

Doctoral Thesis

**Microbiota in Human Diseases**

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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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Bobby Henderson

## **Abstract**

(Microbiome)

(PTB) Section 2 introduces...

(Periodontitis) Section 3 describes...

(Lung)

(Conclusion)

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**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

**SD** Standard deviation

**SEN** Sensitivity

**SPE** Specificity

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

# 1 Introduction

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; C. H. 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 interacts 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 human microbiomes is essential for developing new therapeutic approaches that target these microbial populations to promote health and prevent diseases.

(Diversity indices)

(Classification evaluation)

## 2 Predicting preterm birth using random forest classifier in salivary 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 pathology of PTB has not been entirely elucidated 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 cervico-vaginal 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, hypertension, overweight, 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-zén Gargle, JN Pharm, Pyeongtaek, Gyeonggi, 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 the Department of Biomedical Engineering of the 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 was utilized to evaluate the differences of continuous values between from the PTB participants than the FTB participants;  $\chi^2$ -square test was applied to decide statistical differences of

categorical values. 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 (Mann & Whitney, 1947), 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 Overview of clinical information

In the beginning, 69 volunteer mothers were recruited for this study. However, due to insufficient 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. There were no cases in both groups that had a history of simultaneous periodontal disease or cigarette smoking.

### 2.3.2 Comparison of salivary microbiomes composition

The salivary microbiome composition was composed of 13953804 sequences from 59 study participants, with  $102305.95 \pm 19095.60$  and  $64823.41 \pm 15841.65$  (mean $\pm$ SD) reads/sample before and following the quality-check stage, accordingly. There was not a significant distinction between the PTB and FTB groups with regard to on alpha diversity nor beta diversity metrics (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).

A significant negative correlation was found using Pearson correlation analysis between GW and differences between PTB-enriched DAT and FTB-enriched DAT ( $r = -0.542$  and  $p = 7.8e - 6$ ; Figure 5).

### 2.3.3 Random forest classification to predict PTB risk

To classify PTB according to DAT, random forest classifiers were constructed. The nine most significant DAT were used to obtain the best BA ( $0.765 \pm 0.071$ ; Figure 3a). Moreover, random forest classification model determined each DAT's importance (Figure 3b). We conducted a validation procedure on nine twin pregnancies that were excluded in the initial study design in order to confirm the reliability and dependability of our random forest-based PTB prediction model (Figure 6). Comparable to the PTB prediction model on the 59 initial singleton study participants, the validation classification on PTB risk of these twin participants have an accuracy of 87.5%.

Table 1: **Standard clinical information of study participants.**

Continuous variable for independent *t*-test. Categorical variable for Pearson's  $\chi^2$ -square test. Continuous variable: mean $\pm$ SD. Categorical variable: count (proportion)

