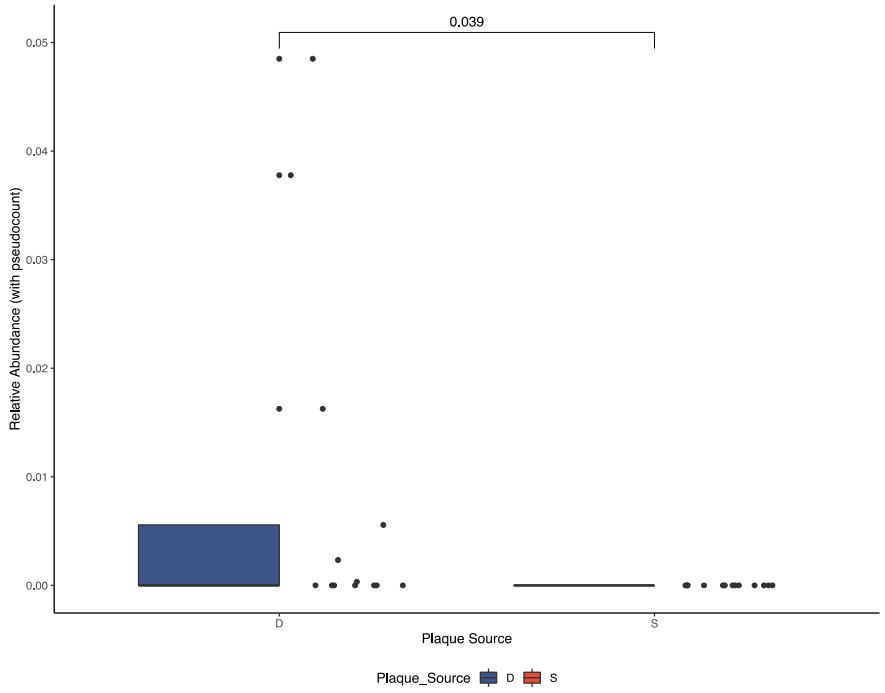


Figure 15: Alpha Diversity of S-ECC Microbiome, Dentin Plaque vs. Supragingival Plaque, E-SECC vs. L-SECC Stratification, DNA

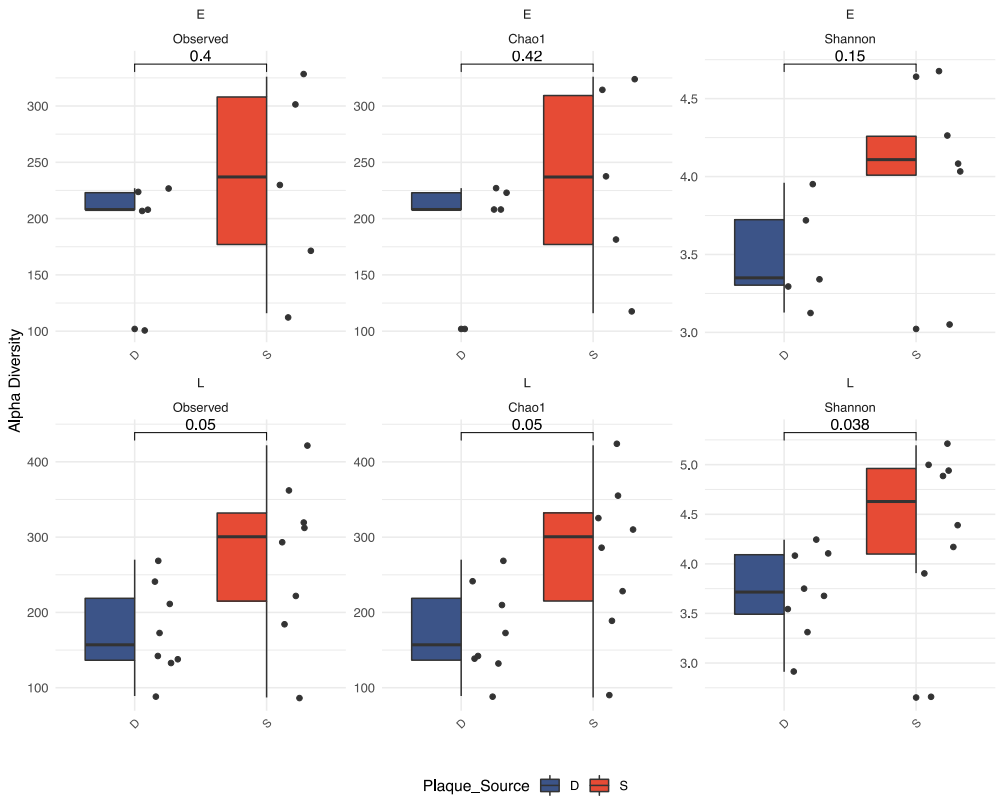


Figure 16: Beta Diversity of S-ECC Microbiome, Principle Coordinate Analysis Using Weighted UniFrac Distances, E-SECC vs. L-SECC Stratification

