December 21, 2017

FINAL REPORT

Type of Award: Orthodontic Faculty Development Fellowship Award (OFDFA) - A126712

Principal Investigator: Andrew Jheon

Title of Project: Alterations in the subgingival microbiome during orthodontic treatment

Period of AAOF Support: 07-01-15 to 06-30-16 (1st NCE to 6-30-17; 2nd NCE to 12-31-17)

Amount of Funding: $20 000

Summary/Abstract of Completed Project Results (250 word maximum):

Introduction: To evaluate changes in the subgingival microbiome before and during fixed appliance orthodontic treatment using 16S rRNA-based high-throughput sequencing. **Methods**: Sixteen patients (10 females and 6 males; ages 12y1m-33y3m) were included in this study. Subgingival microbial samples were collected from 4 teeth of each subject at three different times before and during full-fixed appliance treatment; two patients received clear aligner therapy (i.e., Invisalign). DNA was extracted from the samples, and 16S rRNA-based, next generation sequencing (NGS) was performed catalog global profiles of subgingival microbiome during orthodontic treatment. **Results:** The frequency of T forsythia, C rectus, and P nigrescens significantly increased after placement of orthodontic appliances. For the other species, the frequency tended to increase but no statistically significant differences were noted. The frequency of the change, representing microorganisms not existing at t0 but newly developing at t1 and t2, was higher at the molars than at the incisors. Although sample size was limited, there also appeared to be significant changes in patients receiving clear aligner therapy. **Conclusion:** Orthodontic treatment, whether utilizing full fixed appliances or clear aligners significantly alters subgingival microbial composition and consensus bacterial species that were altered were identified. Our experiments lay a solid foundation for further analysis of subgingival microbiome during orthodontic therapy.

Response to the following questions:

1. Were the original, specific aims of the proposal realized?

The original aim was "to profile changes in microbial composition during fixed orthodontic treatment using 16S rRNA next generation sequencing (NGS). Yes, this original aim has been realized. Our study was the first time that the oral microbiome was globally profiled during orthodontic treatment using NGS.

2. Were the results published?

No not yet. However, the manuscript is in preparation for submission to AJODO (I have attached the current draft). In addition, we will use our data as preliminary results to apply for NIDCR R21 funding for an expanded study.

a. If so, cite reference/s for publication/s including titles, dates, author or co-authors, journal, issue and page numbers.

Isaac Chen, Jennifer Chung, Yanjiao Zhao, Andrew H. Jheon (in preparation) Alterations in the subgingival microbiome during orthodontic treatment. AJODO.

b. Was AAOF support acknowledged? Yes.

c. If not, are there plans to publish? Yes, please see above.

3. Have the results of this proposal been presented? No

a. If so, list titles, author or co-authors of these presentation/s, year and locations: N/A

b. Was AAOF support acknowledged? N/A

c. If not, are there plans to do so? If not, why not? No because we did not budget for any travel to conferences.

4. To what extent have you used, or how do you intend to use, AAOF funding to further your career?

I have applied for BRA funding for 2018 and hope to continue to apply for AAOF funding in the future. It is a valuable source of funding outside of the NIH for my research and laboratory.

Accounting for Project; i.e., any leftover funds, etc.

There are no leftover funds. I apologize that this project took much longer than anticipated. This was largely due to this being my first foray into human microbiome research. Thus, I did not properly account for timing of human research approval and data analysis. Thank you for your patience.

Please return to AAOF via email attachment to aaofevp@aaortho.org

Alterations in the subgingival microbiome during orthodontic treatment

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Highlights

1) Our study is the first to globally profile bacteria using 16S rRNA-based, next generation sequencing (NGS) to catalog changes in subgingival microbiota during orthodontic treatment.

2) There are dramatic alterations in subgingival microbiome during full fixed appliance orthodontic treatment.

3) Although there is little consensus in microbial profile in teeth between subjects and within the same subject, there are several consensus bacterial species that are significantly changed during orthodontic treatment.

4) Although subjects were limited, the use of clear aligners (e.g., Invisalign) also showed significant changes in subgingival microbiome during treatment, perhaps surprising since a company-touted advantage of clear aligners is their perceived neutral effect on oral hygiene.

5) Our study builds a strong foundation to further analyze the role of subgingival microbiome in orthodontic tooth movement utilizing full fixed appliance and clear aligner therapies.

ABSTRACT

Introduction: To evaluate changes in the subgingival microbiome before and during fixed appliance orthodontic treatment using 16S rRNA-based high-throughput sequencing. **Methods**: Sixteen patients (10 females and 6 males; ages 12y1m-33y3m) were included in this study. Subgingival microbial samples were collected from 4 teeth of each subject at three different times before and during full-fixed appliance treatment; two patients received clear aligner therapy (i.e., Invisalign). DNA was extracted from the samples, and 16S rRNA-based, next generation sequencing (NGS) was performed catalog global profiles of subgingival microbiome during orthodontic treatment. **Results:** The frequency of T forsythia, C rectus, and P nigrescens significantly increased after placement of orthodontic appliances. For the other species, the frequency tended to increase but no statistically significant differences were noted. The frequency of the change, representing microorganisms not existing at t0 but newly developing at t1 and t2, was higher at the molars than at the incisors. Although sample size was limited, there also appeared to be significant changes in patients receiving clear aligner therapy. **Conclusion:** Orthodontic treatment, whether utilizing full fixed appliances or clear aligners significantly alters subgingival microbial composition and consensus bacterial species that were altered were identified. Our experiments lay a solid foundation for further analysis of subgingival microbiome during orthodontic therapy.

