

Doctoral Thesis

# Microbiota in Human Diseases

Jaewoong Lee

Department of Biomedical Engineering

Ulsan National Institute of Science and Technology

2024

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# CHURCH OF THE FLYING SPAGHETTI MONSTER

February 09, 2021

## Letter of Good Standing

Dear Sir or Madam:

I am pleased to verify that \_\_\_\_\_

JAEWOONG LEE

is an ordained minister of the Church of the Flying Spaghetti Monster and recognized  
within our organization as a member in good standing.

We hereby consent to this minister performing ceremonies and request that they are  
granted all privileges and respect appropriate to a spiritual leader.

Any questions can be directed to the undersigned.

Representative,  
Church of the Flying Spaghetti Monster  
Bobby Henderson



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## **Abstract**

Your abstract should be here.



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## List of Abbreviations

**ACC** Accuracy

**ASV** Amplicon sequence variant

**AUC** Area-under-curve

**BA** Balanced accuracy

**DAT** Differentially abundant taxa

**F1** F1 score

**Faith PD** Faith's phylogenetic diversity

**FTB** Full-term birth

**GA** Gestational age

**MWU test** Mann-Whitney U-test

**PRE** Precision

**PROM** Prelabor rupture of membrane

**PTB** Preterm birth

**ROC curve** Receiver-operating characteristics curve

**rRNA** Ribosomal RNA

**SEN** Sensitivity

**SPE** Specificity

**t-SNE** t-distributed stochastic neighbor embedding

# 1 Abstract

The microbiome refers to the complex community of microorganisms, including bacteria, viruses, fungi, and other microbes, that inhabit various environments within living organisms (Ursell, Metcalf, Parfrey, & Knight, 2012; Gilbert et al., 2018). In humans, the microbiome plays a crucial role in maintaining health (Lloyd-Price, Abu-Ali, & Huttenhower, 2016), influencing processes such as digestion (Lim, Park, Tong, & Yu, 2020), immune response (Thaiss, Zmora, Levy, & Elinav, 2016; Kogut, Lee, & Santin, 2020; Kim, 2018), and even mental health (Mayer, Tillisch, Gupta, et al., 2015; Zhu et al., 2017; X. Chen, D'Souza, & Hong, 2013). These microbial communities are not static nor constant but rather dynamic ecosystem that interact with their host and respond to environmental changes. Recent studies have revealed that imbalances in the microbiome, known as dysbiosis, can contribute to a wide range of diseases, including obesity (John & Mullin, 2016; Tilg, Kaser, et al., 2011; Castaner et al., 2018), diabetes (Barlow, Yu, & Mathur, 2015; Hartstra, Bouter, Bäckhed, & Nieuwdorp, 2015; Sharma & Tripathi, 2019), infections (Whiteside, Razvi, Dave, Reid, & Burton, 2015; Alverdy, Hyoju, Weigerinck, & Gilbert, 2017), inflammatory conditions (Francescone, Hou, & Grivennikov, 2014; Peirce & Alviña, 2019; Honda & Littman, 2012), and cancers (Helmink, Khan, Hermann, Gopalakrishnan, & Wargo, 2019; Cullin, Antunes, Straussman, Stein-Thoeringer, & Elinav, 2021; Sepich-Poore et al., 2021; Schwabe & Jobin, 2013). Thus, understanding the composition of the microbiome is essential for developing new therapeutic approaches that target these microbial populations to promote health and prevent diseases.

(PTB)

(Periodontitis)

(Lung)

(Conclusion)

**This doctoral dissertation is an addition based on the following papers that the author has already published:**

- Hong, Y. M., Lee, J., Cho, D. H., Jeon, J. H., Kang, J., Kim, M. G., ... & Kim, J. K. (2023). Predicting preterm birth using machine learning techniques in oral microbiome. *Scientific Reports*, 13(1), 21105.

## 2 Predicting preterm birth using machine learning techniques in oral microbiome

**This section includes the published contents:**

Hong, Y. M., Lee, J., Cho, D. H., Jeon, J. H., Kang, J., Kim, M. G., ... & Kim, J. K. (2023). Predicting preterm birth using machine learning techniques in oral microbiome. *Scientific Reports*, 13(1), 21105.

### 2.1 Introduction

Preterm birth (PTB), defined as delivery of newborns before 37 weeks of gestation, is a leading cause of morbidity and mortality in neonates (Blencowe et al., 2012). Established risk factors for PTB include genitourinary tract infections, short cervical length, and multiple pregnancies (Goldenberg, Culhane, Iams, & Romero, 2008). However, there is still disagreement regarding the magnitude of these factors' effects on birth outcomes. Early identification of pregnant women at high risk of PTB can facilitate the implementation of strategies to prolong gestation and improve birth outcomes (Iams & Berghella, 2010).

Despite increased understanding of risk factors contributing to PTB, there remains a considerable lack of sensitivity in predictive models that can serve as a framework for intervention strategies (Sotiriadis, Papatheodorou, Kavvadias, & Makrydimas, 2010). Numerous attempts have been made to predict PTB using machine learning techniques combined with data from health records, inflammatory markers, and vaginal microbiome (Berghella, 2012). Fetal fibronectin is widely used clinically due to its affordability and simplicity. However, it has a low prediction rate with a sensitivity of only 56% (Honest et al., 2009). Cervical length measurement also has limitations due to the hassle and inaccuracy of the procedure and the need for a skilled specialist (Leitich & Kaider, 2003).

Approximately 70% of PTB result from spontaneous onset of preterm labor and preterm pre-labor rupture of membranes (PROM) due to intrauterine infection and inflammation (Romero, Dey, & Fisher, 2014). However, the mechanism of PTB cannot be fully explained by inflammatory and infectious pathways as anti-inflammatory and antibiotic treatments could not reduce PTB incidence rates (Romero, Hassan, et al., 2014). With advancements in molecular genetic technology, studies on maternal microbiomes using 16S ribosomal RNA (rRNA) sequencing have emerged to explore unknown pathways of PTB (Fettweis et al., 2019).

Microorganisms associated with PTB have been postulated to originate from one of two places: the reproductive or genitourinary tract ascending through the cervix or a hematogenous route (Han & Wang, 2013). Recent research has identified vaginal microbial signatures in women who later experience PTB and attempted to predict PTB using cervicovaginal fluid (Kindinger et al., 2017). Although existing reports have verified a potential relationship of vaginal microbiome with PTB, they can only explain an ascending route.

Decades of epidemiological research studies have suggested that periodontitis is an independent risk factor for various adverse birth outcomes, including PTB (Offenbacher et al., 1996). Based on these precedents, it is expected that the oral microbiome can explain another hematogenous route. However,

prenatal oral microbiome is not well understood.

Thus, this study aimed to compare oral microbiome compositions between a PTB group and a full-term birth group, to identify oral microbiome associated with PTB, and to develop a machine learning prediction model of PTB based on oral microbiome compositions.

## **2.2 Materials and methods**

### **2.2.1 Study design and participants**

This study was conducted on singleton pregnant women admitted for delivery at Jeonbuk National University Hospital between 2019 and 2021. This study received approval from the Ethical Research Committee (IRB file No. 2019-01-024). All participants provided written informed consent. Eligible participants included women admitted for induction delivery, elective cesarean section, and those who were hospitalized due to symptoms of preterm labor or preterm pre-labor rupture of membranes.

