Stability of the Oral Microbiome in Children – A Six Month Longitudinal Study

Thesis

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

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Abstract

Introduction: The human oral cavity is home to approximately 700 different species of bacteria that are often represented by multiple strains, and each person harbors a unique subset of these. The microbial community differs between childhood and adulthood; however, it is unclear whether species once acquired are retained or for how long they remain as children accumulate exposures to new species. Purpose: The purpose of this study is to investigate the stability of the oral microbial community at the level of species in children from age 1-13 years, over a six-month period. Materials and Methods: Salivary, supragingival and subgingival plaque samples were collected from children, and then resampled after six months. Bacterial profiles at the species level were compared for the two time points among different age cohorts. Amplification and sequencing of the ribosomal operon 16S rRNA gene was used for species identification. Results: Based on data analyzed from 43 subjects, results indicate measurable stability of the oral microbiome at the level of species over a six-month period in children. This stability was observed in all sampling sites – salivary, supragingival, and subgingival. Future studies will continue to re-sample these patients at six month intervals to confirm our findings. Additionally, the stability of the oral microbiome at the level of strains should also be examined.
Dedication

Dedication to students at The Ohio State University
Acknowledgments

I would like to acknowledge the assistance from my Thesis Committee, Dr. Ann L. Griffen, Dr. Eugene Leys, and Dr. Erin Gross. Thank you so much for all of your support and guidance.
Vita

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2014…………………………………………..DDS University of Michigan (Ann Arbor)

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The Ohio State University

Publications


Soliman AS,1 Hung CW1 Tsodikov A,2 Seifeldin Al,3 Ramadan M,3 Al-Gamal D,3 Schiefelbein, E1 Iyer P,1 Dey S,1 and Ismail K.4 Epidemiologic risk factors of hepatocellular carcinoma in a rural region of Egypt. Hepatol Int. 2010 December; 4(4): 681–690


Fields of Study

Major Field: Dentistry
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Chapter 1: Introduction

The human oral cavity is home to approximately 700 different species of bacteria (represented by multiple strains) and each human harbors a unique subset of these \(1,2\). While some oral microbes serve to establish a bacterial community characteristic of oral health, others have been implicated in oral and systemic diseases \(3\). Shifts in microbial community composition may cause a loss of symbiotic balance between the host and microbiota \(4\), ultimately predisposing the host to disease. Therefore, it is important to understand the baseline stability or variability of the oral microbial community to evaluate the health significance of any changes from baseline that may occur during disease, dietary change, or antibiotic treatment \(6,7,8\).

The Human Microbiome Project has resulted in a good understanding of the composition of adult human microbial composition at the level of species. However, there is limited evidence examining the assembly and stability of oral microbial communities from birth through adolescence. A study profiling the natural history of oral microbial communities in infants from birth through the first year of life found salivary oral microbiota detectible within two weeks after birth and increased in complexity over the course of the infants’ first year of life \(5\). It is still unclear whether strains and species once acquired in the oral microbiome, however, are retained or for how long they remain as children accumulate exposures to new strains.

Most studies of stability have drawn primarily from studying the gut microbiota. Findings from these studies suggest stability of the gut microbiota at the level of strains but not species \(9\). Similarly, studies examining the oral microbial composition have also suggested stability \(10,11,12\). Costello et al analyzed samples from the saliva and tongue dorsum, on two
successive days 3 months apart and showed that variation was less within individuals than
between individuals, suggesting stability (10).

In contrast, other studies analyzing a range of body sites found gut and vagina to be most
stable whereas the oral cavity to be least stable (13,14,15). Thus, the stability of the oral
microbiome is still unclear based on findings from current literature. Moreover, these findings
were based on samples analyzed from adults and may not hold true for children.

The overall purpose of this study is to longitudinally investigate the stability of the oral
microbial community at the species level in children from age 1 through 13 years - over a six-
month period. We hypothesize that many species will be retained and that with increasing age,
children will develop a more complex microbiota as these species accumulate.
Chapter 2: Materials and Methods

Clinical Methods

Recruitment

Subjects were recruited from patients presenting for exam and hygiene visits at the Dental Clinic at Nationwide Children’s Hospital. IRB approval was obtained for the study, and parents gave informed consent form and children over the age of eight years gave assent. In this study, data from 43 subjects were analyzed to support our findings.