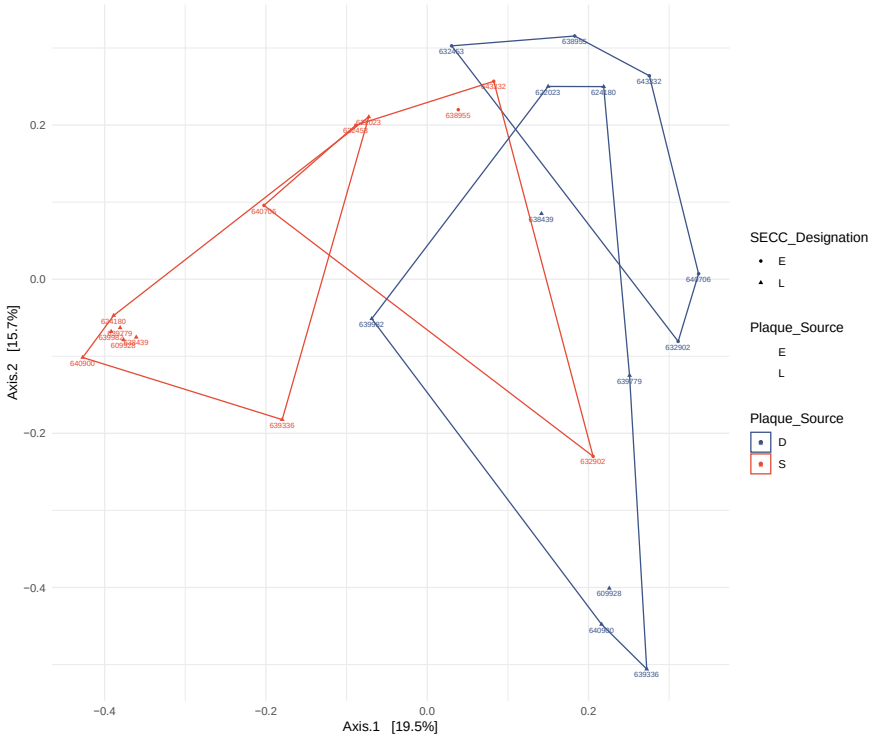


Figure 17: Phylum Relative Abundance in the S-ECC Microbiome, E-SECC vs. L-SECC Stratification

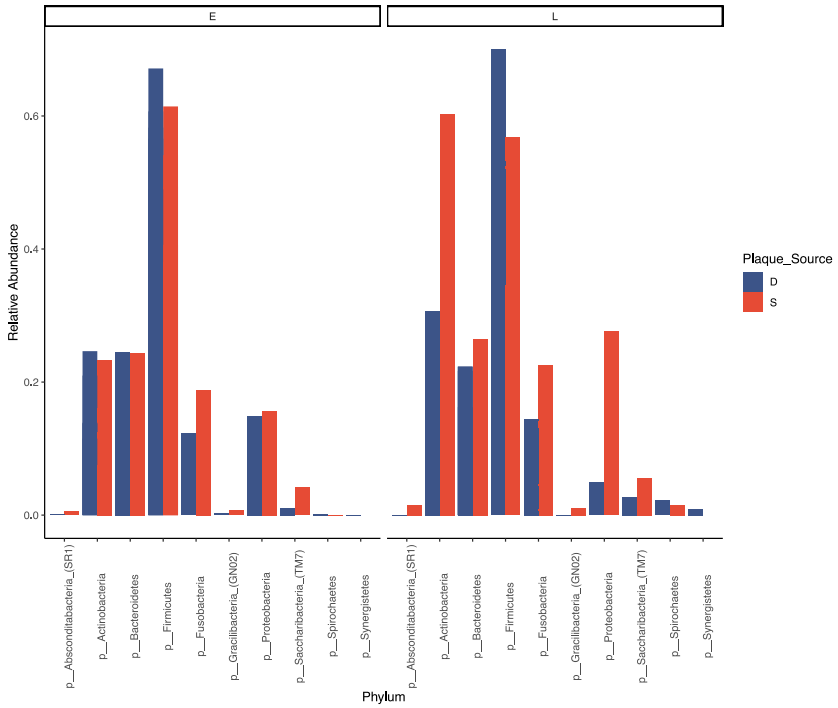


Figure 18: Phylum of Firmicutes Relative Abundance Supragingival Plaque vs. Dentin Plaque DNA, E-SECC vs. L-SECC Stratification