### **2.2.2 Data collection and grouping**

Data on current and historical pregnancy outcomes were collected from questionnaires and electronic medical records. This information encompassed demographic factors (gestational age, birth weight, sex) and maternal risk factors (maternal age at delivery, cesarean section, preterm pre-labor rupture of membranes, previous preterm delivery history, gestational or overt diabetes mellitus, pregnancy-induced or chronic hypertension, and pre-pregnancy overweight or gestational weight gain). All subjects were divided into a PTB group or a full-term birth (FTB) group, with PTB defined as delivery before 37 weeks of gestation.

### **2.2.3 Oral microbiome sample collection**

Oral microbiome samples were collected using mouthwash within 24 h before delivery. Standard sterile techniques were employed. Medical staff supervised all sample collection procedures. Participants were instructed to avoid brushing their teeth, eating, or drinking 30 min before sampling. Saliva samples were obtained by rinsing the mouth with 12 mL of a gargle solution (E-zen Gargle, JN Pharm, Pyeongtaek, Korea) for 30 s. Sample were labeled with the subject's anonymous ID and stored at 4 °C until further processing. The resuspended sample was transferred to a microcentrifuge tube. Genomic DNA was extracted using an ExgeneTM Clinic SV kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions and stored at -20 °C.

### **2.2.4 16s rRNA gene sequencing and taxonomy assignment**

Specimens were sent to Department of Biomedical Engineering, Ulsan National Institute of Science and Technology for taxonomy assignment. Then 16S rRNA sequencing was performed using an Illumina MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA) commissioned by Macrogen (Macrogen, Seoul, Korea). Library protocols for amplifying V3 and V4 regions were used. Pooled library was sequenced using a v3 600 cycle chemistry after diluting the sample to a final concentration of 6 pM with 20% PhiX control to generate 300 bp paired end reads.

## **2.2.5 Bioinformatics and statistical analysis**

Independent t-test and chi-square test were used to compare differences between the PTB group and the FTB group. SPSS (version 20.0) was used for all data analyses (Spss et al., 2011). Statistical significance was considered at  $p < 0.05$ .

16S rRNA sequences from study subjects were imported with QIIME2 (version 2022.2) for further processing (Bolyen et al., 2019). Sequences were filtered with DADA2 (Callahan et al., 2016). Amplicon sequence variants (ASV) were assigned taxonomy with the Human Oral Microbiome Database (version 15.22) (T. Chen et al., 2010). To measure richness of microbiomes, two diversity indices were calculated. Alpha diversity, a measure of species within a particular community, was calculated using the Faith's phylogenetic diversity (Faith PD) index within the QIIME2 platform. Communities numerically dominated by a few species will exhibit a low Faith PD index, whereas communities in which abundance is distributed equally among species will exhibit a high evenness. Mann–Whitney–Wilcoxon test was used to find statistically significant differences in Faith PD index. Beta diversity measures dissimilarity between pairs of communities. It was calculated using the Hamming diversity index. PERMANOVA multivariate test was used to calculate statistically significant differences in the Hamming diversity index.

To identify differentially abundant taxa (DAT) with distinct abundance differences between the PTB group and the FTB group, DESeq2 was implemented using `DESeqDataSetFromMatrix` method as described in the package tutorial (Love, Huber, & Anders, 2014). Taxa with  $|\log_2 \text{FoldChange}| > 1$  and adjusted  $p < 0.05$  are considered as significant different.

## **2.2.6 Machine learning prediction model development**

Following qualitative and quantitative analyses of associations between the PTB group and each bacterium, a random forest classifier was used to find the criteria to predict PTB with oral microbiome data. Random forest classification is an ensemble machine learning algorithm that summarizes many decision trees to improve classification evaluations and robustness (Breiman, 2001). Random forest classifier was implemented to predict PTB based on oral microbiome compositions. To assure consistence and reliable classification results (Wong & Yeh, 2019), we performed stratified  $k$ -fold cross-validation ( $k = 5$ ). Moreover, to decide the best features that could maximize classification evaluations, we performed random forest classification evaluations only with some DAT selected by their importances. Evaluations for classification included accuracy (ACC), balanced accuracy (BA), precision (PRE), sensitivity (SEN), and specificity (SPE).

## **2.2.7 Ethics approval and consent to participate**

The research protocol was approved by the Institutional Review Board of Jeonbuk National University Hospital (No. 2019-01-024) and was performed according to the Declaration of Helsinki (Goodyear, Krleza-Jeric, & Lemmens, 2007).

## 2.3 Results

### 2.3.1 Study participant demographics

In this study, a total of 69 volunteer mothers were initially recruited. However, one participant with incomplete data and nine individuals with twin pregnancies were excluded from the study cohort. As a result, 59 women (30 in the PTB group and 29 in the FTB group) were included in the final analysis. Demographic and clinical characteristics of subjects in the PTB group and the FTB group are summarized in Table 1. Because PROM is a major cause of preterm birth, it was significantly higher in PTB group. There was no significant difference in other maternal clinical characteristics between the PTB group and the FTB group. There were no cases with a history of smoking or concurrent periodontal disease in both groups.

### 2.3.2 Comparison of oral microbiomes

The oral microbiome comprised 13,953,804 sequences from 59 oral microbiome samples, with  $102,305.95 \pm 19,095.60$  and  $64,823.41 \pm 15,841.65$  reads per sample before and after filtering low-quality reads and trimming extra-long tails, respectively. After filtering low-quality reads and trimming extra-long tails, remaining representative reads were clustered in ASVs with their exact sequence match. There were no significant differences in measures of alpha diversity (Faith PD) and beta diversity (Hamming distance) indices for samples between the PTB group and the FTB group (Figure 4).

Of 465 genera and species analyzed, 32 DAT between the PTB group and the FTB group were selected by DESeq2 (Love et al., 2014), including 26 FTB-enriched DAT and six PTB-enriched DAT. In order to mitigate the confounding effect of PROM, we excluded 7 PROM-related DATs from these 32 DAT (Figure 5). There were a total of 25 DATs between the PTB group and the FTB group, with 22 DATs enriched in the FTB group and three in the PTB group, as depicted in Figure 1.

Figure 2 displays DAT volcano plot in the oral microbiome sorted by differences between FTB-enriched DAT and PTB-enriched DAT proportions, indicating a decrease in gestational age for FTB-enriched DAT proportions. Pearson correlation analysis revealed a strong negative correlation ( $r = -0.542$  and  $p = 7.8e - 6$ ) between gestational age and difference between PTB-enriched DAT and FTB-enriched DAT proportions.

### 2.3.3 Random forest classification

Random forest classifiers were established to classify PTB based on DAT. The best BA( $0.765 \pm 0.071$ ) was achieved using the nine most important taxa (Figure 3a). The Random forest model calculated the importance of each DAT (Figure 3b). Overall ACC, PRE, SEN, and SPE were  $0.714 \pm 0.061$ ,  $0.700 \pm 0.194$ ,  $0.728 \pm 0.058$ , and  $0.743 \pm 0.138$ , respectively. To validate the performance of our machine learning prediction model, we conducted a validation test on nine twin pregnancies that were excluded from the paper (Figure 6). On PTB subjects of these twin samples, the machine learning classifications have 87.5% accuracy, comparable to the machine learning classification on the singleton study subjects.

