

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

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 (Helminck, 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 human 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.

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List of Abbreviations

ACC Accuracy

ASV Amplicon sequence variant

AUC Area-under-curve

BA Balanced accuracy

C-section Cesarean section

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 Introduction

(Diversity indices)

(Classification evaluation)

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), characterized by the delivery of neonates prior to 37 weeks of gestation, is one of the major cause to neonatal mortality and morbidity (Blencowe et al., 2012). Multiple pregnancies including twins, short cervical length, and infection on genitourinary tract are known risk factor for PTB (Goldenberg, Culhane, Iams, & Romero, 2008). Nevertheless, the extent to which these aspects affect birth outcomes is still up for debate. Henceforth, strategies to boost gestation and enhance delivery outcomes can be more conveniently implemented when pregnant women at high risk of PTB are identified early (Iams & Berghella, 2010).

Prediction models that can be utilized as a foundation for intervention methods still have an unacceptable amount of classification evaluations, including accuracy, sensitivity, and specificity, despite a great awareness of the risk factors that trigger PTB (Sotiriadis, Papatheodorou, Kavvadias, & Makrydima, 2010). Several attempts have been made to predict PTB through integrating data such as human microbiome composition, inflammatory markers, and prior clinical data with predictive machine learning methods (Berghella, 2012). Because it is affordable and straightforward to use, fetal fibronectin is commonly used in medical applications. However, with a sensitivity of only 56% that merely similar to random prediction, it has a low classification evaluation (Honest et al., 2009). Due to the difficulty and imprecision of the method in general, as well as the requirement for a qualified specialist cervical length measuring is also restricted (Leitich & Kaider, 2003).

Preterm prelabor rupture of membranes (PROM) brought on by gestational inflammation and infection contribute to about 70% of PTB cases (Romero, Dey, & Fisher, 2014). Nevertheless, as antibiotics and anti-inflammatory therapeutic strategies were ineffective to decrease PTB occurrence rates, the mechanism of PTB cannot be completely explained by inflammatory and infectious pathways (Romero, Hassan, et al., 2014). Recent researches on maternal microbiomes were beginning to examine unidentified connections of PTB as a consequence of developmental processes in molecular biological technology (Fettweis et al., 2019).

However, as anti-inflammatory and antibiotic therapies were insufficient to lower PTB occurrence rates, infectious and inflammatory processes are insufficient to exhaustively clarify the pathogenesis and pathophysiology of PTB. It has been hypothesized that the microbiota linked to PTB originate from either a hematogenous pathway or the female genitourinary tract increasing through the vagina and/or cervix. (Han & Wang, 2013). Vaginal microbiome compositions have been found in women who eventually acquire PTB, and recent studies have tried to predict PTB risk using cervicovaginal fluid (Kindinger et

al., 2017). Even though previous investigation have confirmed the potential relationships between the vaginal microbiome compositions and PTB, these studies are only able to clarify an upward trajectory.

Multiple unfavorable birth outcomes, including PROM and PTB, have been linked to periodontitis as an independence risk factor, according to numerous epidemiological researches (Offenbacher et al., 1996). It is expected that the oral microbiome will be able to explain additional hematogenous pathways in light of these precedents; however, the oral microbiome composition of fetuses is limited understood.

Hence, in order to identify the salivary microbiome linked to PTB and to establish a machine learning prediction model of PTB determined by oral microbiome compositions, this study examined the salivary microbiome compositions of PTB study participants with a full-term birth (FTB) study participants.

2.2 Materials and methods

2.2.1 Study design and study participants

Between 2019 and 2021, singleton pregnant women who received treatment to Jeonbuk National University Hospital for childbirth were the participants of this study. This study was conducted according to the Declaration of Helsinki (Goodyear, Krleza-Jeric, & Lemmens, 2007). The Institutional Review Board authorized this study (IRB file No. 2019-01-024). Participants who were admitted for elective cesarean sections (C-sections) or induction births, as well as those who had written informed consent obtained with premature labor or PROM, were eligible.

2.2.2 Clinical data collection and grouping

Questionnaires and electronic medical records were implemented to gather information on both previous and current pregnancy outcomes. These clinical data included known risk factors, namely maternal age at delivery, diabetes mellitus (gestational or overt), hypertension (pregnancy-induced or chronic), overweight (pre-pregnancy or gestational weight gain), C-section, PROM, and history of PTB, along with demographic neonatal factors, such as gestational week on birth, weight, and sex.

2.2.3 Salivary microbiome sample collection

Salivary microbiome samples were collected 24 hours before to delivery using mouthwash. The standard methods of sterilizing were performed. Medical experts oversaw each stage of the sample collecting procedure. Participants received instruction not to eat, drink, or brush their teeth for 30 minutes before sampling salivary microbiome. Saliva samples were gathered by washing the mouth for 30 seconds with 12 mL of a mouthwash solution (E-zen Gargle, JN Pharm, Pyeongtaek, Korea). The samples were tagged with the anonymous ID for each participant and kept at 4 °C until they underwent further processing. Genomic DNA was extracted using an ExgeneTM Clinic SV kit (GeneAll Biotechnology, Seoul, Korea) following with the manufacturer instructions and store at -20 °C.