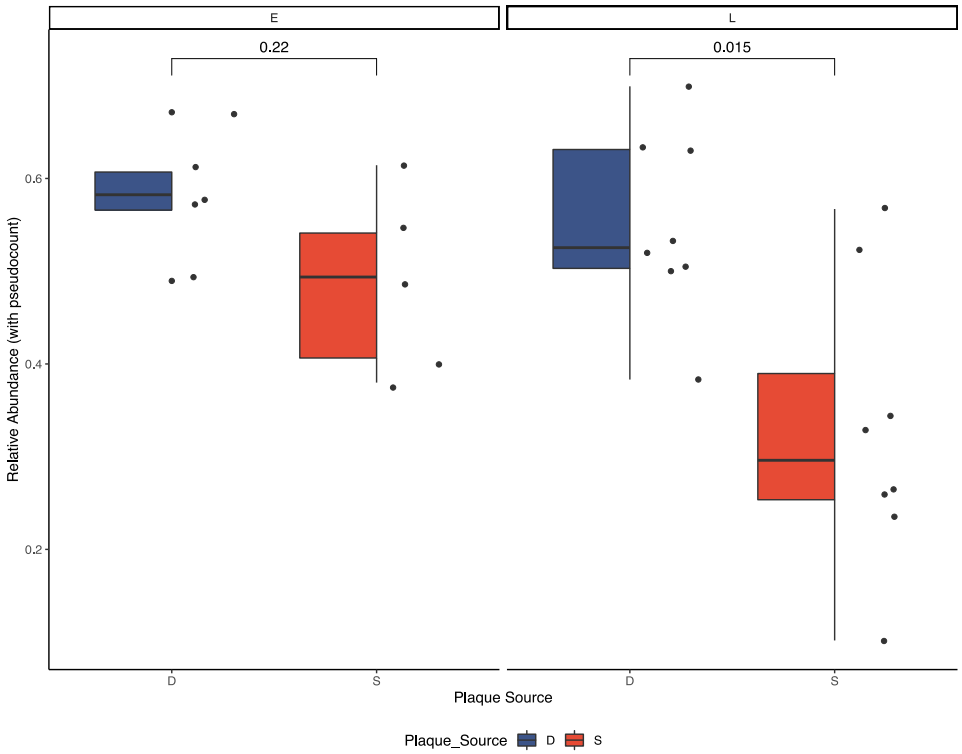


Figure 19: Phylum of Proteobacteria Relative Abundance Supragingival Plaque vs. Dentin Plaque DNA, E-SECC vs. L-SECC Stratification

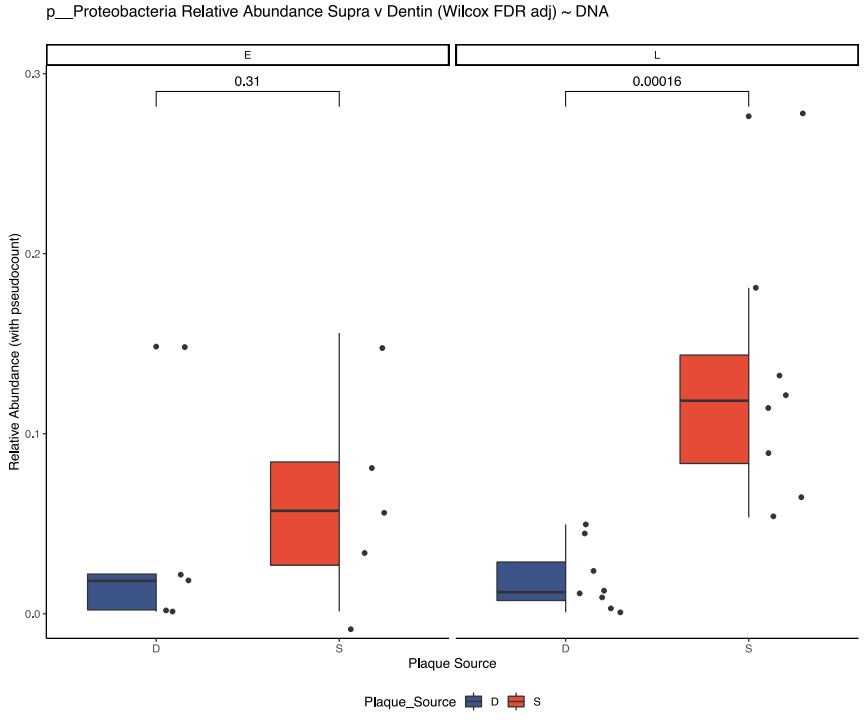


Figure 20: Phylum of Fusobacteria Relative Abundance Supragingival Plaque vs. Dentin Plaque DNA, E-SECC vs. L-SECC Stratification