Table 1: **Baseline clinical characteristics of study subjects.** Continuous variable: Mean  $\pm$  standard deviation. Categorical variable: count (proportion). Continuous variable for independent *t*-test. Categorical variable for Pearson's  $\chi^2$ -square test.

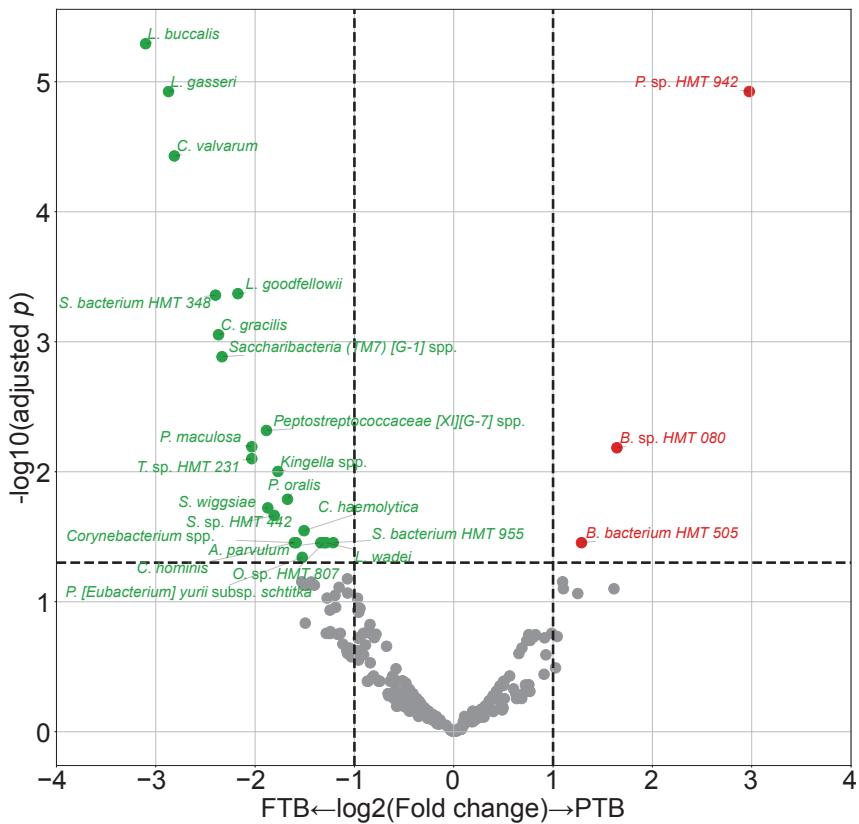
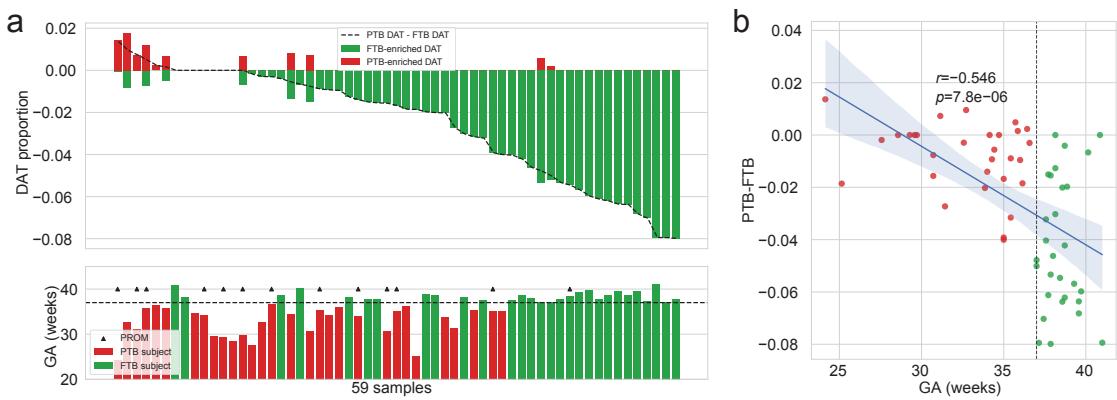


Figure 1: **DAT volcano plot.** DAT volcano plot shows DAT, with PTB-enriched DAT shown as red dots and FTB-enriched DAT shown as green dots. Taxa with  $|\log_2 \text{FoldChange}| > 1$  and adjusted  $p < 0.05$  are considered as significant different.



**Figure 2: Oral microbiome compositions over DAT.** (a) Proportions of DAT of study subjects. Samples are sorted by difference between PTB-enriched DAT proportion and FTB-enriched DAT proportion. GA of samples are shown, matched with order of upper panel. PTB: red bar, FTB: green bar. PROM: arrow head. (b) Correlation plot with GA and difference between PTB-enriched DAT proportion and FTB-enriched DAT proportion. Pearson correlation shows strong negative coefficient ( $r = -0.542$  and  $p = 7.8e - 06$ ) between GA and difference between PTB-enriched DAT proportion and FTB-enriched DAT proportion.

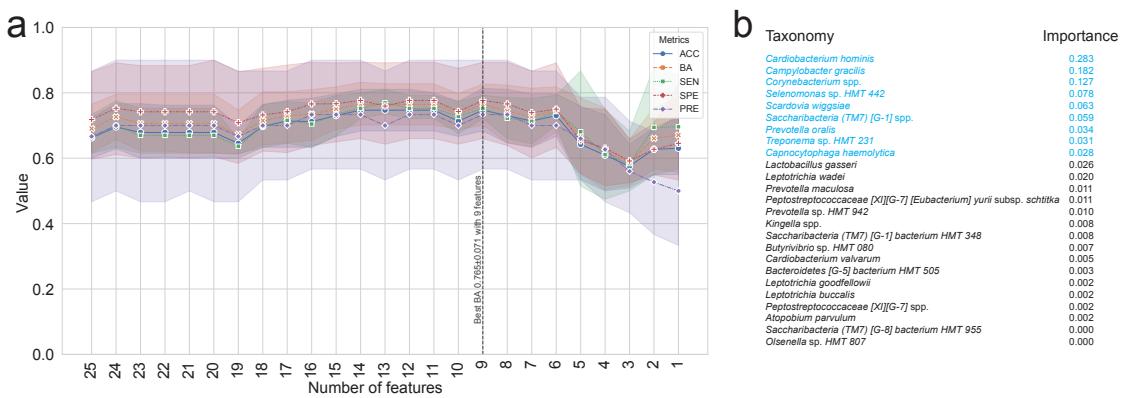
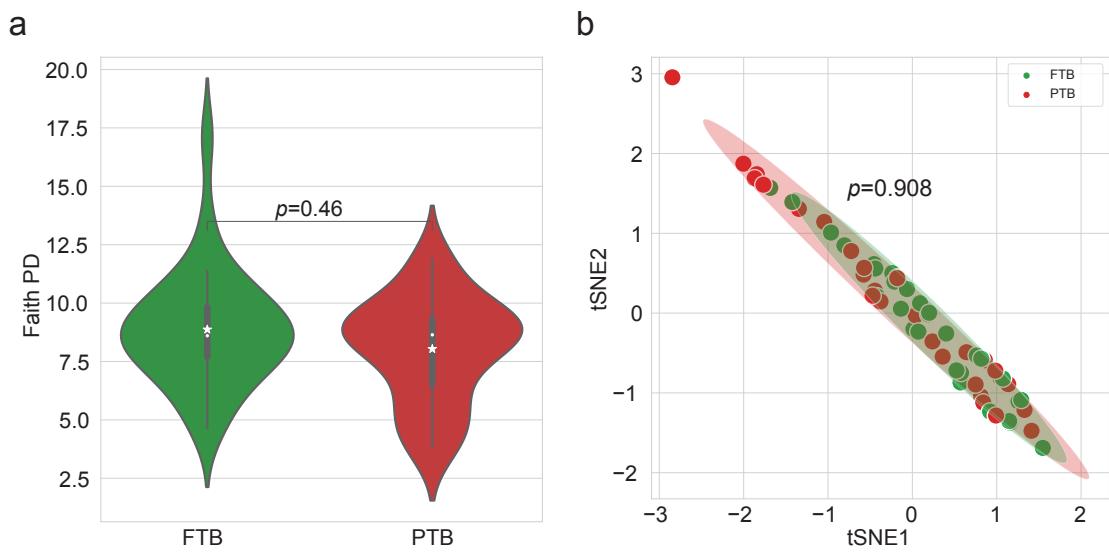