2.2.4 16s rRNA gene sequencing

Salivary microbiome samples were transported to Department of Biomedical Engineering of Ulsan National Institute of Science and Technology . 16S rRNA sequencing was then carried out using a commissioned Illumina MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA). Library methods were utilized to amplify the V3-V4 areas. 300 base-pair paired end reads were produced by sequencing the pooled library using a v3 600 cycle chemistry after the samples had been diluted to a final concentration of 6 pM with a 20% PhiX control.

2.2.5 Bioinformatics analysis

The independent *t*-test and χ^2 -square test were utilized to evaluate the differences between the PTB and FTB participants. Clinical measurement comparisons were conducted using SPSS (version 20.0) (Spss et

al., 2011). At $p < 0.05$, statistical significance was taken into consideration.

QIIME2 (version 2022.2) was implemented to import 16S rRNA gene sequences from salivary microbiome samples of study participants for additional bioinformatics processing (Bolyen et al., 2019). DADA2 was used to verify the qualities of raw sequences (Callahan et al., 2016). The remain sequences were clustered into amplicon sequence variants (ASVs). Diversity indices, namely Faith PD for alpha diversity index (Faith, 1992) and Hamming distance for beta diversity index (Hamming, 1950), were calculated. Mann–Whitney–Wilcoxon U -test and PERMANOVA multivariate test were evaluated for measuring statistical significance (Anderson, 2014; Kelly et al., 2015).

Taxonomic assignment were implemented with HOMD (version 15.22) (T. Chen et al., 2010). Afterward, DESeq2 was implemented to identify differentially abundant taxa (DAT) that could distinguish between salivary microbiome from PTB and FTB participants (Love, Huber, & Anders, 2014). Taxa with $|\log_2 \text{FoldChange}| > 1$ and $p < 0.05$ were considered as statistically significant.

The taxa for predicting PTB using salivary microbiome data were determined using a random forest classifier (Breiman, 2001). Through stratified k -fold cross-validation ($k = 5$) that preserves the existence rate of PTB and FTB participants, consistency and trust worthy classification were ensured (Wong & Yeh, 2019).

2.3 Results

2.3.1 Study participant demographics

In this study, a total of 69 volunteer mothers were initially recruited. However, due to incomplete clinical information or twin pregnancies, 10 participants were excluded from the study participants. Demographic and clinical information of the study participants are displayed in Table 1. Because PROM is one of the leading factors of PTB, it was prevalent in the PTB group than the FTB group. Other maternal clinical factors did not significantly differ between the FTB and PTB groups. In neither group were there any cases with a history of cigarette smoking or concomitant periodontal disease.

2.3.2 Comparison of salivary microbiomes composition

The salivary microbiome composition consisted of 13953804 sequences from 59 salivary samples, with 102305.95 ± 19095.60 and 64823.41 ± 15841.65 reads per sample before and after quality-check, respectively. Alpha diversity (Faith PD) and beta diversity (Hamming distance) indices did not significantly differ between the PTB and FTB groups (Figure 4).

DESeq2 was used to select 32 DAT that distinguish between the PTB and FTB groups out of the 465 species that were examined (Love et al., 2014): 26 FTB-enriched DAT and six PTB-enriched DAT. Seven PROM-related DAT were removed from these 32 PTB-related DAT to lessen the confounding effect of PROM (Figure 5). Therefore, there were a total of 25 PTB-related DAT: 22 FTB-enriched DAT and three PTB-enriched DAT (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 ± 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 ± 0.061 , 0.700 ± 0.194 , 0.728 ± 0.058 , and 0.743 ± 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: **Standard clinical characteristics of study participants.** Continuous variable are displayed as Mean±standard deviation. Categorical variable are displayed as count (proportion). Continuous variable for independent *t*-test. Categorical variable for Pearson's χ^2 -square test.