Introduction

In 2007, the National Institutes of Health (NIH) funded the human microbiome project (HMP) to map microbial makeup of healthy humans in an effort to better understand correlation of the microbiome with human health [1]. It was discovered that nearly everyone routinely carries pathogens and microorganisms known to cause illnesses. However, in healthy individuals pathogens cause no disease and simply coexist with their host and the rest of the microbiome [1]. Recently, Sender et al. [2] published a revised estimate of bacteria to human cell ratio to be nearly 1:1, an estimated 38 trillion bacteria to 30 trillion human cells. Every human body contains personalized microbiomes that are essential to maintain health, but also capable of eliciting disease. For example, there is a concentration of 10^{11} bacteria/ml in the gut that provide both beneficial and harmful effects [2]. This gut flora enhances our immune system, helps absorb vitamins, and utilizes the food consumed. Conversely, the gut flora is also associated with obesity, intestinal and systemic inflammation, and even cancer and autism [3].

Bacterial diversity in dental plaque and oral biofilm is estimated to include at least 800 different species, consisting of a wide variety of gram-positive and gram-negative bacteria [4]. The number of species is expected to rise into the thousands with the application of next-generation sequencing (NGS) techniques [5, 6]. Within the oral cavity, there are three main types of surfaces for bacteria to colonize: the hard surfaces of teeth (i.e., supra- and sub-gingival), the soft tissues of the oral mucosa, and saliva [7]. Furthermore, the oral microbiome is extremely dynamic because of the oral cavity's

continual exposure with the external environment [8]. Saliva has a bacterial concentration of 10^9 bacteria/ml whereas dental plaque has an equivalent bacterial concentration as the colon (i.e., 10^{11} bacteria/ml) [2]. Studies have shown the oral cavity's microbiome to be a key source in the etiology of many oral and systemic diseases [9, 10].

Dental plaque is a dynamic, complex biofilm and a microbial ecosystem [11]. Bacterial diversity in dental plaque or oral biofilm is estimated to include at least 800 different species, consisting of a wide variety of gram-positive and gram-negative bacteria [4, 12-15]. The number of species is expected to rise into the thousands with the application of next-generation sequencing (NGS) [5, 6]. In addition, many species in kingdom Monera, also known as archaebacteria, have been associated with oral disease such as chronic periodontitis [16, 17]. On top of the staggering number of bacteria and archaebacteria, dental plaque comprises an assortment of micro-niches, metabolic functions, and a web of inter- and intra-species interactions that accumulates through sequential and ordered colonization by different bacterial strains and species [18]. Biofilms develop under a wide range of different conditions, environments, and factors (e.g., pH, availability of oxygen and nutrients, and interbacterial co-adhesion), and these differences all have been demonstrated to lead to compositional changes in oral biofilm [18]. Oral biofilm profiling has been hampered because it is extremely difficult to define normal microbiota because of individual variation [19]. However, dental health has been associated with the absence [20, 21] or elevation [22-24] of certain species.

The composition of subgingival microbiota can be influenced by several factors including oral hygiene regimens, dental restoratives, and orthodontic appliances. The etiology of gingivitis and periodontitis is microbial infection, resulting in an imbalance between the host and subgingival microorganisms [17]. Fixed appliances can change the subgingival microbial environment by increasing plaque accumulation and deepening the gingival sulcus [25, 26]. Orthodontic appliances generally increased the level of periodontopathogens in subgingival plaques [27-29], however, several studies have reported no significant difference [30] or a decrease in the level of periodontopathogens decreased during orthodontic treatment due to metal corrosion, which imposed toxic effects on the microorganism [31]. Thus, previous studies regarding changes in periodontopathogens during orthodontic treatment have been inconsistent.

All previous studies involving orthodontic full fixed appliances have used microbial analysis methods such as candidate-based PCR or bacterial culture methods [30], but these techniques suffer from selection biases or difficulty in growing cultures. 16S rRNA combined with next generation sequencing (NGS) has been used previously to profile the microbiome in health and disease including periodontal diseases [32-36], but never during orthodontic treatment. 16S rRNA/NGS catalogs all human and microbial DNA in each of the samples, which is then sorted to identify specific genetic signals found only in bacteria — such as the variable genes of bacterial ribosomal RNA called 16S rRNA that can identify the presence of different microbial species [1]. The purpose of this study is, for the first time, to track global changes in subgingival bacterial

composition before and after the placement of patients undergoing traditional full fixed appliance orthodontic treatment.