Subjects were examined and sampled at baseline and at six months (coinciding with routine dental recall visits). Children from age 1 through 13 years were followed for 6 months. Recruitment and sampling occurred before a dental prophylaxis was performed.

Inclusion/Exclusion Criteria:

Patients had to have an English-speaking primary caregiver accompanying them and patient had to be ASA I or II (no serious medical problems). Children with ASA status III or greater with chronic disease affecting the immune system or requiring chronic use of antibiotics, early onset periodontitis (shown to alter microbial profile), or diabetes were excluded from the study. Additionally, sampling was not conducted within 30 days of any antibiotic therap. Each age cohort will be balanced on gender (40-60% male) and race (AA 15-25%).

Clinical Data Collection and DNA sampling

The initial charting at each visit included tooth presence, caries, gingivitis (none, localized, generalized), plaque level (light, moderate, heavy), presence of visible tongue biofilm or orthodontic appliances, restorations, sealants, and soft tissue lesions. These values were collectively scored and recorded by a dental hygienist and verified by an attending dentist at the
dental clinic. Parents or Caregivers completed a survey to obtain history of antibiotic use, oral hygiene practices, number of persons residing in household, daycare or school arrangements, fluoride exposure (water and topical), a simple diet survey focusing on carbohydrate frequency, and tobacco exposure/use history was also collected by interview. Additionally, baseline information on breast feeding and delivery mode were obtained. For subjects under 3 yrs of age, information on pacifier, bottle or sippy cup use were also recorded.

Subjects were sampled at least 1 hr after home oral hygiene or consuming food or drink, and prior to any dental prophylaxis or other dental procedure. Samples were collected in a non-invasive manner and aseptically to avoid contamination of sampling supplies - including no touching with ungloved hands. Saliva was collected by placing a Copan swab in the right and left sublingual lingual vestibule for a minimum of 30 seconds each side. The swab was placed in a 15 ml tube containing 2 ml of ATL buffer and stored under refrigeration until transported to the lab for storage under -20 C until DNA isolation. Supragingival plaque was collected from the buccal surfaces of all teeth on the right side using sterile microbrushes. The brushes were placed in a 15 ml tube containing 2 ml of ATL buffer and stored under refrigeration or frozen until being transported to the lab. Subgingival samples were obtained from the mesiobuccal sulcus of each tooth on the right side using sterile paper points. Paper points were gently inserted and removed after a few seconds and placed in a 1.5 ml microfuge tube containing 0.5 ml of ATL buffer. Supra and subgingival samples were pooled separately and stored at -80 C. Dental prophylaxis was then performed at this point or at any subsequent time.

**Laboratory Methods**

**DNA isolation and amplification**
DNA was isolated and 16S rRNA gene amplification and Illumina MiSeq sequencing was performed. Sequences were identified at the level of species by blast of the CORE database. Species-level counts were tabulated and relative abundance was calculated for each sample.
Chapter 3: Results

Study Participants

Samples from forty-three subjects were analyzed, however, the current study is in progress and recall samples will be collected longitudinally for two more years. The age distribution for our sample size is depicted in Figure 1, where the mean age is 8.9 years (age 4-13). As shown in Figure 2, the majority of our sample size consisted of Black or African American race (67%), followed by White (20%), Other (1%), and Asian (<1%). Overall, 51% of the sample size was female and 49% male (also see Figure 2).

Data Analysis

Data was analyzed using Bray Curtis multidimensional scaling and Env-Fit test. For each sample, the Euclidian distances from each collection time point (initial and six months) were calculated and a single value bound between 0 and 1 measuring the level of dissimilarity was mapped (where 0 means the two sites share the same species 1 means the two sites do not share any). The three different samples based on sampling site (salivary, supragingival, subgingival) collected from each subject at the two different time points were analyzed and results are shown in Figures 3a,b,c (respectively). Each figure (based on sampling site) depicts two box plots: one mapping the measures of dissimilarity within the same subject and the other mapping the measure of dissimilarity between subjects. To determine the measure of dissimilarity between subjects, an initial sample from a subject was compared to a different subject’s (within one year of age) recall sample (at six months). The mean measure of dissimilarity comparing within subject and between subject values was statistically significant (P-value<.05; .02[saliva], .006[supragingival], .02[subgingival]) at all three sampling sites, suggesting stability of the oral microbiome. To further
illustrate these findings, see (Figures 4a,b,c). The middle line represents the normalized mean measure of dissimilarity that was calculated from “between-subject samples”. The measures of dissimilarity comparing samples from the two different time points for samples within subjects were then mapped in relation to the normalized mean. As seen in each figure (4a,b,c, the majority of the points fall below the normalized mean measure of dissimilarity.