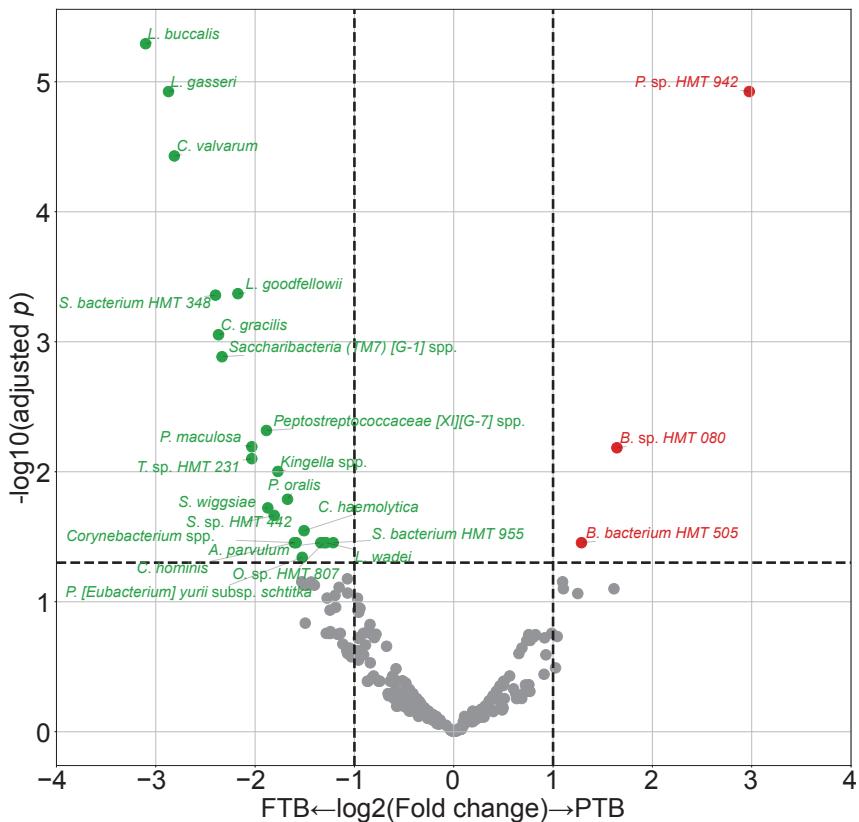


Figure 1: **DAT volcano plot.** PTB-enriched DAT shown as red dots and FTB-enriched DAT shown as green dots. Taxa with $|\log_2 \text{FoldChange}| > 1$ and adjusted- $p < 0.05$ are considered as significant different.

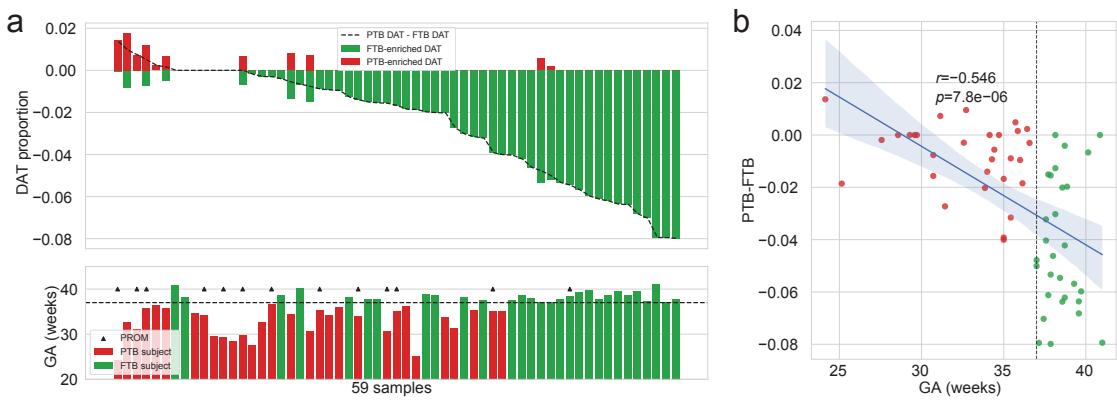


Figure 2: Oral microbiome compositions over DAT. **(a)** Proportions of DAT of study subjects. Samples are sorted by difference between PTB-enriched DAT proportion and FTB-enriched DAT proportion. GA of samples are shown, matched with order of upper panel. PTB: red bar, FTB: green bar. PROM: arrow head. **(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.

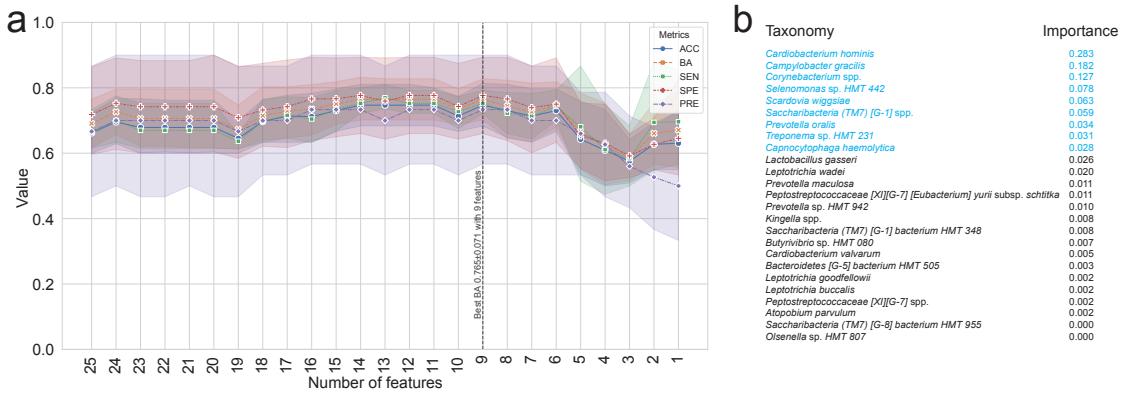


Figure 3: Machine learning evaluations over DAT. (a) Machine learning evaluation upon number of features (DAT). Random Forest classifier has the best balanced accuracy (mean \pm standard deviation.; 0.765 ± 0.071) with the nine most important DAT. **(b)** Importance of DAT is shown. Note that $0 \leq$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