Materials and Methods

Subjects

Sixteen subjects (10 Females and 6 Males; ages 16.2-17.5) were selected among patients who arrived for orthodontic treatment at the Department of Orthodontics, University of California San Francisco. Six patients failed to complete the entire study. The study design was approved by the Ethics Committee (IRB 15-17868). Subjects were enrolled according to the following criteria: (1) no known systemic disease; (2) no use of antimicrobial, antifungal or anti-inflammatory drugs within 3 months before the baseline examination; (3) no craniofacial anomalies; (4) not-pregnant; and (5) would be undergoing treatment with fixed orthodontic appliances in the mandibular arch. Five subjects (4 female and 1 male; Age ??) not undergoing orthodontic treatment were selected as a control group.

Orthodontic bonding

All subjects received oral hygiene instructions by the same clinician in the form of both verbal instructions and an instructional video by Dolphin Aquarium (Patterson Technology). Subjects were instructed to continue their routine oral hygiene regiment. All subjects received orthodontic therapy with fixed buccal appliances. For orthodontic treatment, metal brackets (3M Unitek, GAC, Opal or GAC innovation) were bonded directly with composite resin (find brand of composite) onto incisors and premolars. Bands (Unitek and GAC) were cemented with polyacid-modified composite resin (Ultra Band-Lok) onto molars. The arch wires were tied using o-chains (find brand of o-chains) and in one case self-ligated.

Clear aligner therapy

Two patients received clear aligner trays (Invisalign) and treatment was administered following instruction from Invisalign.

Collection of subgingival microbiota

Subgingival crevicular fluid samples were collected from the straight buccal gingival crevice of the lower right first molar (30B), lower right central incisor (25B), lower left first premolar (21B) and the straight lingual of lower left first premolar (21L). Samples were collected at three different time points: before appliance placement (t0), 6 weeks (t1) and 12 weeks (t2) into treatment. The sampling sites were isolated using full mouth isolators (Dentsply, GAC) and the tongue held back with a mouth mirror when sampling tooth 21L. Two sterile paper points size 30 (Dentsply, GAC) were inserted into the gingival crevice and were left *in situ* for 60 s. These paper points were transferred immediately into Eppendorf tubes containing 250 mL of 0.9% saline solution (Baxter, Grainger) and frozen at -80 degrees F.

Periodontal Assessment

Immediately after sampling the sites, the probing depths (PD), GM-CEJ measurement, clinical attachment loss (CAL), and bleeding on probing (BOP) were measured and recorded in Axium periodontal charting system for all three time points. A sterilized periodontal probe (find brand) was used to measurement PD and GM-CEJ. BOP was observed and classified as present or not present after the periodontal measurements. All sampling and measurements were performed by the same clinician. Once all the

samples were collected, they were sent to Jackson Laboratory in Bar Harbor, ME for 16S rRNA gene sequencing and analysis.

16S rRNA gene sequencing: Metagenomic DNA from the sampled paper points was extracted using a PowerSoil ® DNA Isolation Kit. 16S rRNA V1-3 regions from the metagenomic DNA were amplified using primers 27F and 534R (27F:5'-AGAGTTTGATCCTGGCTCAG-3' and 534R: 5'- ATTACCGCGGCTGCTGG-3'). Sequencing reads were processed by removing the sequences with low quality (average qual <35) and ambiguous nucleotides (N's); chimeric amplicons were removed using UChime software. OTU was generated from the processed reads using an automated pipeline. Each OTU was classified from phylum to genus level using the most updated RDP classifier and training set. A taxonomic abundance table was generated with each row as bacterial taxonomic classification, each column as sample ID, and each field with taxonomic abundance. The abundance of a given taxon in a sample was presented as relative abundance (the read counts from a given taxon divided by total reads in the sample).

Statistical Analysis

Each sample was subsampled to the lowest number of read counts among samples in the dataset and rarefied to 5000 read counts. The abundance of a taxon in a sample was indicated as the relative abundance, which was calculated by dividing the number of reads for a taxon by the total read counts of the sample. Alpha diversity indices including richness and Shannon diversity were calculated as previously described. To

examine difference between control and bracket samples, we performed non-metric multidimensial scaling (NMDS) plots with Bray-curtis distance. Microbiome stability in both groups was determined by calculating the Bray-curtis dissimilarity from the same samples across different time points.

Results (in preparation)

We obtained a mean of 23,755 sequences per sample and aggregated to a total of 402 operational taxonomic units (OTUs). In our braces cohort, we observed that there was a significant increase in microbial richness between samples obtained before braces intervention and six weeks after intervention among three of the four sites. Richness stayed at a similarly high microbial richness by 12 weeks. This increase in richness was accompanied by the decrease in Streptococcus, the most abundant genera found across all samples. Similar trends were observed when looking at diversity among samples by Bray Curtis. Samples taken before bracket treatment clustered more tightly and differently compared to the two time points after, which clustered similarly among each other. In addition, over 30% of our bracket cohort developed periodontitis compared to 15% of our controls by six weeks of intervention. Overall samples that were associated with periodontitis had a lower relative abundance of Streptococcus. We also observed that there were fewer OTUs shared among the four sites at t0 relative to t1 and t2, suggesting that the whole oral microbiome becomes more uniformed with bracket treatment. Interestingly, the unique OTUs to each site over time did not remain the same, suggesting the uniqueness of each tooth's bacterial profile may be transient.