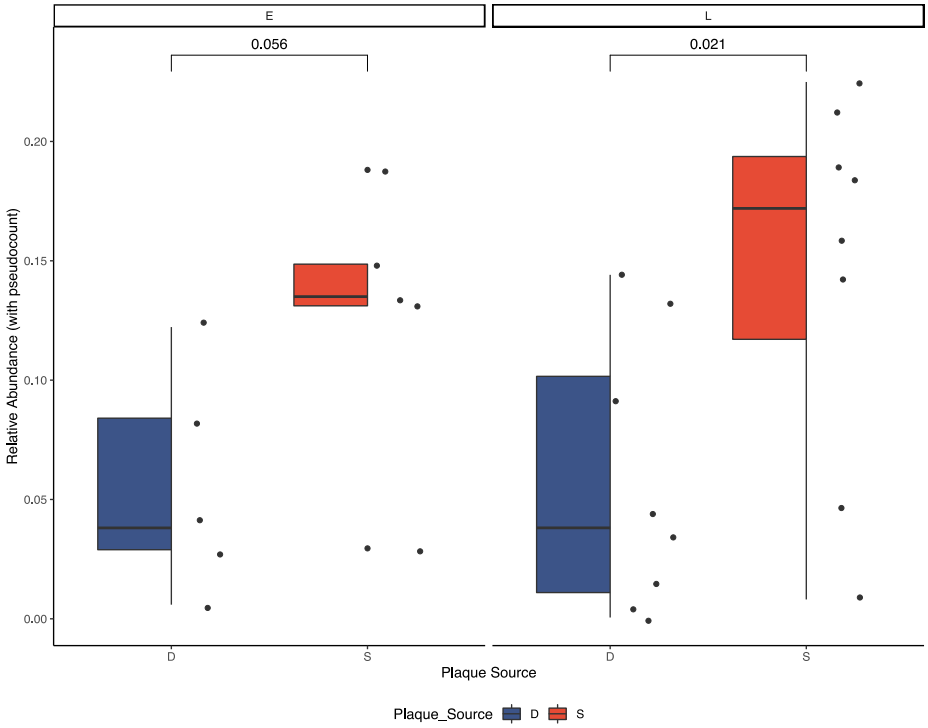


Figure 21: Genera Within the Firmicutes Phyla, Relative Abundance in the S-ECC Microbiome, E-SECC vs. L-SECC Stratification

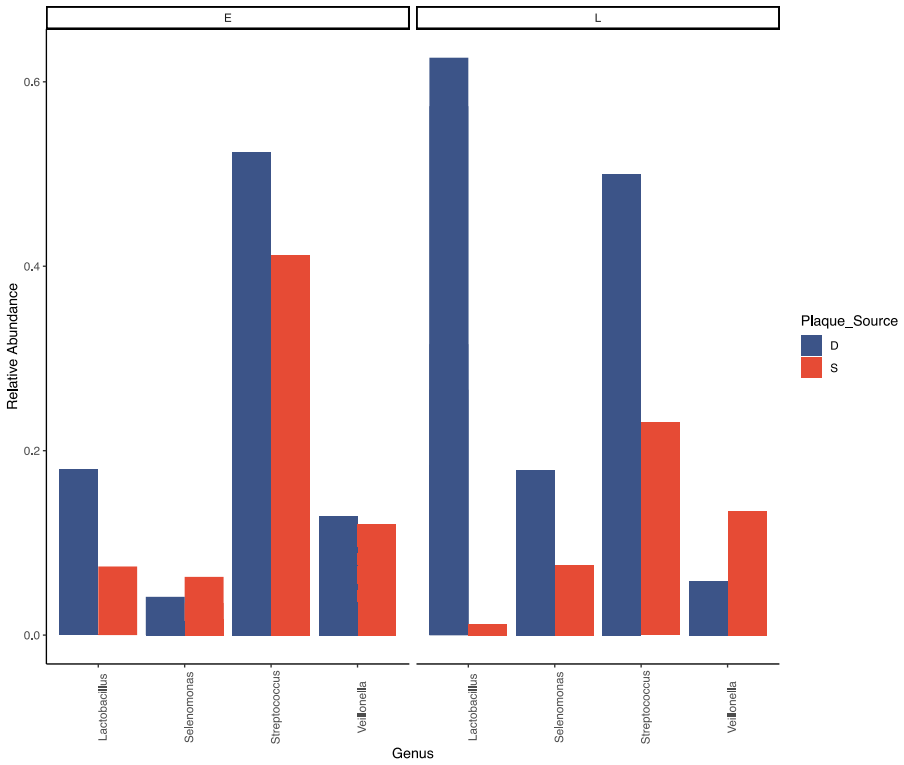


Figure 22: Lactobacillus Genera, Relative Abundance in the S-ECC Microbiome, E-SECC vs. L-SECC Stratification