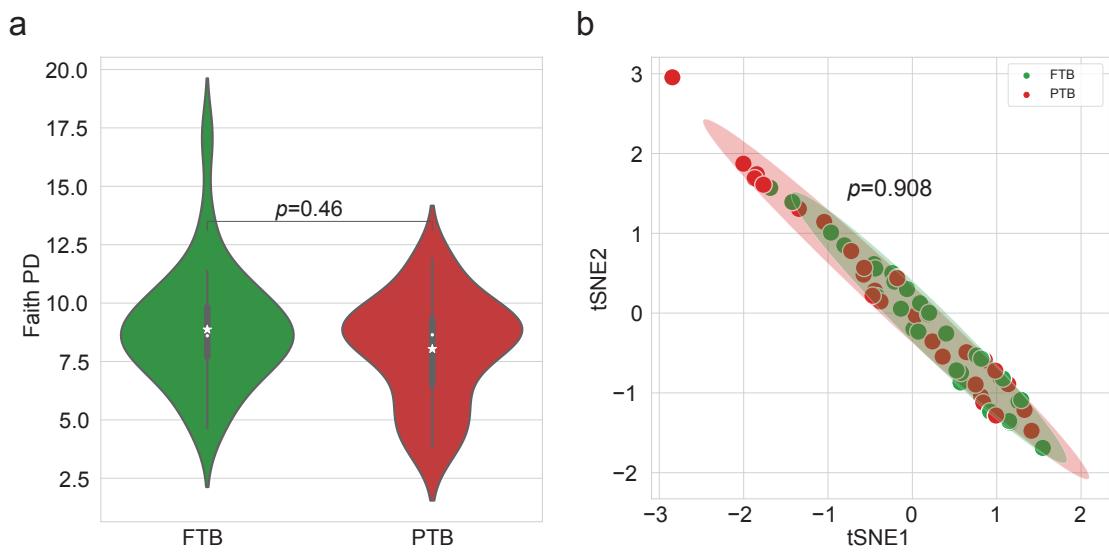


Figure 4: Diversity indices. **(a)** Alpha diversity index (Faith PD). Mann-Whitney-Wilcoxon test did not find statistically significant difference between the PTB group and the FTB group. Mean values are marked with star-point. **(b)** t-SNE plot with beta diversity index (Hamming distance). PERMANOVA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

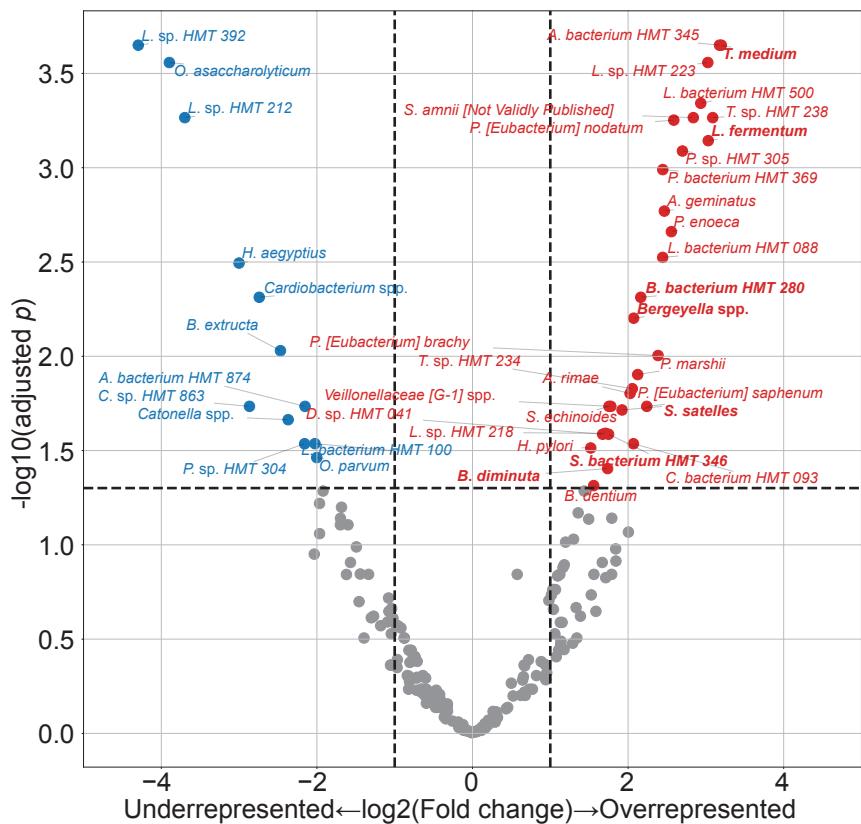


Figure 5: PROM-related DAT volcano plot. This is a subgroup analysis between 12 participants in PTB group with PROM and 18 participants in PTB group without PROM, 42 PROM-related DAT were selected between these two groups. Out of these 42 PROM-related DAT, only 7 DAT overlapped with PTB-related DAT, 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.