Figure 3: **Machine learning evaluations over DAT.** **(a)** Machine learning evaluation upon number of features (DAT). Random Forest classifier has the best balanced accuracy (mean  $\pm$  standard deviation.;  $0.765 \pm 0.071$ ) with the nine most important DAT. **(b)** Importance of DAT is shown. Note that  $0 \leq$  importance  $\leq 1$ , and  $\sum$  importance = 1. The 20 most important DAT that give the best balanced accuracy is marked with blue.



**Figure 4: Diversity indices.** **(a)** Alpha diversity index (Faith PD). Mann-Whitney-Wilcoxon test did not find statistically significant difference between the PTB group and the FTB group. Mean values are marked with star-point. **(b)** t-SNE plot with beta diversity index (Hamming distance). PERMANOVA multivariate test did not find statistically significant difference between the PTB group and the FTB group.

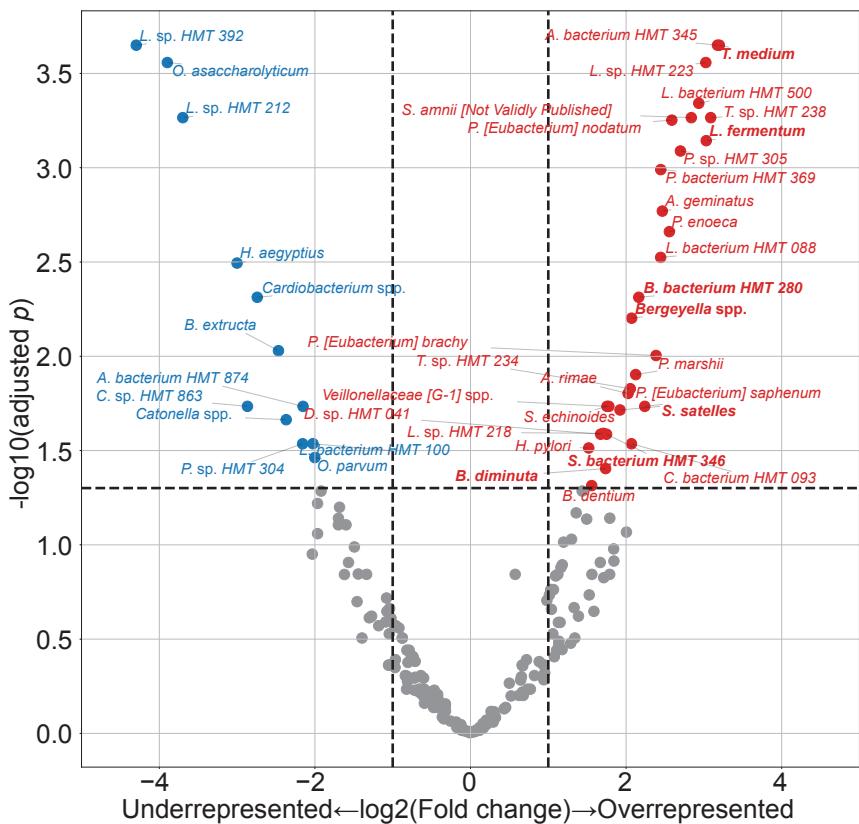


Figure 5: **PROM-related DAT volcano plot.** This is a subgroup analysis between 12 participants in PTB group with PROM and 18 participants in PTB group without PROM, 42 PROM-related DAT were selected between these two groups. Out of these 42 PROM-related DAT, only 7 DAT overlapped with PTB-related DAT, as it indicated by the bold marking. Volcano plot shows PROM-related DAT, with 12 PROM-underrepresented DAT shown as blue dots and 30 PROM-overrepresented DAT shown as red dots. Taxa with  $|\log_2 \text{FoldChange}| > 1$  and adjusted  $p < 0.05$  are considered as significant different.

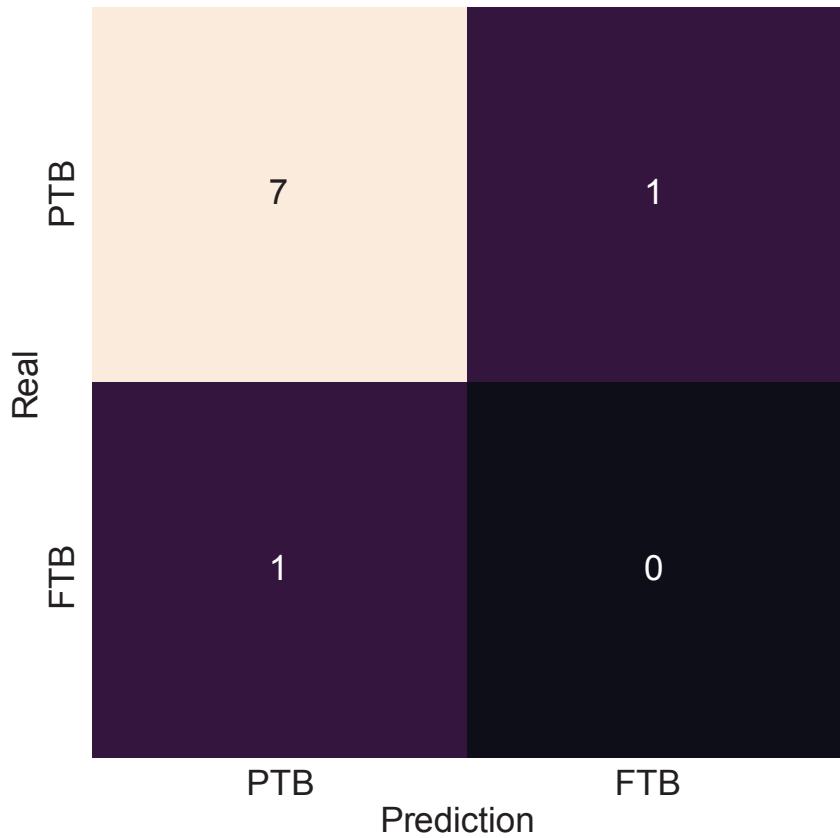


Figure 6: **Heatmap plot of PTB classification with validation data.** A validation test on 9 twin pregnancies that had excluded from the paper was conducted. They consist of 8 PTB subjects and 1 FTB subject. As twin pregnancies have a 7-10 times higher PTB rate than singleton pregnancies, resulting in a majority of the test data falling into the PTB group. The machine learning classifications have 87.5% accuracy, comparable to the machine learning classification on the singleton study subjects (Mean  $\pm$  standard deviation.  $0.714 \pm 0.061$ )