There may be greater stability of the oral microbiome among supragingival sites based on findings depicted in Figure 3b and 4b (p-value is most significant at this site and supragingival samples had more points “below the line”, respectively).
Chapter 4: Discussion

To date, this is the first study to provide longitudinal analysis of the stability of the oral microbiome in children. Our findings support recent studies in this field suggesting stability of oral microbiome, but also provide a longitudinal analysis of oral microbial succession at the species level among children from birth through adolescence.

The two main conclusions based on the preliminary data analyzed from the current study are as follows:

1) There is measurable stability of the oral microbiome at the level of species over a six-month period in children.
2) This was observed in salivary, subgingival and supragingival sites.

Additionally, our data suggest that the oral microbiome may be more stable than the gut since our findings suggest stability at the level of species. In contrast, studies examining the stability of gut microbiota are based on stability at the level of strains. Understanding the stability of the oral microbiome and factors that may cause shifts in microbial composition could add to our ability to develop preventive and treatment strategies to guide the formation of health-promoting oral bacterial communities.

Limitations and Future Directions

The small sample size in this pilot study limited the power of our statistical analyses, and going forward the sample size and the length of time subjects are monitored will be increased. Also in this study sample, white subjects were under-represented and the age distribution was uneven and very young subjects were not included. These issues will be addressed as additional
subjects are recruited. Limitations regarding sample collection and reporting were also present in this study. Sample collection was performed by dental students who could have introduced variation in collection methods and consistency in collection timing.

Expanding the longitudinal study to follow subject for two more years, meanwhile collecting recall samples at six-month intervals will allow us to confirm the preliminary results of this study. Additionally, stability of samples collected from the three different sampling sites need to be further analyzed to determine if supragingival sites exhibit the most stability (as our data suggests). Analysis of the stability of the oral microbiome will enable us to better understand shifts of community composition, which may predispose hosts to disease.
References


   *Genome Biol.* 15(7):R89


Figure 1: The age distribution among recall patients ($N=43$). The x-axis represents the age of patients in years and the y-axis represents the number of subjects within each age group. The mean age was 8.8 years ($min = 4; max = 13$).

Figure 2: The race and gender distribution among recall patients ($N=43$). The x-axis represents the race - Black or African American ($N_{tot}=29; 16\ females, 13\ males$); White ($N_{tot}=9; 5\ females, 4\ males$); Asian ($N_{tot}=1; 0\ females, 1\ male$); Other ($N_{tot}=4; 1\ females, 3\ males$). The y-axis represents the number of patients. The number of females and males within each race category is also depicted above.
Figure 3: Bray-Curtis Analysis, Mean measure of dissimilarity among “Same” within sample comparison and “Different” between-subjects’ samples. 3a) Saliva samples p-value = .02, (b) Supragingival samples (p-value = .005), (c) subgingival samples (p-value = .02).

Figure 4: Bray-Curtis Analysis, Middle line is the normalized mean of measure of dissimilarity from “between-subject” analysis. 4(a) Saliva, 4(b) Supragingival 4(c) Subgingival. Red points depict points with values above the mean (more dissimilar) and blue points below mean (less dissimilar).
Appendix A: Study Information Sheet
Research Study Information for Participants and Parents

We are conducting a study to find out how normal oral bacteria are acquired in children. Earlier studies have shown that babies are born without bacteria in their mouths, but they slowly and steadily acquire different kinds of bacteria during their first year of life. Understanding more about the acquisition of different kinds of bacteria throughout childhood will help us determine the path from a relatively simple set of bacteria in infants to the complex set of bacteria that live in adult mouths.