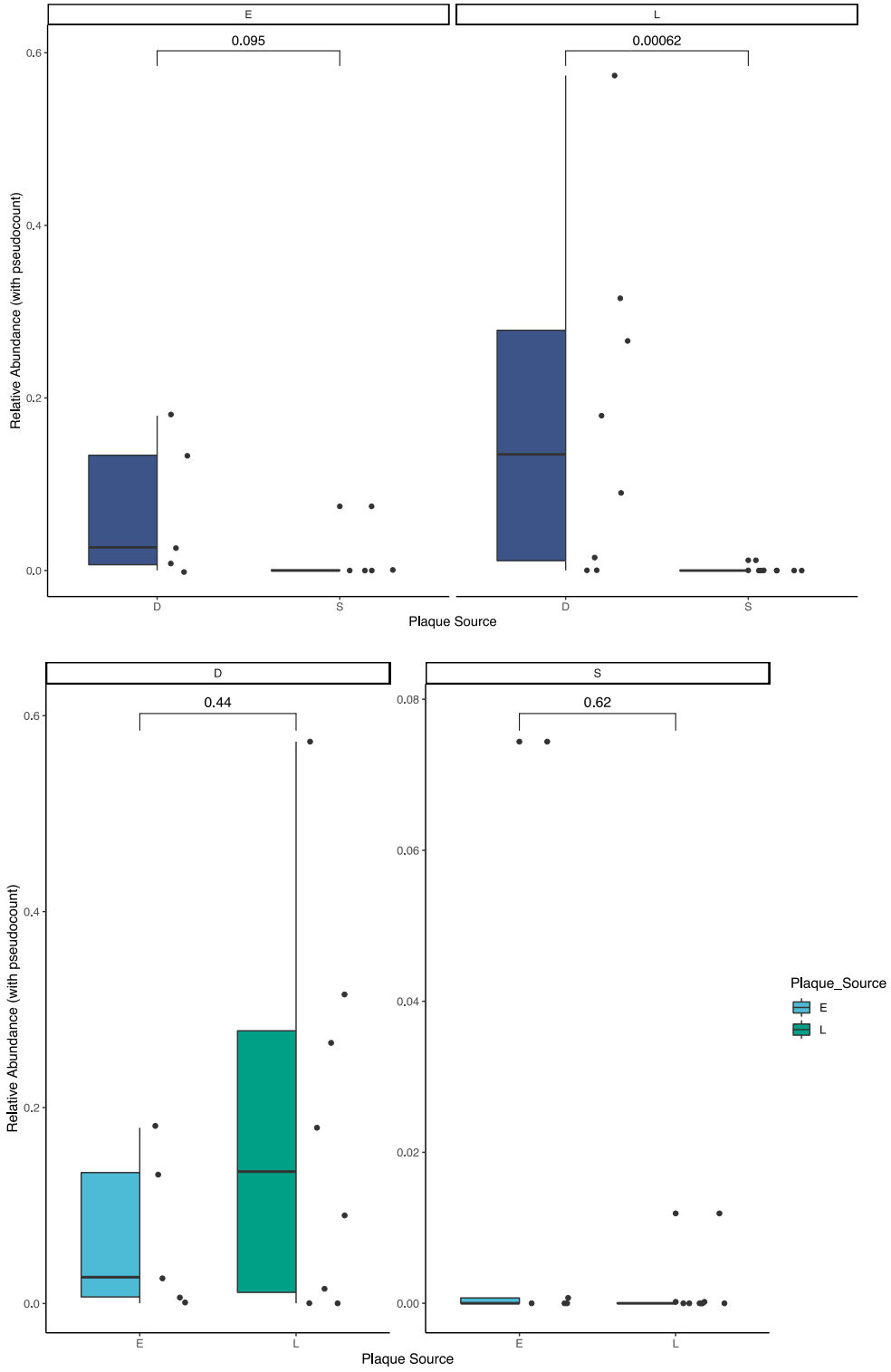


Figure 23: Streptococcus Genera, Relative Abundance in the S-ECC Microbiome, E-SECC vs. L-SECC Stratification