## 2.4 Discussion

In this study, we developed a method for predicting PTB based on random forest classifier using oral microbiome compositions. Recently, several sporadic reports have suggested a bidirectional relationship between oral microbiome and pregnancy (Han & Wang, 2013). However, prenatal oral microbiome is not well understood yet. Some research has shown that oral microbial dysbiosis combined with gingival inflammation can lead to adverse pregnancy outcomes, including low birth weight, PTB, pre-eclampsia, and miscarriages (Ide & Papapanou, 2013). Nevertheless, these results have been inconsistent due to methodologies employed in studies that only target known pathogens.

*Fusobacterium nucleatum* is the most prevalent oral microbiome studied (Han, 2015; Brennan & Garrett, 2019; Bolstad, Jensen, & Bakken, 1996). *Fusobacterium nucleatum* is a Gramnegative, anaerobic, filamentous oral microbiome. It is considered one of the most abundant species in the oral microbiome. It can also be isolated from vaginal microbiome (Vander Haar, So, Gyamfi-Bannerman, & Han, 2018; Witkin, 2019). Intra-amniotic *Fusobacterium nucleatum* infection leading to PTB has been reported in human and animal studies (Doyle et al., 2014). Other studies have shown that other oral pathogens including *Porphyromonas gingivalis* and intrauterine *Bergeyella* spp. can be isolated from the placenta of women who deliver prematurely (León et al., 2007; Katz, Chegini, Shiverick, & Lamont, 2009). In the present study, although *Bergeyella* spp. was overrepresented in the PTB group with PROM, it was excluded in the finally selected 25 DAT. Furthermore, *Campylobacter gracilis* was one of the FTB-enriched DAT that can aid colonization by periodontal pathogens including *Porphyromonas gingivalis* in subgingival microbiome (Yang et al., 2022). *Lactobacillus gasseri* was also one of the FTB-enriched DAT. It is known that *Lactobacillus gasseri* in vaginal microbiome can decrease early PTB risk (Basavaprabhu, Sonu, & Prabha, 2020; Payne et al., 2021).

We found that decisive species differentiating between two groups were mainly abundant in the FTB group, with DAT consisting of 22 FTB-enriched DAT and three PTB-enriched DAT. We hypothesize that deficiency of species having a protective impact might have triggered the pathophysiology of PTB. Two different mechanisms have been proposed to explain the relationship between unhealthy microbiota composition and adverse pregnancy outcomes. The first mechanism proposed that periodontal bacteria originating in the gingival biofilm could translocate from the unhealthy oral cavity and cross the placenta, reach the intra-amniotic fluid and fetal circulation and directly affect the fetoplacental unit, resulting in bacteremia (Hajishengallis, 2015). The second mechanisms proposed that systemic dissemination of endotoxins and/or inflammatory mediators derived from periodontal plaque and secreted by the subgingival inflammatory site could be carried to the fetoplacental unit (Stout et al., 2013; Aagaard et al., 2014). Although certain microbiota has the same species, their subgroups can have both positive and negative influences on pregnancy outcomes. Following this line of thought, we believe that composition or dysbiosis of the oral microbiome is more important than the presence of specific microbiota.

It is worth noting that microbial changes occurring during pregnancy might be nature consequences of a healthy pregnancy. Three reasons can explain the susceptibility to oral diseases such as periodontitis during pregnancy. These diseases are common in pregnant women due to hormonally driven hyper-

reactivity of the gingiva to bacteria in the subgingival biofilm. Other factors that increase the risk of poor oral health during pregnancy include changes in dietary habits (frequent snacking or increased consumption of carbohydrate-rich foods), stomach acids from nausea and vomiting that contribute to the breakdown of tooth enamel, and a decreased likelihood of seeking dental care during pregnancy. We plan to implement pathway analyses to investigate direct link between the microbiome and PTB.

Even though there was limited power resulting from a small number of participants and restricted validation sample size, our study verified that oral microbiota might provide potential biomarkers for predicting pregnancy complications using machine learning methods including random forest classification. Additionally, the fact that the entire microbiome was not analyzed was a limitation of this study because our analysis only used relative values measured by 16S rRNA sequencing, not 16S metagenome sequencing. We did not investigate other factors could impact the oral microbiome, such as participant's diet and socioeconomic status.

Despite these limitations, this prospective study demonstrated the potential of a PTB prediction model using oral microbiome in mouthwash. Further multi-center and larger-scale studies are needed to confirm our results before applying techniques developed in this study in the clinical field.

### **3 Prediction model for periodontitis severity based on the salivary microbiomes**

This section includes the published contents:

#### **3.1 Introduction**

## **3.2 Materials and methods**

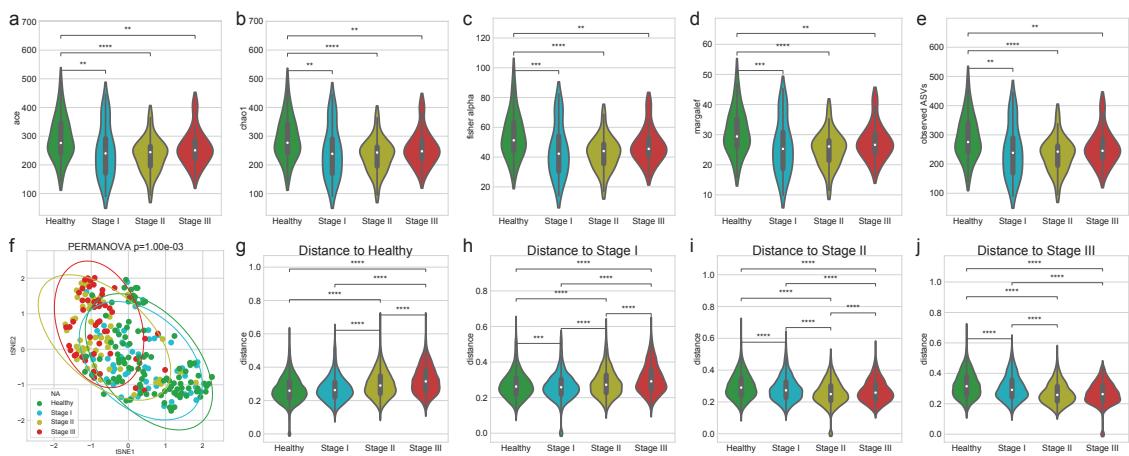
### **3.2.1 Study subjects and clinical procedure**

### **3.2.2 Bioinformatics analysis**

### **3.3 Results**

- 3.3.1 Summary of study subjects and sequencing data**
- 3.3.2 Diversity indices reveal differences in diversity among the periodontal statuses**
- 3.3.3 Differentially abundant taxa among healthy and different periodontitis stages and their correlation**
- 3.3.4 Classification of periodontitis stages by random forest models**

Table 2: **Clinical characteristics of the study subjects.** Significant differences were assessed using the Kruskal-Wallis test. NA: Not applicable.