Official title of the Study: The Natural History and Stability of Acquisition of the Oral Microbiome from Infancy to Adolescence

Who and where: The study is being conducted at the Ohio State University (OSU) and Nationwide Children’s Hospital in Columbus, Ohio. A team of researchers from the two institutions is conducting the study. The study has been funded by the National Institutes of Health, an agency of the federal government.

Why we need you: In this study we will identify the bacteria present in the mouths of children and adolescents, to determine what bacteria are acquired, and when. We are recruiting children, who are currently 0-12 years old, and who expect to remain in the Columbus area for at least the next 4 years. To make it easy we are recruiting from community groups where children and their parents come together for group activities.

What would be involved: Every 6 months for the next 4 years, we will collect saliva using soft swabs, and dental plaque using small, soft brushes and paper points. We will also do a brief dental exam and ask several questions about your child’s oral care, diet, habits etc (total time 10-15 mins). We will schedule sampling for a time when your group is meeting so that we can see as many participants as possible at once. However, we can make arrangements to see participants who are not able to be present for the group activity at another time at the OSU College of Dentistry, or at another location of your choosing.

What we will do with the samples: We will analyze the bacteria that are present in the samples by looking at bacterial DNA.

What you will receive for participation: A $10 gift card each time you are sampled.

Safeguards: Only the clinical personnel involved in the study would have access to participants’ individually identifiable private information (e.g., name, address). All protocols have been reviewed and received institutional approval for research involving human subjects, in order to ensure participants’ privacy, confidentiality, and protection.

Please contact us for more details:

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The Ohio State University College of Dentistry
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Columbus, OH 43210-1267
Appendix B: Study Protocol Forms
Clinical Protocol for Initial Visit

Study Title: The natural history and stability of acquisition of the oral microbiome from infancy to adolescence

Study ID: IRB14-00184

Sample numbering system:

AQ prefix for Acquisition study
1xxx is subject # starting at 1001
y is visit # starting at 0

z indicates sample type
- Y soft tissue swab
- P supragingival plaque
- S subgingival plaque

Study kits are stored in the OSU faculty area on the first floor in a labeled drawer.

Study kit contents:

Forms
- Study Information Sheet
- Consent Form (2 copies)
- Assent Form (2 copies)
- 1) Exam & Sampling Form
- 2) Contact Info & History Form
- 3) Beverage Survey Form

Supplies
a. Saliva & soft tissue swab (numbered as AQ1xxx.y,Y)
   - 1 Copan swab
   - 15 ml tube containing 200 µl ATL buffer
b. supragingival plaque (numbered as AQ1xxx.y,P)
   - 6 microbrushes
   - 15 ml tube containing 200 µl ATL buffer
c. subgingival plaque (numbered as AQ1xxx.y,B)
   - cotton rolls
   - 4 packs of medium paper points (total 20 paper points)
   - 1/4 ml tube containing 200 µl ATL buffer
Recruitment

- Subjects will be recruited from patients presenting for exam visits at the Dental Clinic at Nationwide Children's Hospital.
- Receptionist or care provider must first ask parents if it's okay for study personnel to approach them about the study.
- The Study Information Sheet can be used to inform parents about the study.
- Once parent agrees, study personnel can approach them directly.

Confirm that patient meets inclusion criteria:

- Child between 1 and 14 years of age at start of study
- Sampling must occur before prophylaxis is performed
- English-speaking primary caregiver who accompanies the child to dental visits
- Family intends to remain in Columbus for next 4 years and is willing to return for 6-month check-up visits on a regular basis
- No antibiotic treatment in the previous 30 days
- No chronic disease affecting the immune system or requiring chronic use of antibiotics
- No indication for antibiotic prophylaxis for infective endocarditis
- No early onset periodontitis
- No diabetes

Obtain consent:

- Explain study and answer questions
- You may use the Study Information Sheet to inform parents about the study. Use of this form is optional.
- Parents must sign 2 copies of the Consent Form and you must give them one of the copies.
- Children 8 yrs of age or older must sign 2 copies of the Assent Form and you must give them one of the copies.