Discussion (in preparation)

One of the major problems associated with phase II, fixed appliance treatment is its long duration, on average of 29 months [37]. There is empirical, clinical evidence that increased bacterial plaque may decrease the rate of orthodontic tooth movement and overall treatment times. In addition, the microbiome has recently been shown to possess critical, much larger roles in health and disease, most notably with studies on the effects of the gut microbiome on obesity [38], intestinal cancer [39], diabetes [40], and autism [3]. Finally, the biological and mechanical changes induced by orthodontic treatment would likely lead to changes in subgingival dental plaque. Thus, the contribution of the microbiome, and specifically subgingival plaque, during orthodontic therapy is an exciting possibility that has yet to be tested. Ultimately, manipulation of the microbiome may be a therapeutic option to accelerate orthodontic tooth movement and shorten treatment times.

We observed significant microbial changes in the oral microbiome of patients with bracket intervention and the composition of these communities may contribute to periodontitis.

Acknowledgment

We would like to thank the American Association of Orthodontics Foundation for supporting this work through the Orthodontic Faculty Development Fellowship Award (OFDFA) to A.H.J..

Figure Legends

Figure 1. Subgingival microbial profiles pooled for control (no treatment), full fixed appliance (bracket), and clear aligner (invisalign) therapy. For all groups, OTU_1.streptococcus is the most common bacterial genus, followed by OTU_3. Streptococcus and OTU_2. Veillonella. In the bracket group, tooth 30B showed an overall decrease in OTU_1. Streptococcus, whereas 25B and 21B showed decreases at t1 followed by increases at t2 although not to the same levels at t0. 21L profile remained relatively the same at all three time points. In the control group, the levels of OTU_1. Streptococcus had similar abundance at all three time points for 30B and 25B, an overall decrease in 21B but an increase in 21L. The bracket group showed an overall decrease in OTU_3. Streptococcus from t0 to t2, whereas the control group showed an overall increase in OTU_3. Streptococcus, except for tooth 30B. OTU_2. Veillonella increased for bracket tooth 25B and 21L but decreased in 30B and 21B. In the control group, OTU_2. Veillonella increased in tooth 25B, decreased in 30B and 21B, and was barely present in 21L. The amount of “other” bacteria was higher on the bracket group than in the control group.

Figure. 2. Bray-Curtis, Richness, and Shannan diversity analyses. (A) The Bray-Curtis analysis provides a measure of community composition differences between samples based on OTU counts regardless of taxonomic assignment. Ordinations based on this metric demonstrated a clear separation between control, bracket, and Invisalign groups for tooth 30B. 25B and 21L were slightly more similar and 21B showed

significant separation between t0-t1 but were more similar at t1-t2. (B) The bracket group demonstrated a significant increase in microbiome richness from t0 to t2, whereas the control group only showed slight increases in microbiome richness from t0 to t2. (C) The Shannon diversity index (H) is commonly used to characterize species diversity in a community. At t0 both bracket and control groups started with similar diversity indexes. The bracket group showed an increase in diversity for tooth 30B and 21L at t1 and t2, but tooth 25B and 21B showed increases at t1 followed by decreases at t3. In the control group, tooth 30B and 21B showed minimal changes in diversity, 25B showed a decrease in diversity at t1 and an increase at t2, whereas tooth 21L showed a decrease in diversity at t2.

Figure 3. Non-metric Multidimensial Scaling (NMDS) ordination analysis. To evaluate the relationship between bacterial community composition and various environmental factors, we performed NMDS analysis to represent pairwise Bray-Curtis similarities between bacterial communities.

Figure 4. Global subgingival microbial profiles of individual subjects and teeth.

Figure 5. Venn diagram showing shared OTU or consensus bacterial species in all teeth and time.

Figure 6. Venn diagram showing shared OTU or consensus bacterial species in all teeth at the three time points.

Supplemental Figure 1. Top 10 up- and down-regulated bacterial species for each tooth and subject at t0-t1 and t1-t2. Subjects are listed as A-O.