**Figure 7: Diversity indices.** Comparisons of salivary microbiomes among healthy controls and patients with various periodontitis stages. Alpha-diversity indices (**a-e**) indicate that healthy controls have increased heterogeneity than periodontitis stages as measured by: **(a)** ace **(b)** chao1 **(c)** Fisher alpha **(d)** Margalef, and **(e)** observed ASVs. **(f)** The beta-diversity index (weighted UniFrac) was visualized using a tSNE-transformed plot. The confidence ellipses are shown to display the distribution of each periodontitis stage. The distance to each stage demonstrated that each periodontitis stage was distinguished from the other periodontitis stages: **(g)** distance to Healthy **(h)** distance to Stage I **(i)** distance to Stage II, and **(j)** distance to Stage III. Statistical significance determined by the Mann-Whitney U-test (MWU) test:  $p \leq 0.01$  (\*\*) and  $p \leq 0.0001$  (\*\*\*\*).

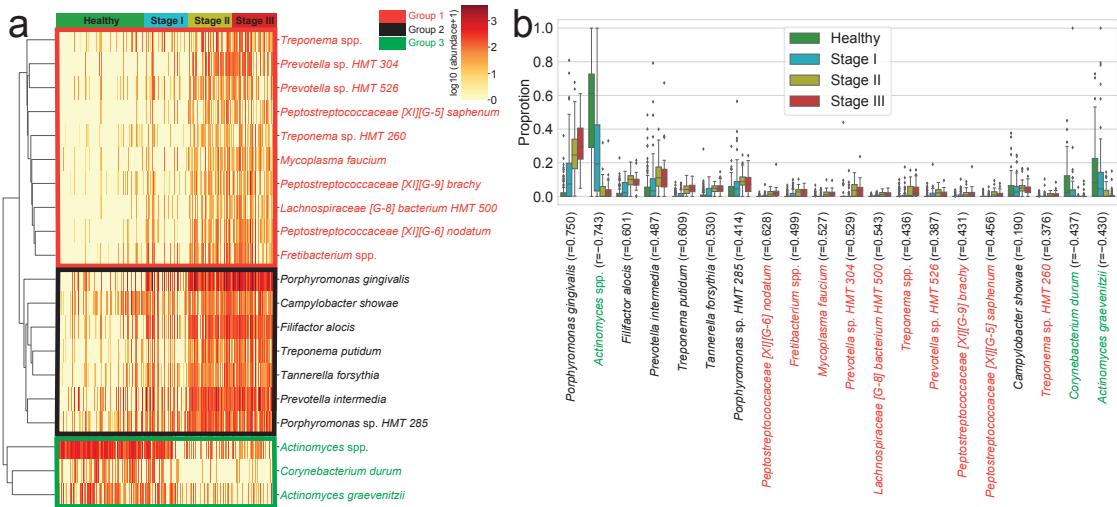


Figure 8: **Differentially abundant taxa**. Differentially abundant taxa (DAT) that were identified by ANCOM. **(a)** Heatmap of clustered DAT with similar distribution among subjects. Group 1, Group 2, and Group 3 are marked in red, black, and green, respectively. **(b)** Box plots showing the proportions of DAT. Taxa were sorted by their importance according to ANCOM.