Confirm the following & record on the “1) Exam & Sampling Form”:

- no dental prophylaxis within 30 days
- no antibiotics within 30 days

Give the parent the 2) Contact Info & History Form to fill out while you are sampling the patient.

Collect samples & exam data using “1) Exam & Sampling Form” before prophylaxis is performed:

1. Collect the following samples aseptically, avoiding contamination of sampling supplies (no touching with ungloved hands). Place the tubes upright in a disposable cup.
   a. **Saliva and soft tissue swab** will be collected by stroking a Copan swab across the right and left buccal mucosa and the dorsum of the tongue for a minimum of 15 seconds. The swab will be placed in a yellow label 15 ml tube containing ATL buffer.
   b. All visible **supragingival plaque** will be collected from the buccal surfaces of all teeth on the right side using sterile microbrushes. The brushes will be placed in a pink label 15 ml tube containing ATL buffer.
c. **Subgingival samples** will be obtained from the mesiobuccal sulcus of each tooth on the right side using sterile paper points. Paper points will be gently inserted and removed after 5 seconds and placed in a **blue label** 1.5 ml microfuge tube containing ATL buffer.

2. Score the presence of visible biofilm (plaque) on tongue.

3. Record the presence of any orthodontic appliances.

*Dental prophylaxis may be performed at this point or at any subsequent time*

While the child is being examined and sampled, parents will be filling out the **2) Contact Info & History Form**. After you have completed sample collection you will review the **2) Contact Info & History Form** with the parent, clarifying any questions and completing blanks or missing data.

After the dental visit is completed and the patient can join you and the parent, you will conduct the sugar-containing beverage interview with both the parent and child together using the **3) Beverage Survey Form**.

**Patient incentives**

When data collection is complete distribute

- Gift card (log in book)
- Dental gift bag
- Parking voucher

Sample tubes will be placed upright in the rack in the freezer compartment of the study refrigerator in the dental academic offices on the first floor within 2 hours of collection. Samples will be transported to the lab at OSU in batches.

The completed study forms will be placed in the bag they came from and stored in the locked, labeled cabinet in the OSU faculty area on the first floor.

After the entire research encounter is completed, data will be extracted from EPIC and recorded to complete the final sections of the "**1) Exam and Sampling Form**." This does not have to be completed on the day of the visit.

4. plaque levels
5. gingival health
6. calculus
7. current medications
8. medical conditions
9. other findings
10. tooth exam
   - tooth presence
   - white spot lesions
   - caries
   - restorations
   - sealants
Appendix C: Data Collection Form
<table>
<thead>
<tr>
<th>Confirm</th>
<th>4. Plaque levels</th>
<th>7. Current medications</th>
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<tbody>
<tr>
<td>[ ] no antibiotics within 30 days</td>
<td>[ ] None</td>
<td></td>
</tr>
<tr>
<td>[ ] no prophylaxis within 30 days</td>
<td>[ ] Light</td>
<td></td>
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<tr>
<td>[ ] no prophylaxis within 30 days</td>
<td>[ ] Moderate</td>
<td></td>
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**Clinical exam**

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<th>1. Samples (check off)</th>
<th>5. Gingivitis</th>
<th>8. Medical conditions</th>
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<tbody>
<tr>
<td>□ saliva &amp; soft tissue swab</td>
<td>□ None</td>
<td></td>
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<tr>
<td>□ supragingival plaque buccal right side</td>
<td>□ Localized</td>
<td></td>
</tr>
<tr>
<td>□ subgingival plaque mesiobuccal right side</td>
<td>□ Generalized</td>
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<th>2. Tongue biofilm</th>
<th>6. Calculus</th>
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<tr>
<td>[ ] no</td>
<td>[ ] Present</td>
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<td>[ ] yes</td>
<td>[ ] None noted</td>
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<th>3. Ortho appliances</th>
<th>Describe:</th>
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<td>[ ] no</td>
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<td>[ ] yes</td>
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**OK to prophylaxis now or later**

**Tooth exam**

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<th>10. Tooth exam</th>
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<tr>
<td>[ ] Circle teeth present</td>
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<tr>
<td>[ ] Whitestones &quot;I&quot;</td>
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<td>[ ] Caries red</td>
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<td>[ ] Restorations black</td>
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**Teeth numbers:**

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