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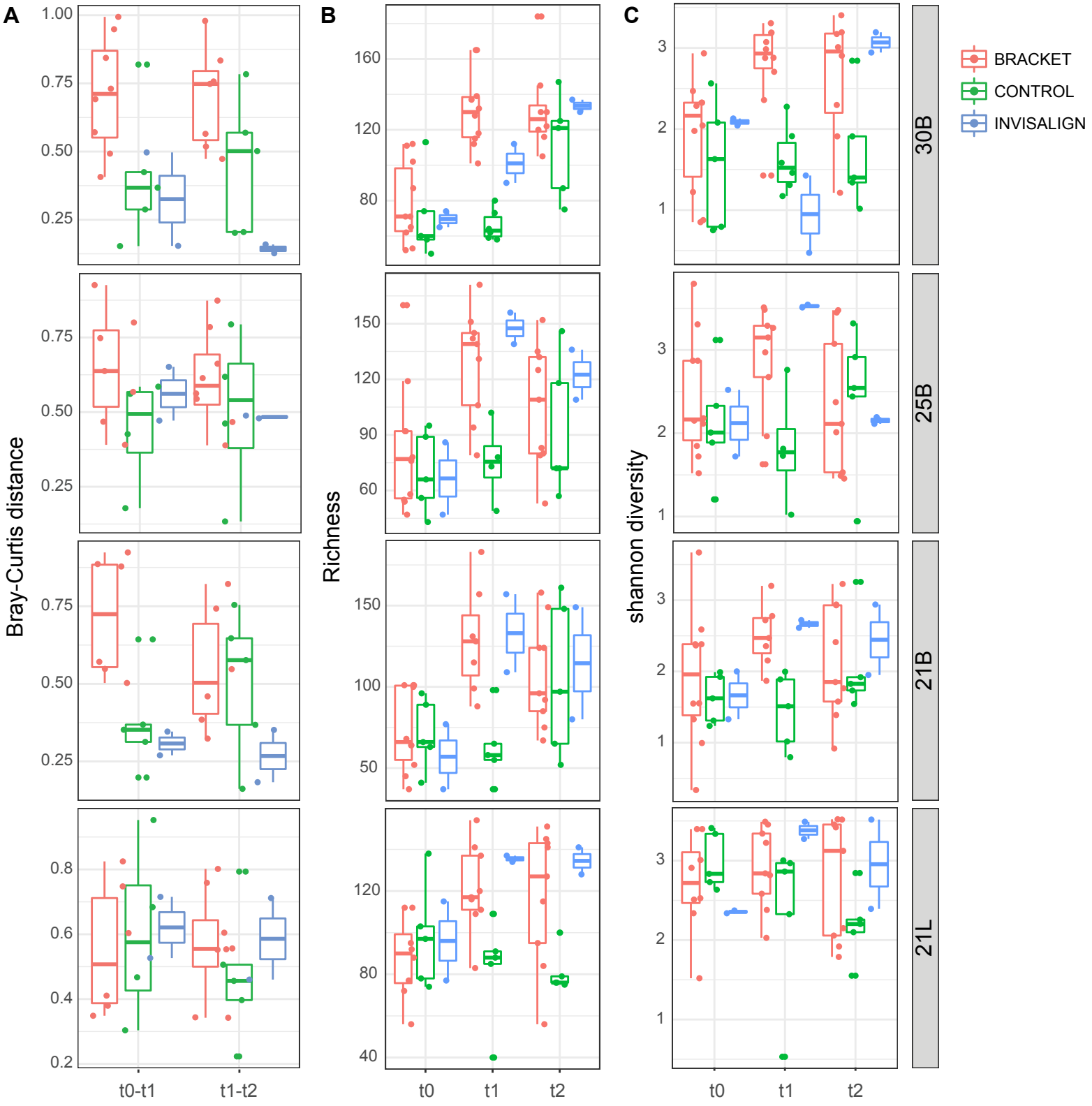
OTU_1.Streptococcus
OTU_27.Prevotella
OTU_18.Leptotrichia
OTU_28.Streptococcus
OTU_5.Kingella
OTU_61.Capnocytophaga
OTU_36.Leptotrichia
OTU_11.Neisseria
OTU_67.Streptococcus
OTU_9.Neisseria
OTU_26.Capnocytophaga
OTU_7.Neisseria
OTU_29.Leptotrichia
OTU_55.Prevotella
OTU_12.Streptococcus
OTU_20.Granulicatella
OTU_25.Neisseria
OTU_210.Fusobacterium
OTU_10.Fusobacterium
OTU_33.Porphyromonas
OTU_350.Streptococcus
OTU_37.unclassified_Prevotellaceae
OTU_17.Granulicatella
OTU_8.Rothia
OTU_389.Streptococcus
OTU_14.Streptococcus
OTU_15.Gemella
OTU_294.Streptococcus
OTU_19.Saccharibacteria_genera_incertae_sedis
OTU_13.Fusobacterium
OTU_4.unclassified_Burkholderiales
OTU_2.Veillonella
OTU_3.Streptococcus
Others