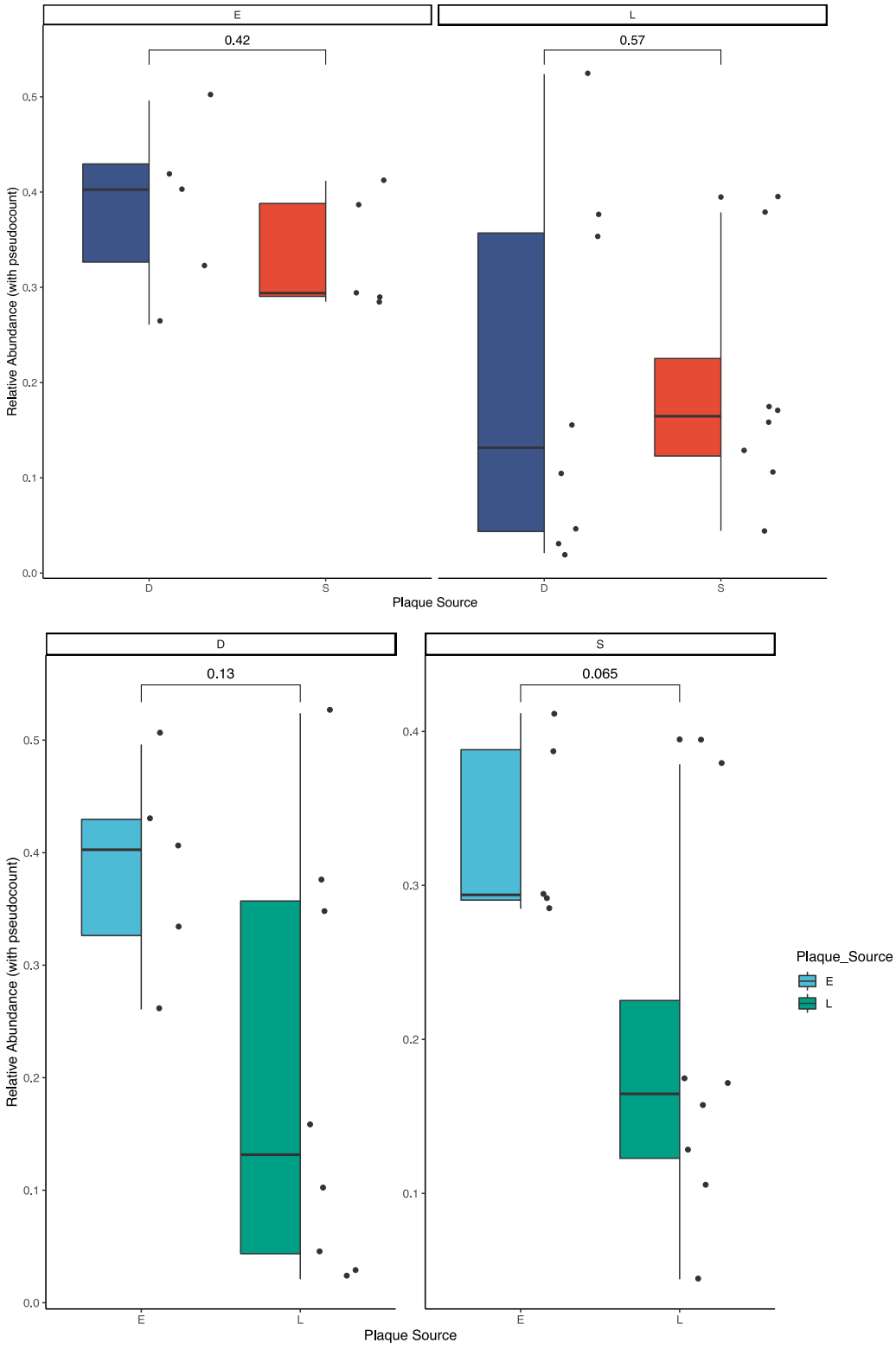


Figure 24: Species within Firmicutes Phyla Relative Abundance, E-SECC vs L-SECC Stratification