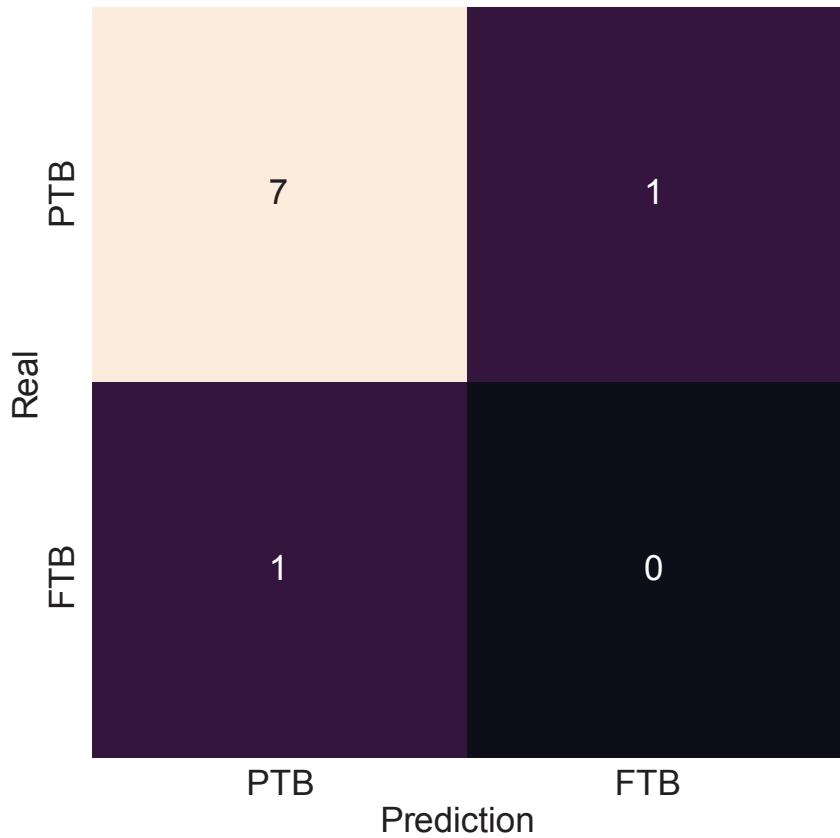


Figure 6: **Heatmap plot of PTB classification with validation data.** A validation test on 9 twin pregnancies that had excluded from the paper was conducted. They consist of 8 PTB subjects and 1 FTB subject. As twin pregnancies have a 7-10 times higher PTB rate than singleton pregnancies, resulting 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 ± 0.061)

2.4 Discussion

In this study, we developed a method for predicting PTB based on random forest classifier using salivary 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 hormonal 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.

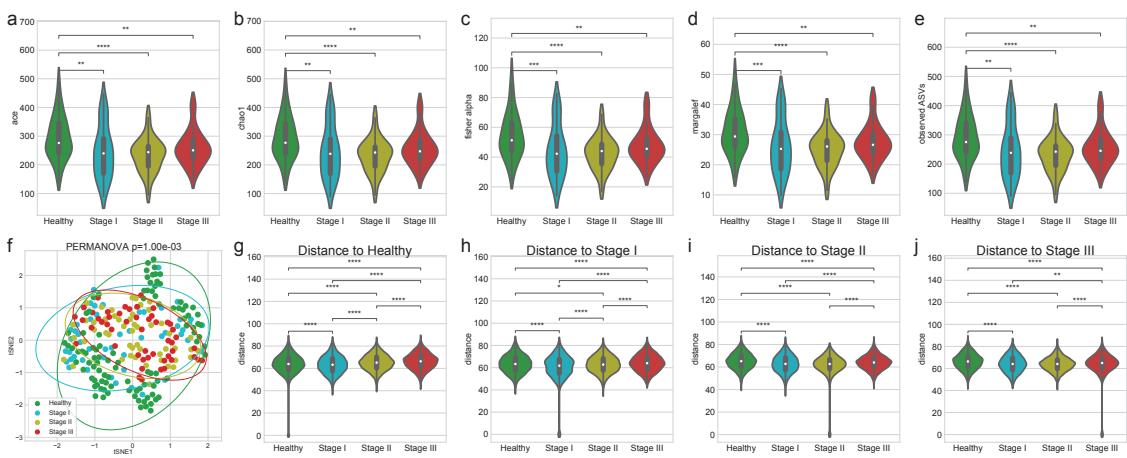


Figure 7: Diversity indices. Comparisons of salivary microbiomes among healthy controls and patients with various periodontitis stages. Alpha-diversity indices (**a-e**) indicate that healthy controls have increased heterogeneity than periodontitis stages as measured by: **(a)** ace **(b)** chao1 **(c)** 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 $p \leq 0.0001$ (****).