Figure 2


A
B
C
Bray-Curtis distance
Richness
shannon diversity
BRACKET
CONTROL
INVISALIGN
30B
25B
21B
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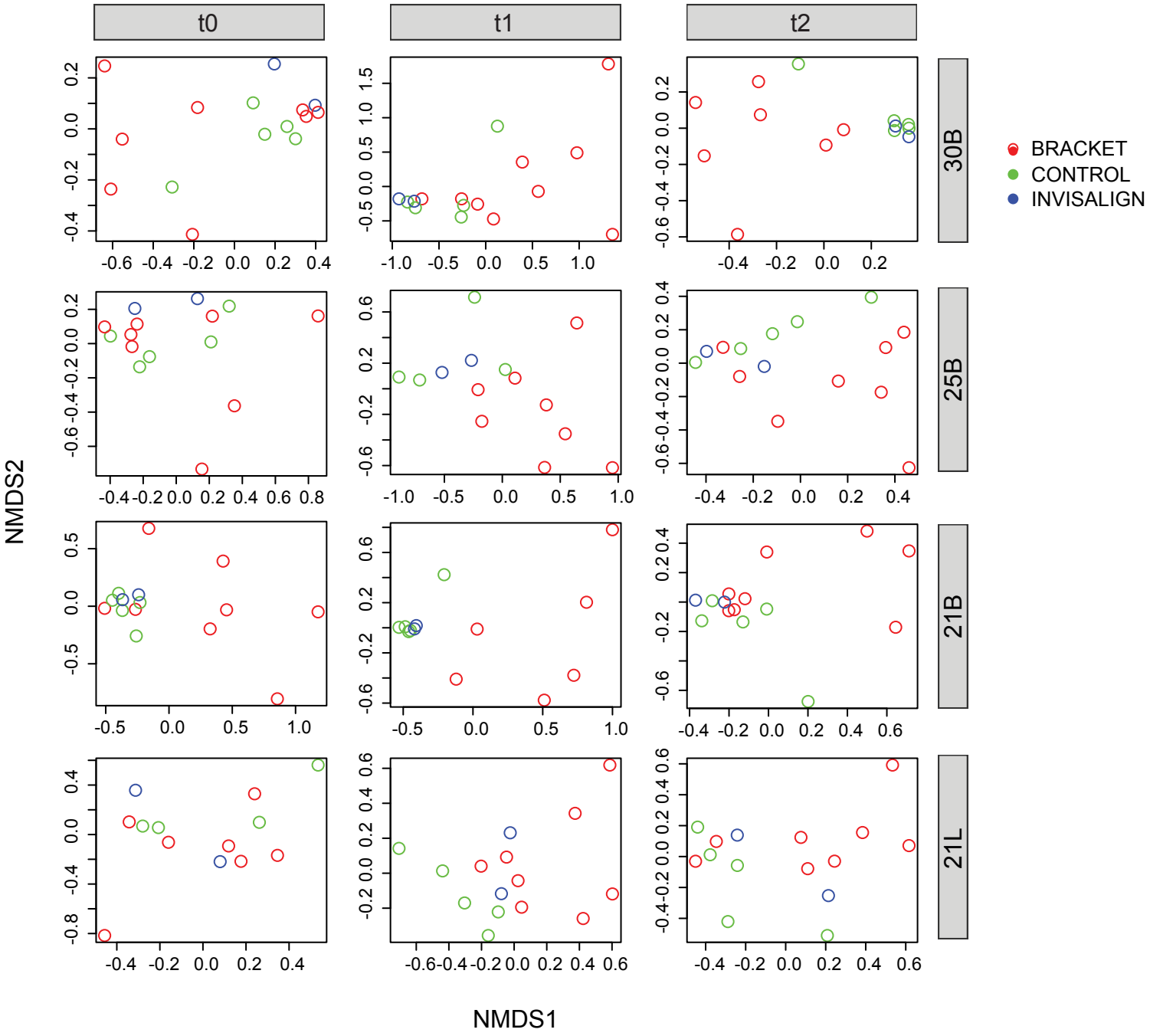
t0-t1
t1-t2
t0
t1
t2