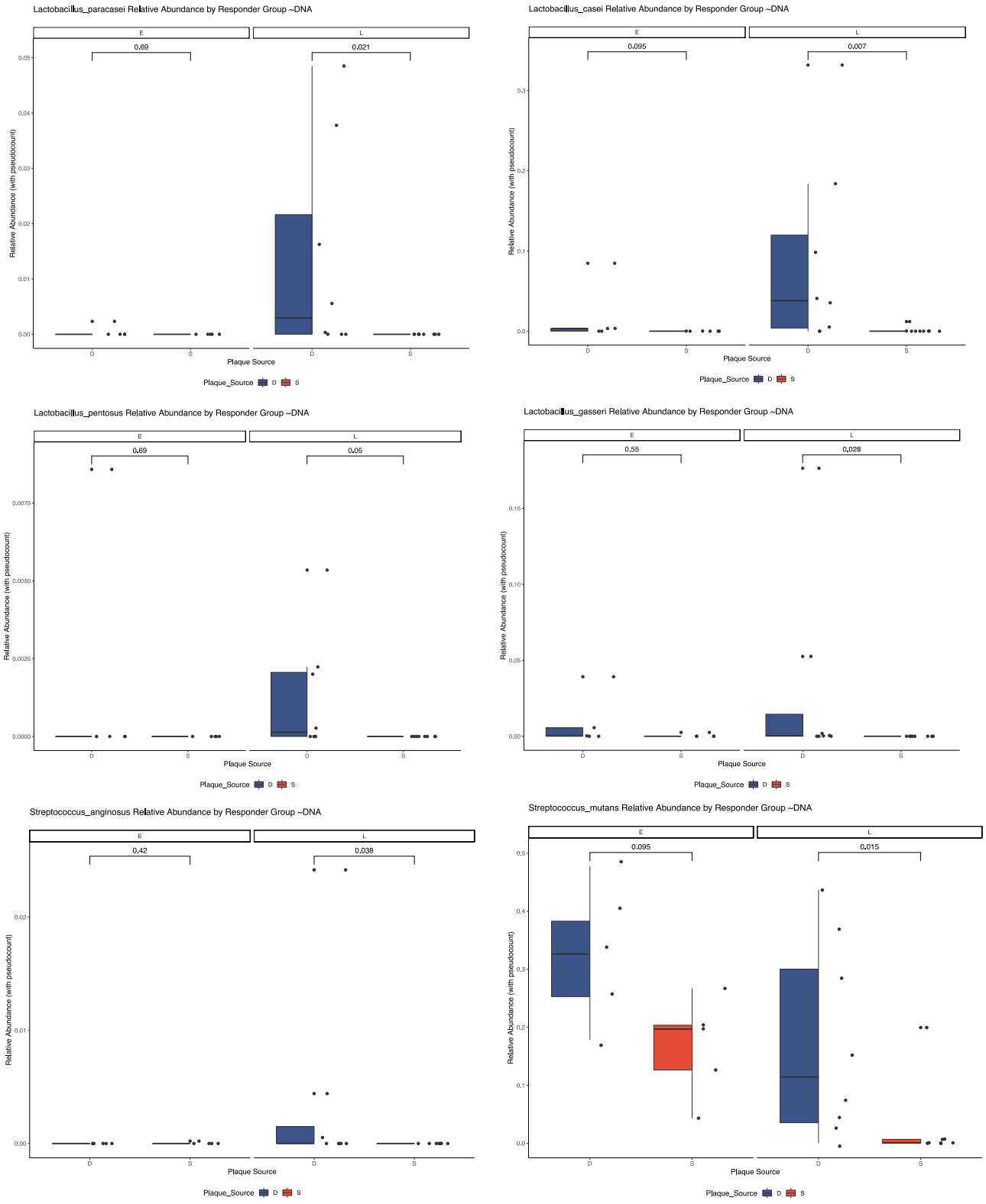


Figure 25:

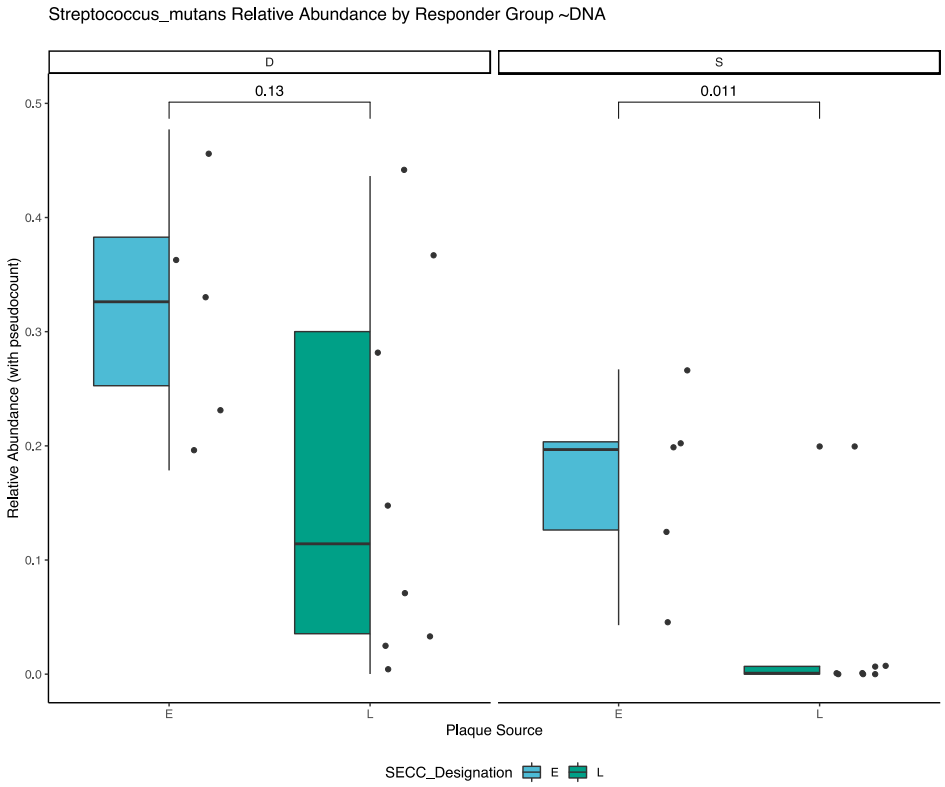


Figure 26:

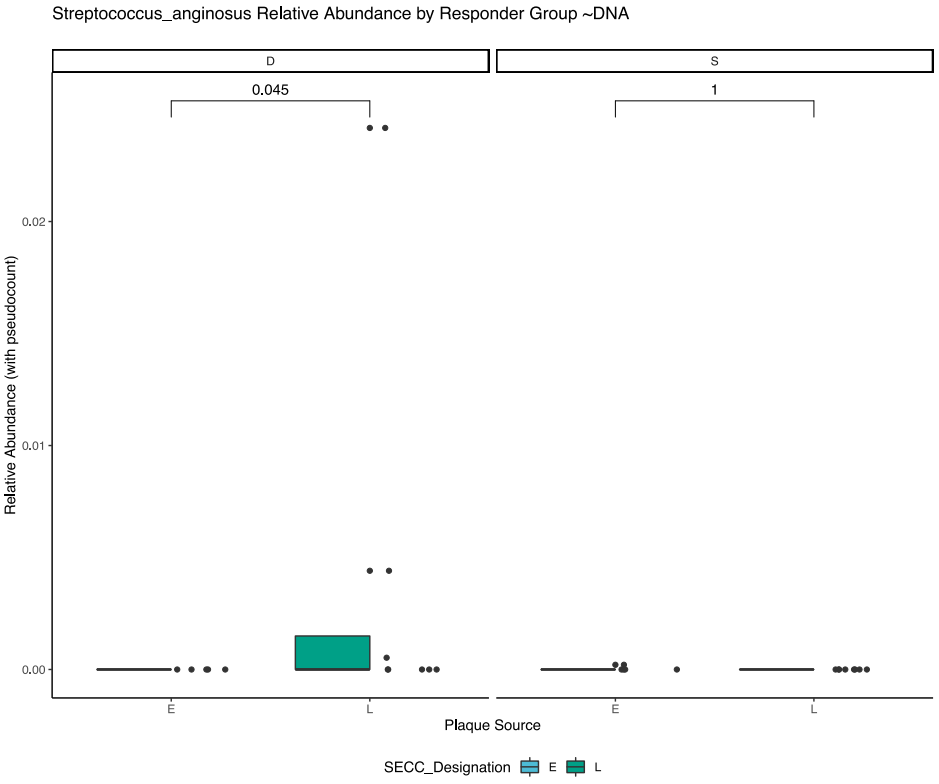
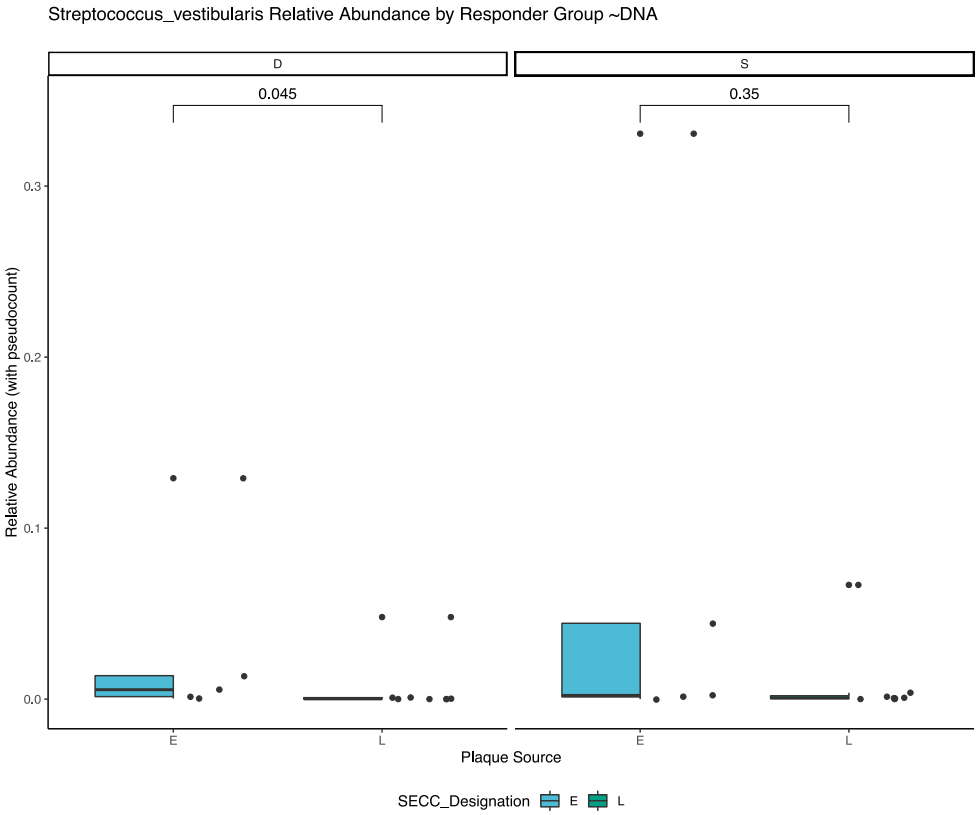


Figure 27:


Streptococcus_vestibularis Relative Abundance by Responder Group ~DNA
D
S
0.045
0.35
0.3
0.2
0.1
0.0
Relative Abundance (with pseudocount)
E
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SECC_Designation
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