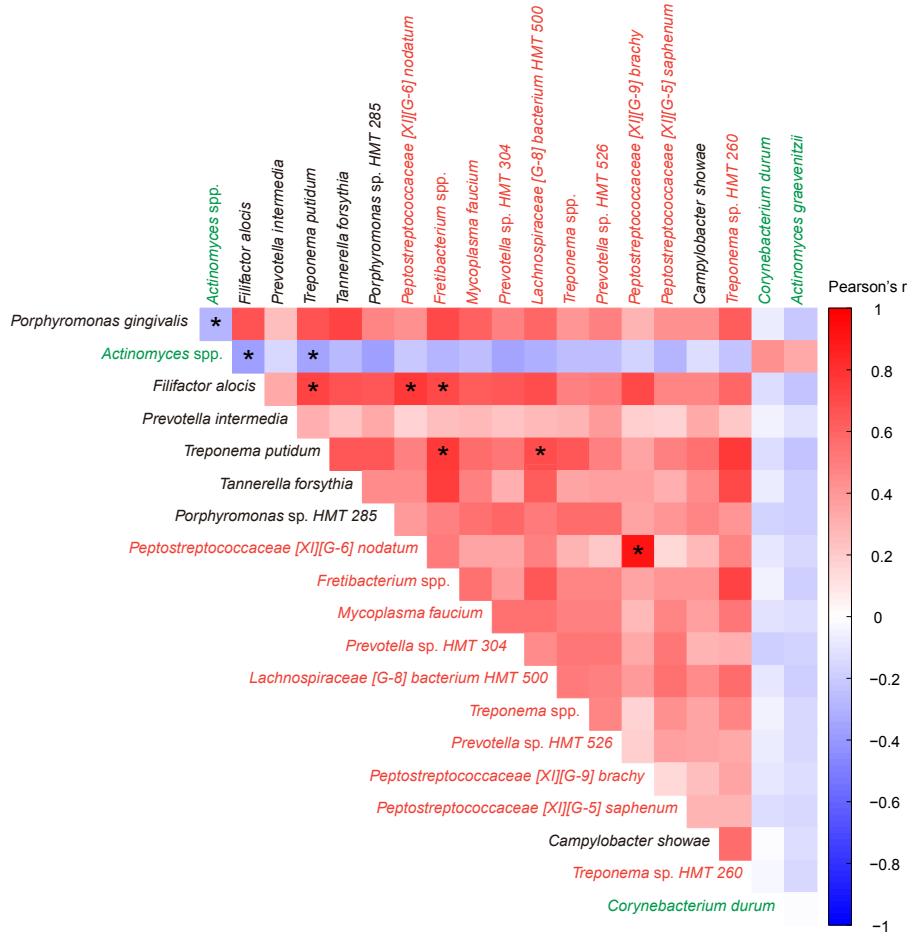
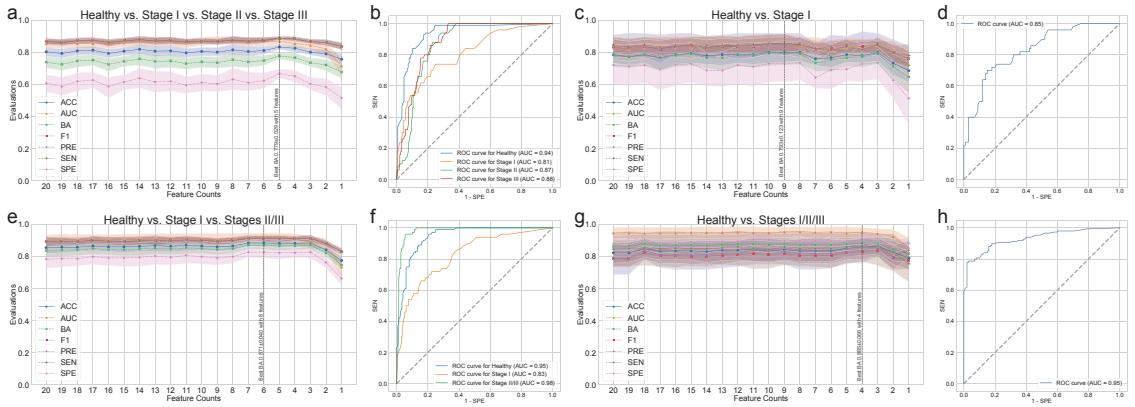
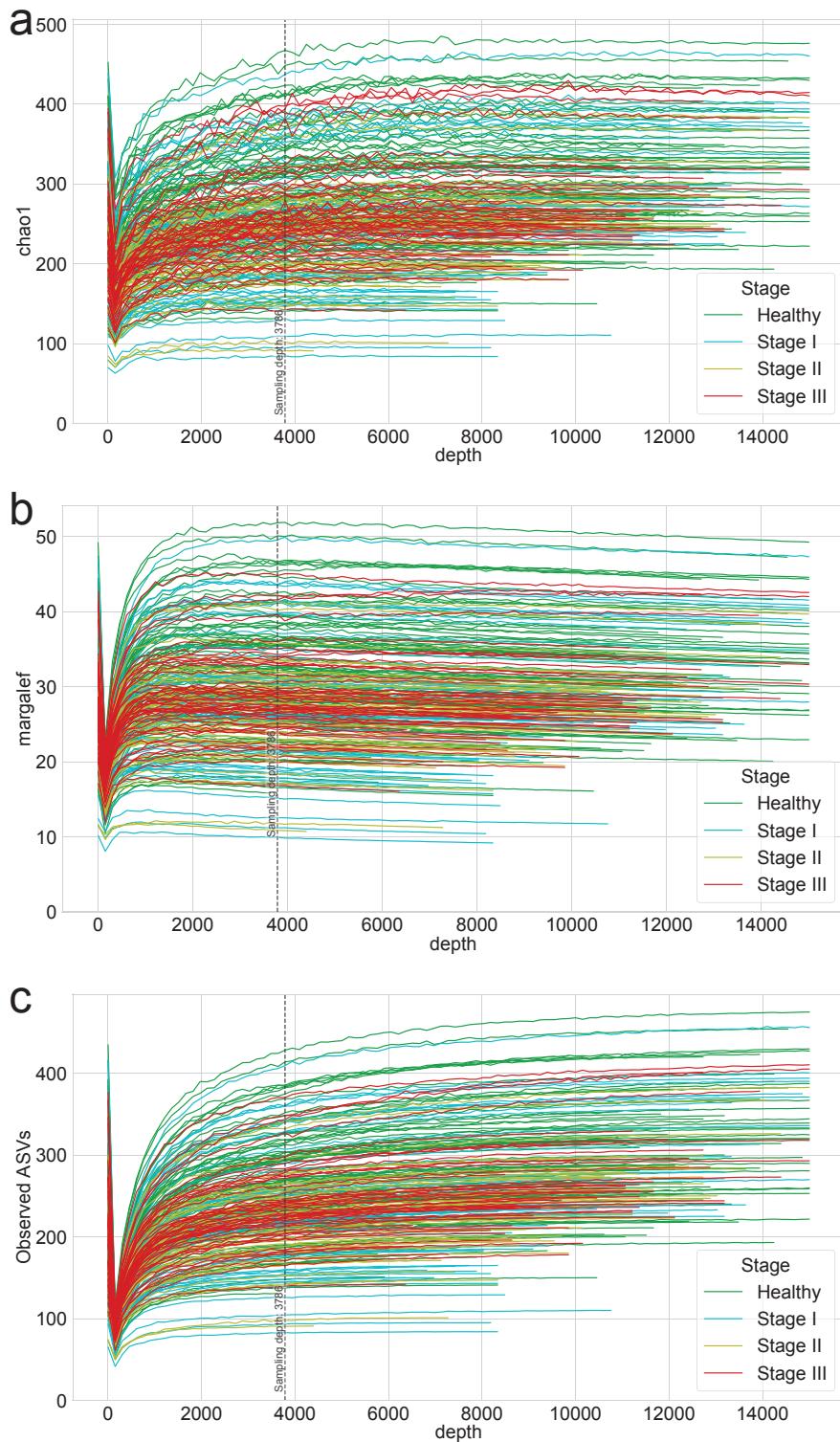


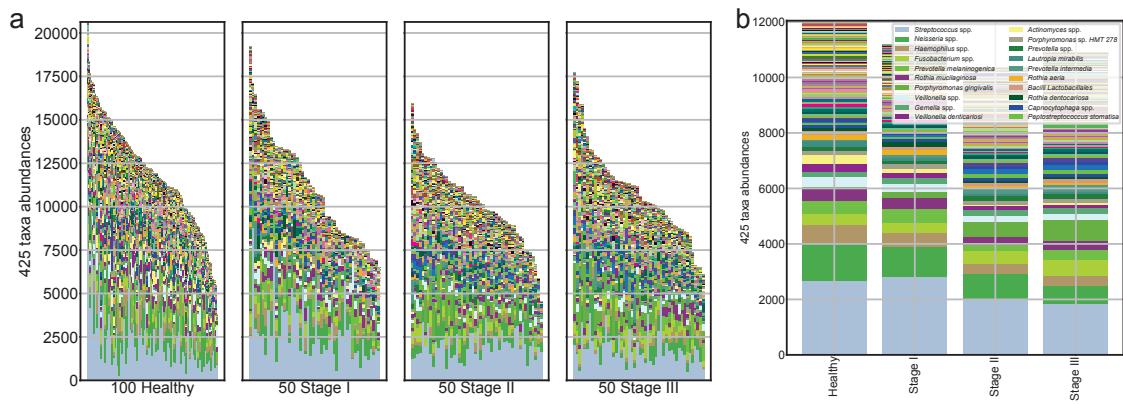
Figure 9: **Correlation heatmap.** Pearson's correlations between differentially abundant taxa in healthy status and periodontitis stages. Statistical significance was determined by strong correlation, i.e.,  $| \text{coefficient} | \geq 0.5$  (\*).