Figure 3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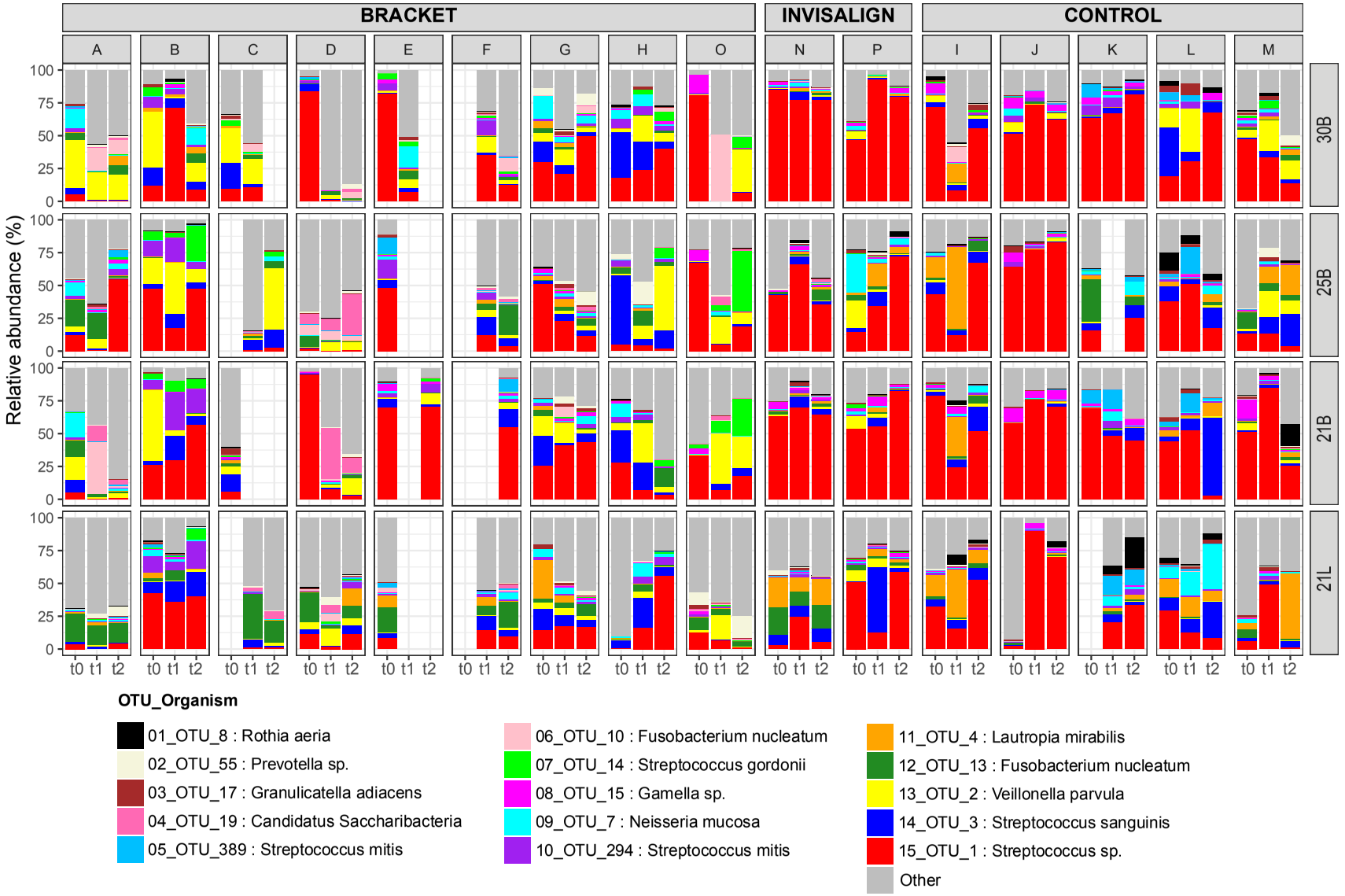
NMDS2