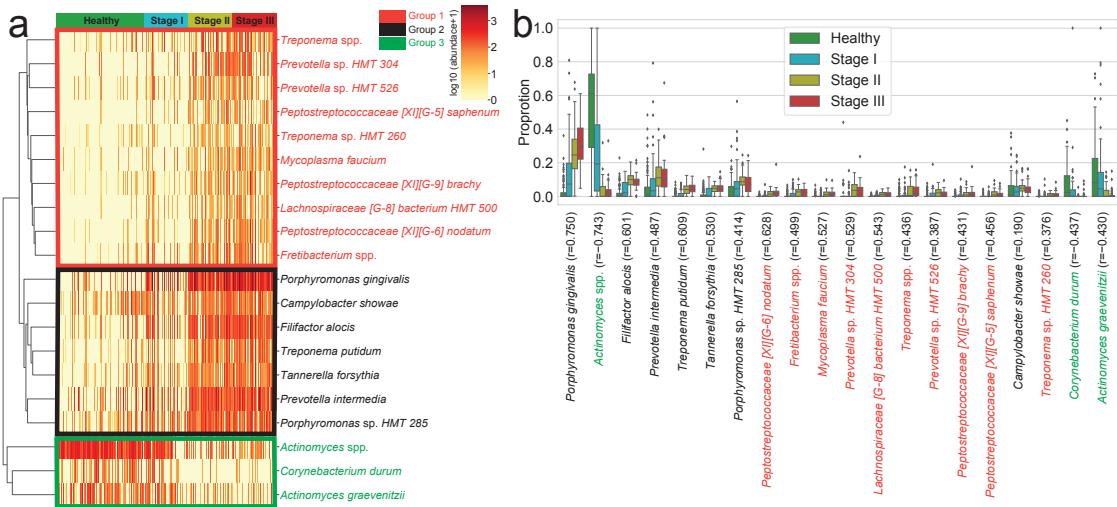


Figure 8: Differentially abundant taxa. Differentially abundant taxa (DAT) that were identified by ANCOM. **(a)** Heatmap of clustered DAT with similar distribution among subjects. Group 1, Group 2, and Group 3 are marked in red, black, and green, respectively. **(b)** Box plots showing the proportions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

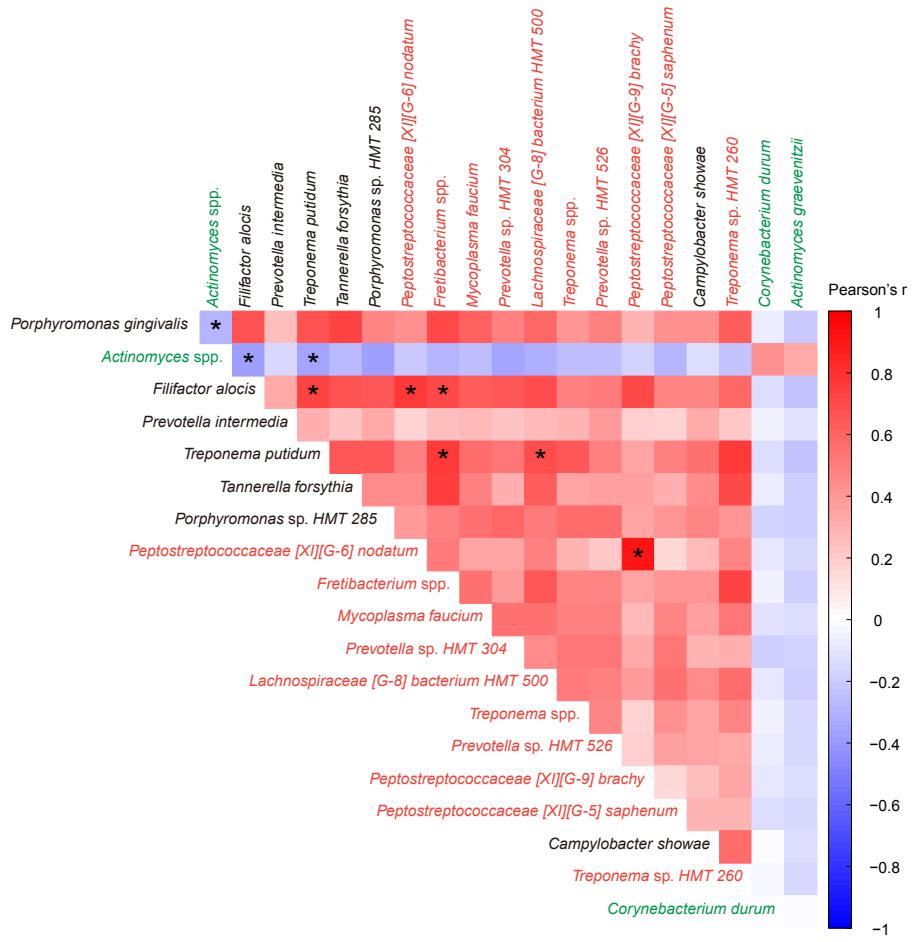


Figure 9: **Correlation heatmap.** Pearson's correlations between differentially abundant taxa in healthy status and periodontitis stages. Statistical significance was determined by strong correlation, i.e., $| \text{coefficient} | \geq 0.5$ (*).