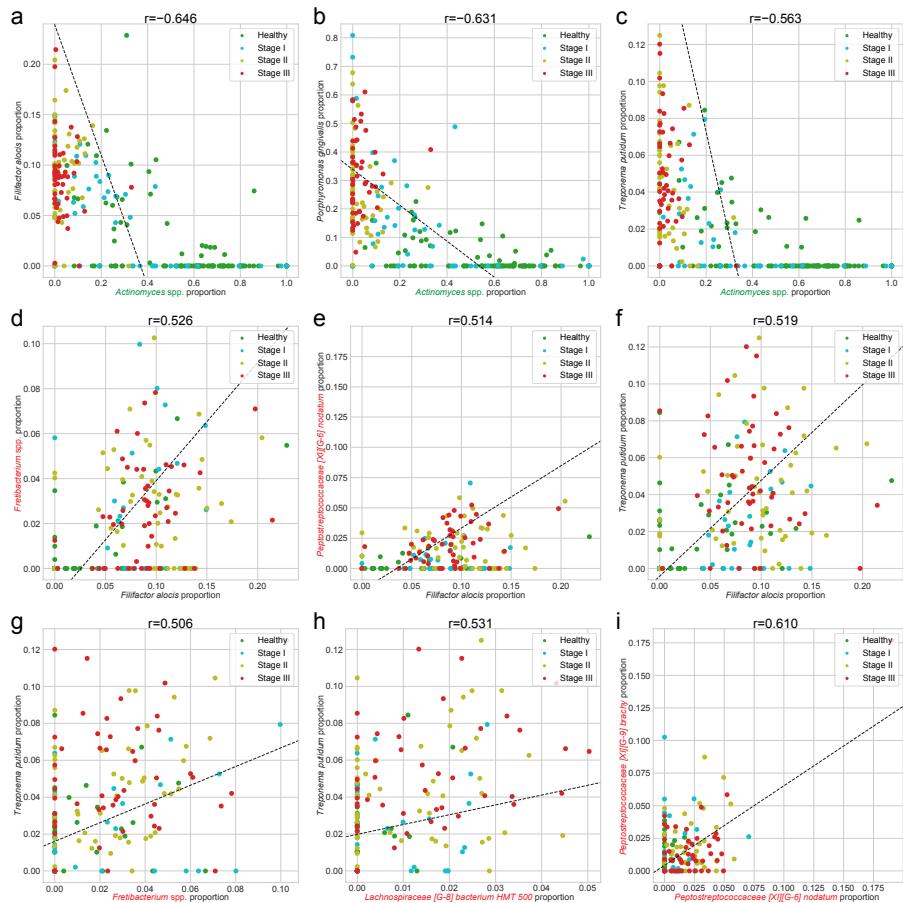
**Figure 10: Random forest classification metrics.** The classification metrics in the random forest classifications were as follows: accuracy (ACC), area-under-curve (AUC), balanced accuracy (BA), F1 score (F1), precision (PRE), sensitivity (SEN), and specificity (SPE). Every classification metric ranges from [0, 1], with higher values indicating better performance. **(a)** Classification performance for healthy vs. stage I vs. stage II vs. stage III. **(b)** Receiver-operating characteristics (ROC) curve for the highest BA of (a). **(c)** Classification performance for healthy vs. stage I. **(d)** ROC curve on the highest BA of (c). **(e)** Classification performance for healthy vs. stage I vs. stages II/III. **(f)** ROC curve for the highest BA of (e). **(g)** Classification performance for healthy vs. stages I/II/III. **(h)** ROC curve for the highest BA of (h).



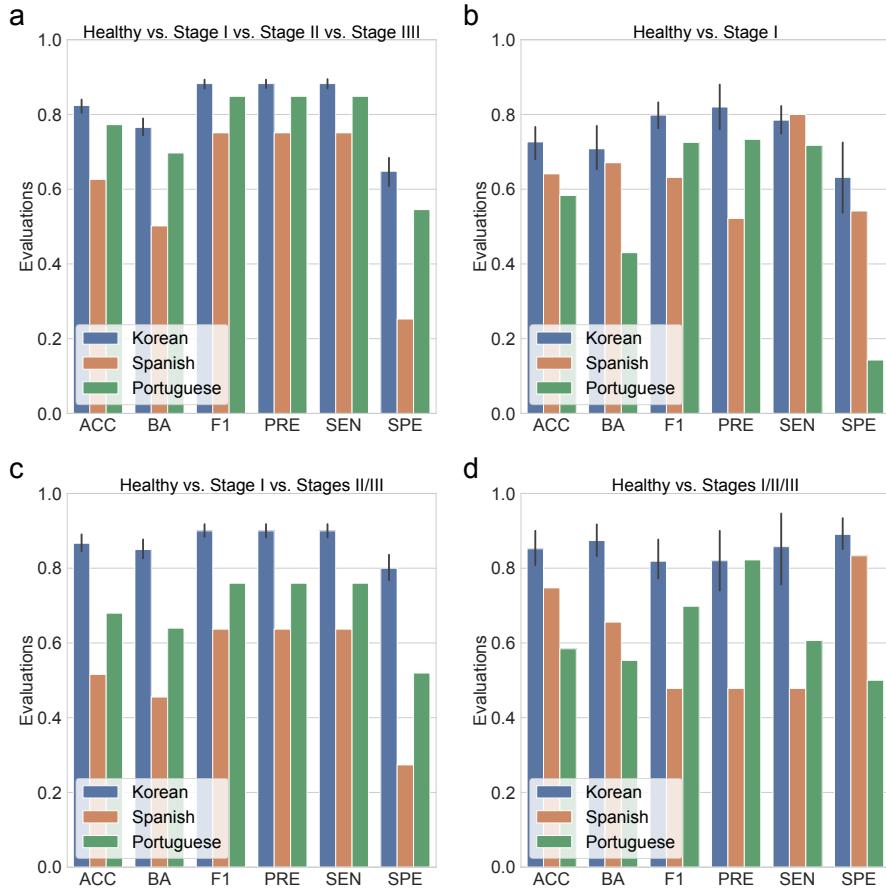
**Figure 11: Rarefaction curves for alpha diversity indices.** Rarefaction of (a) chao1 (b) margalef, and (c) observed ASVs were generated to measure species richness and determine the sampling depth of each sample.



**Figure 12: Absolute abundance of salivary bacterial taxa in the different periodontal statuses at the species level.** Stacked bar plot of the absolute abundance of bacterial species for all samples **(a)** and the mean absolute abundance of bacterial species in the healthy, stage I, stage II, and stage III groups **(b)**.



**Figure 13: Correlation plots for differentially abundant taxa.** We selected the combinations of DAT with absolute Spearman correlation coefficients greater than 0.5. The color represents periodontal healthy periodontal statuses (green: healthy, cyan: stage I, yellow: stage II, and red: stage III).



**Figure 14: Random forest classification metrics from external datasets.** The classification metrics in the random forest classification included accuracy (ACC), balanced accuracy (BA), F1 score (F1), precision (PRE), sensitivity (SEN), and specificity (SPE). Every classification metric ranges from [0, 1], with higher values indicating better performance. **(a)** Classification performance for healthy vs. stage I vs. stage II vs. stage III. **(b)** Classification performance for healthy vs. stage I. **(c)** Classification performance for healthy vs. stage I vs. stages II/III. **(d)** Classification performance for healthy vs. stages I/II/III.

### **3.4 Discussion**

## **4 Lung microbiome**

### **4.1 Introduction**

## **4.2 Materials and methods**

### **4.3 Results**

#### **4.3.1 Discussion**

## **5 General conclusion and future perspective**

### **5.1 General conclusions**

In conclusion, the research described in this doctoral dissertation was conducted to identify significant

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In the Section 2, I show that

## **5.2 Plan for future**

### **5.3 Future perspective**

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My Lord, *the Flying Spaghetti Monster*,  
give us grace to accept with serenity the things that cannot be changed,  
courage to change the things that should be changed,  
and the wisdom to distinguish the one from the other.

Glory be to *the Meatball*, to *the Sauce*, and to *the Holy Noodle*.

As it was in the beginning, is now, and ever shall be.

R'Amen.