Figure 4


BRACKET
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A
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L
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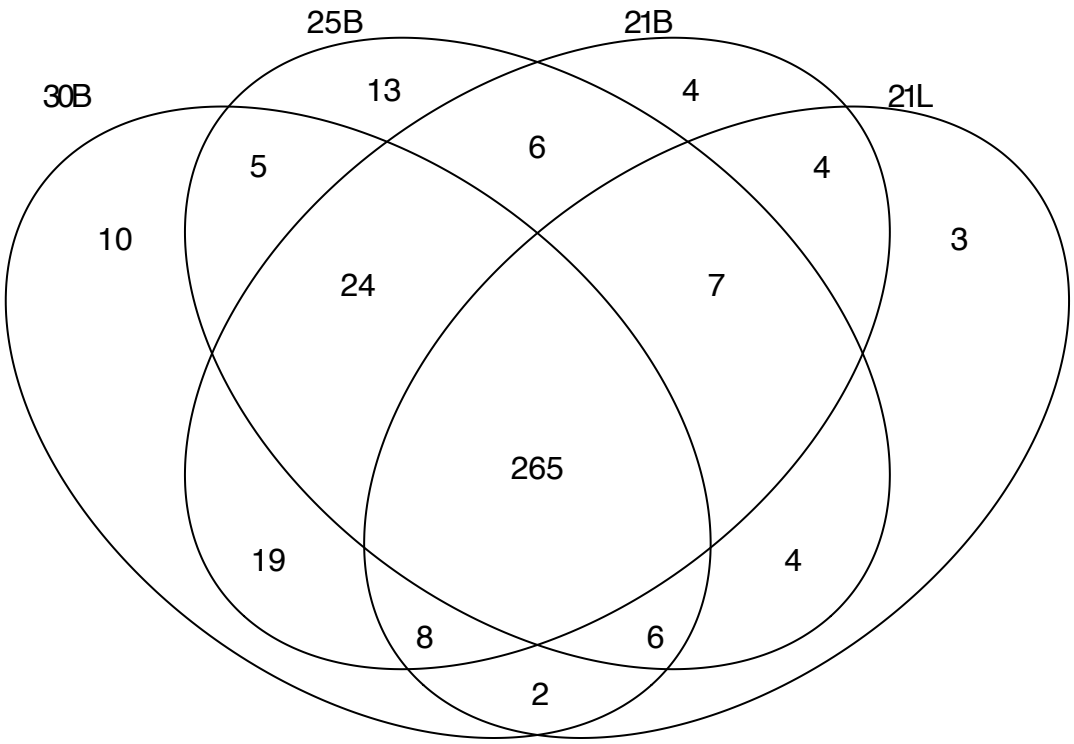
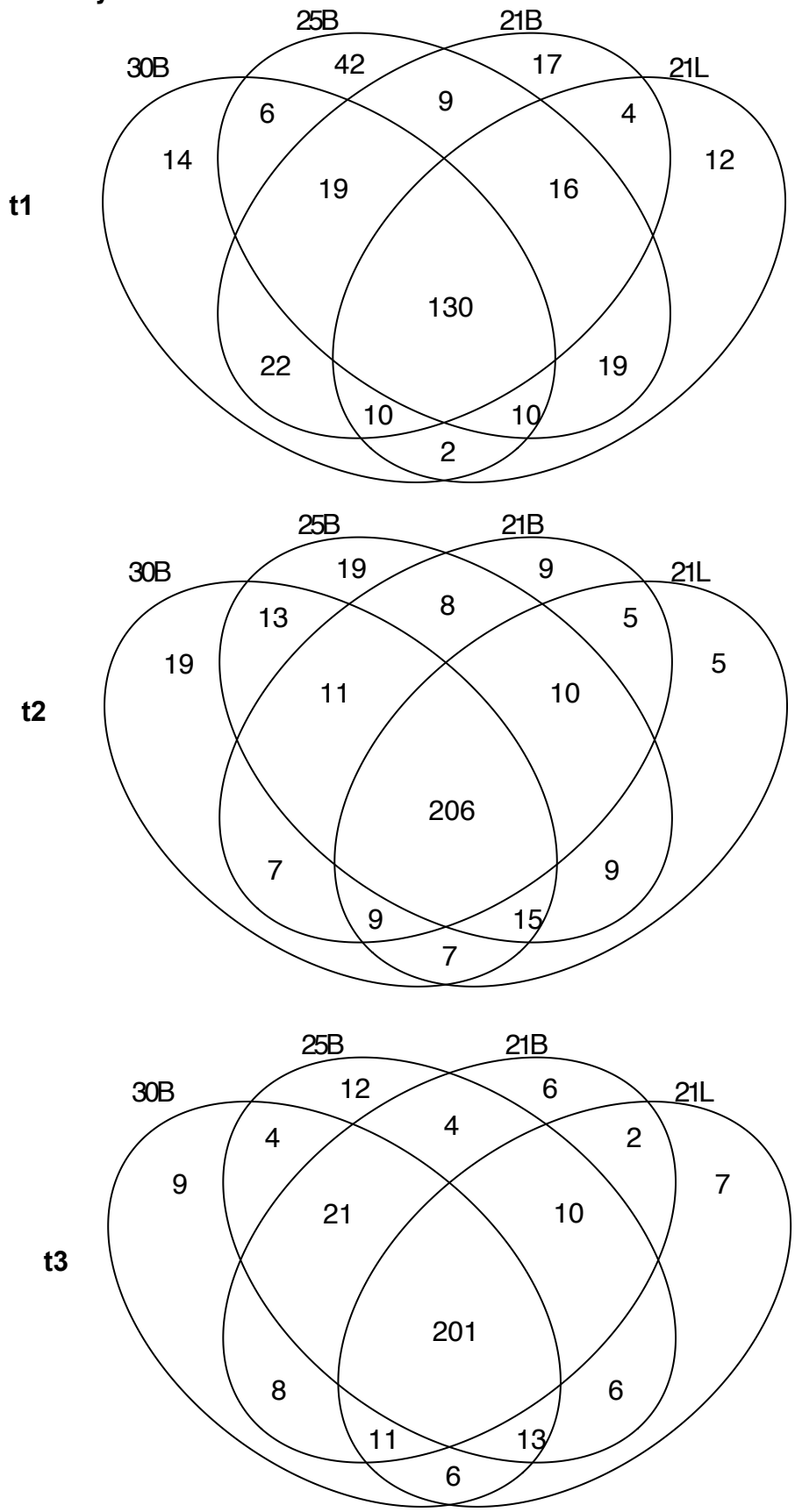
30B
25B
21B
21L
Relative abundance (%)
0
25
50
75
100
t0 t1 t2
OTU_Organism
01_OTU_8 : Rothia aeria
02_OTU_55 : Prevotella sp.
03_OTU_17 : Granulicatella adiacens
04_OTU_19 : Candidatus Saccharibacteria
05_OTU_389 : Streptococcus mitis
06_OTU_10 : Fusobacterium nucleatum
07_OTU_14 : Streptococcus gordonii
08_OTU_15 : Gamella sp.
09_OTU_7 : Neisseria mucosa
10_OTU_294 : Streptococcus mitis
11_OTU_4 : Lautropia mirabilis
12_OTU_13 : Fusobacterium nucleatum
13_OTU_2 : Veillonella parvula
14_OTU_3 : Streptococcus sanguinis
15_OTU_1 : Streptococcus sp.
Other

BRACKET only: Shared otu in among all teeth among all timepoints



BRACKET only



SUPPLEMENTAL TABLE 1