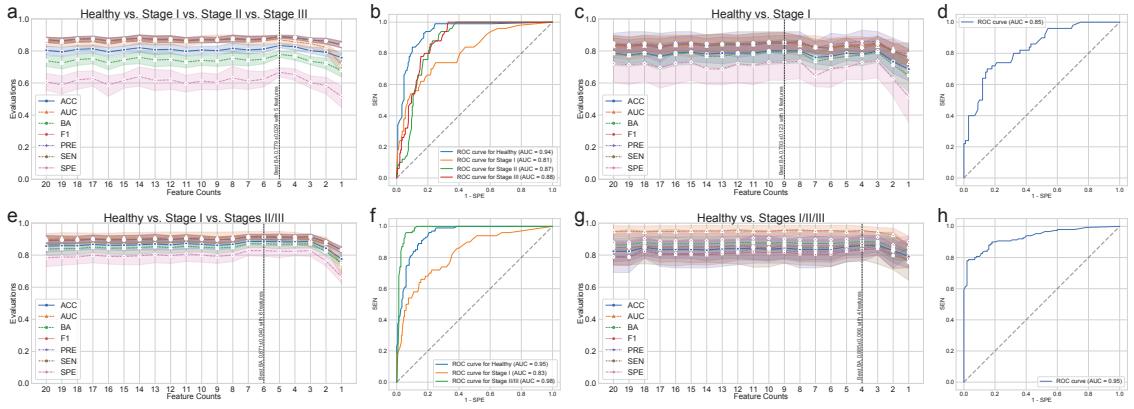


Figure 10: Random forest classification metrics. The classification metrics in the random forest classifications were as follows: accuracy (ACC), area-under-curve (AUC), balanced accuracy (BA), F1 score (F1), precision (PRE), sensitivity (SEN), and specificity (SPE). Every classification metric ranges 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).

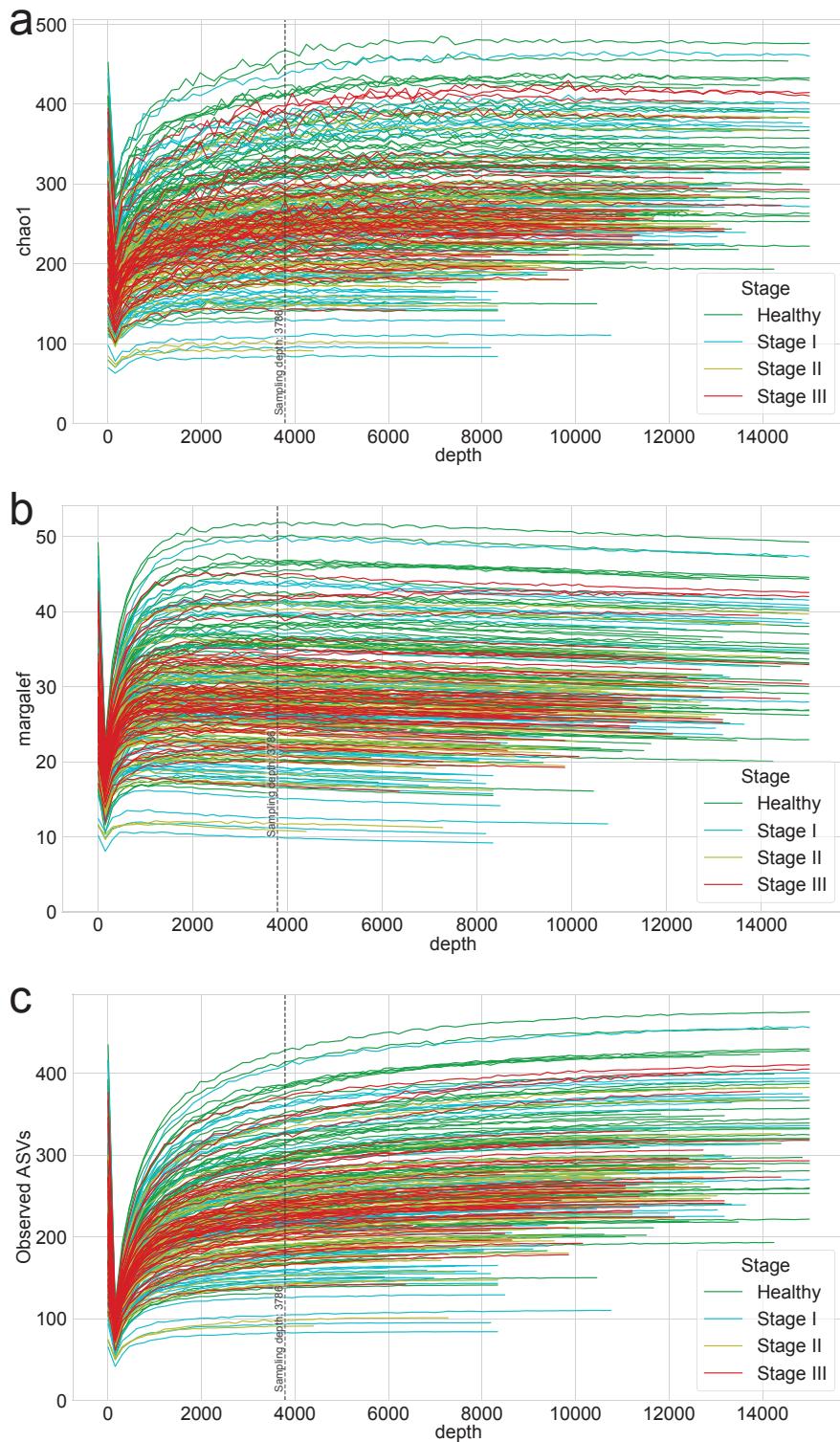


Figure 11: Rarefaction curves for alpha diversity indices. Rarefaction of (a) chao1 (b) margalef, and (c) observed ASVs were generated to measure species richness and determine the sampling depth of each sample.

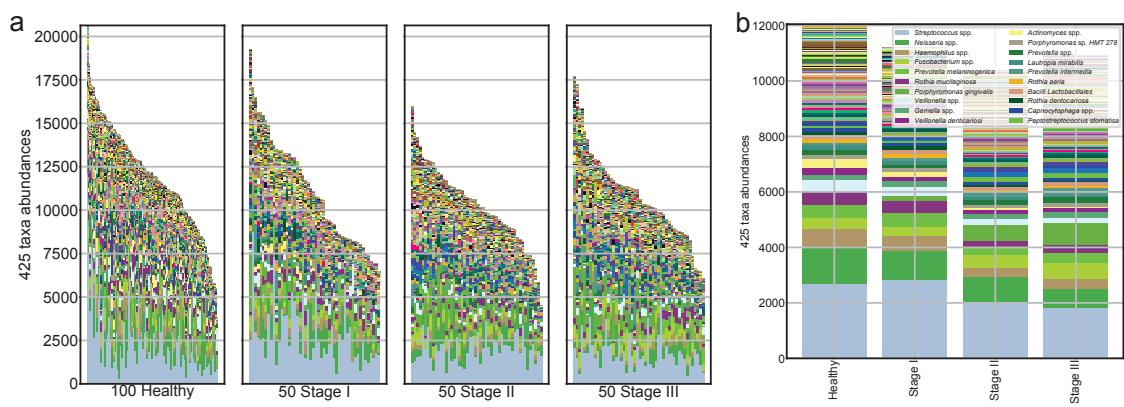


Figure 12: Absolute abundance of salivary bacterial taxa in the different periodontal statuses at the species level. Stacked bar plot of the absolute abundance of bacterial species for all samples (**a**) and the mean absolute abundance of bacterial species in the healthy, stage I, stage II, and stage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

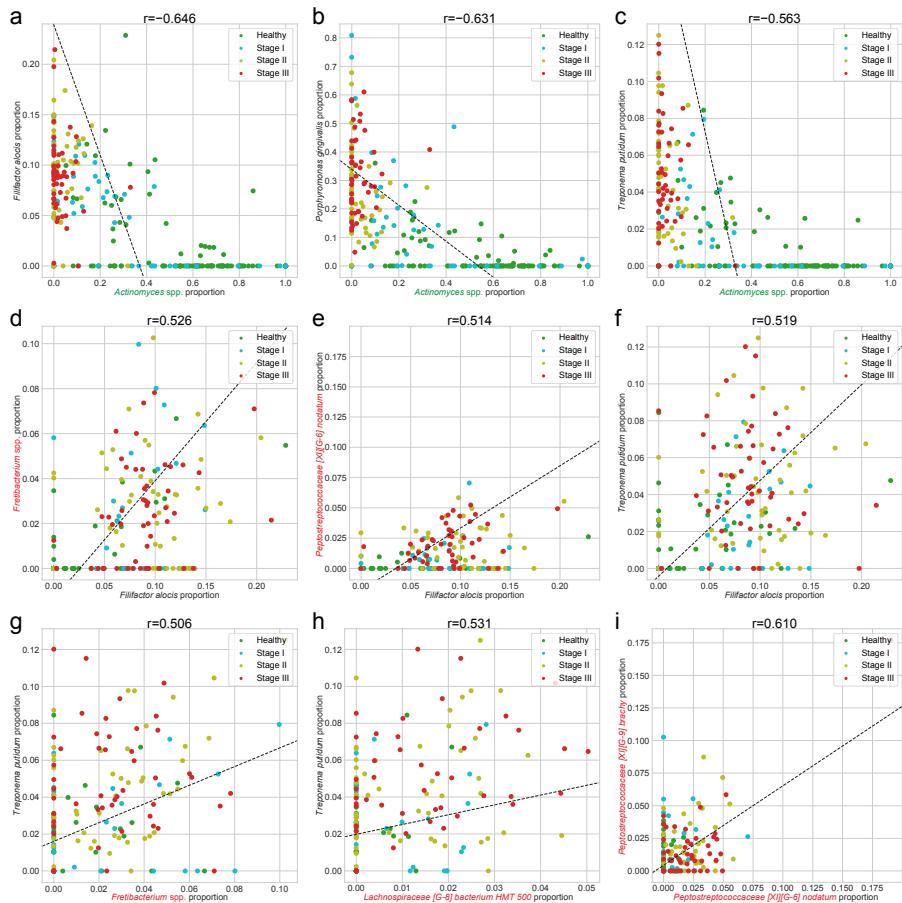


Figure 13: Correlation plots for differentially abundant taxa. We selected the combinations of DAT with absolute Spearman correlation coefficients greater than 0.5. The color represents periodontal healthy periodontal statuses (green: healthy, cyan: stage I, yellow: stage II, and red: stage III).

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 ...

In the Section 2, I show that

5.2 Plan for future

5.3 Future perspective

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