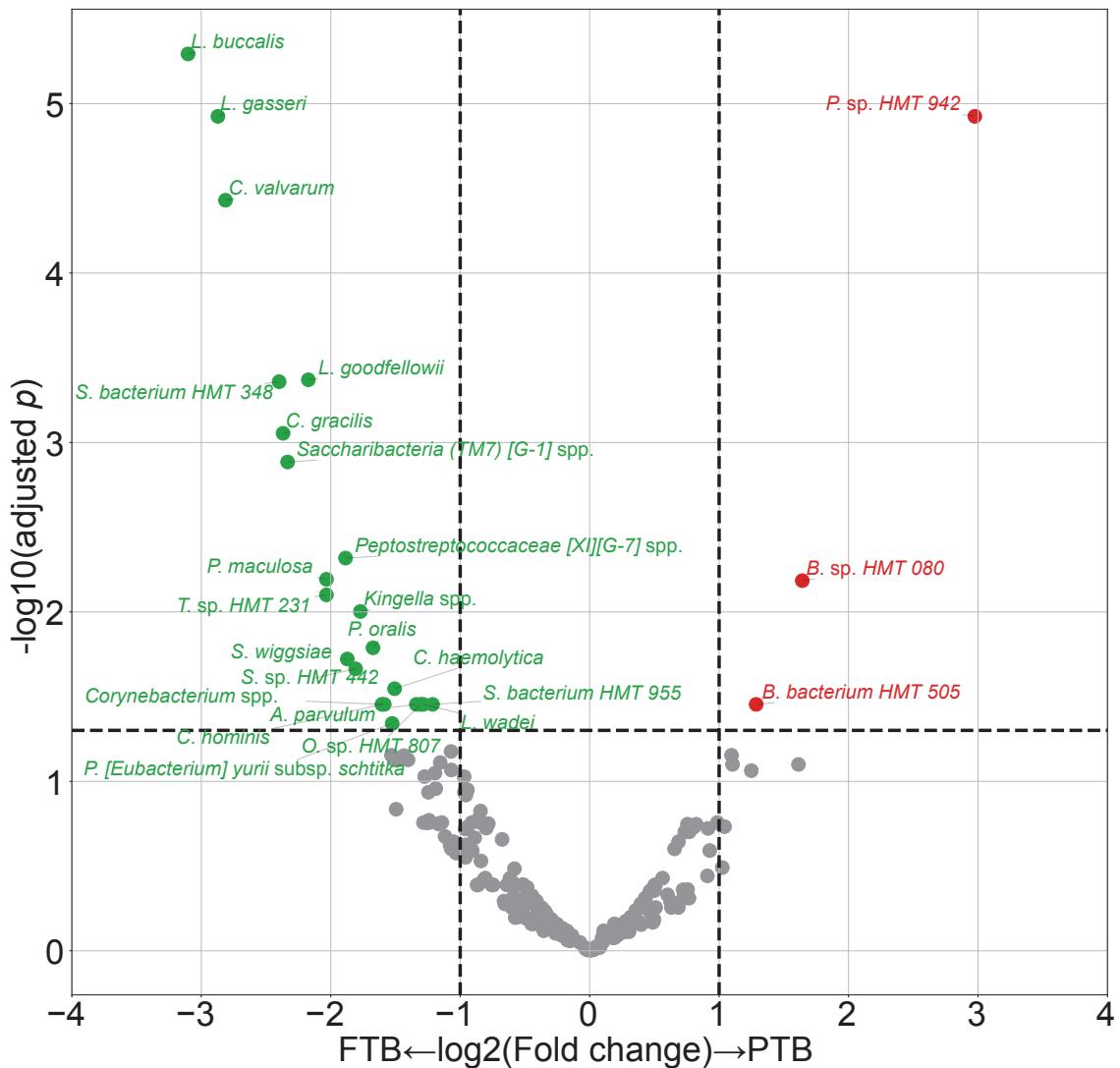


Figure 1: DAT volcano plot.

Red dots represent PTB-enriched DAT, while green dots represent FTB-enriched DAT.

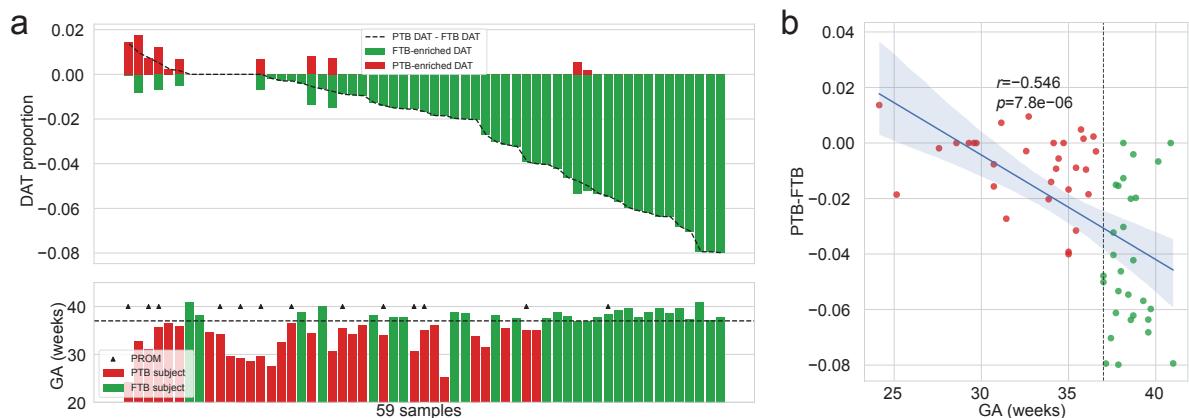


Figure 2: **Salivary microbiome compositions over DAT.**

**(a)** Frequencies of DAT of study subjects. The study participants are arranged in respect of (PTB-enriched DAT – FTB-enriched DAT). The study participants' GA is displayed in accordance with the upper panel's order (PTB: red bar, FTB: green bar. PROM: arrow head.) **(b)** Correlation plot with GA and (PTB-enriched DAT – FTB-enriched DAT). Strong negative correlation is found with Pearson correlation.

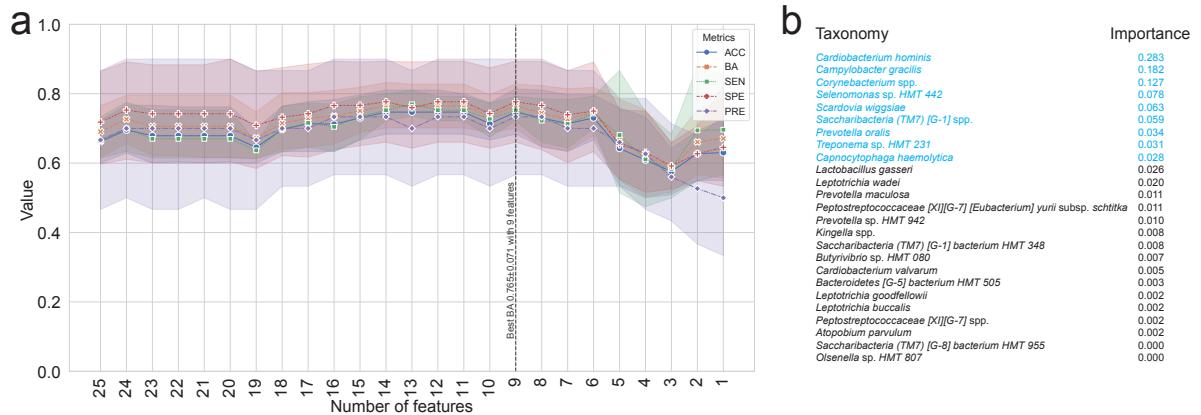


Figure 3: **Random forest-based PTB prediction model.**

**(a)** Machine learning evaluations upon number of features (DAT). Random Forest classifier has the best BA ( $0.765 \pm 0.071$ ; Mean $\pm$ SD) with the nine most important DAT. **(b)** Importance of DAT.

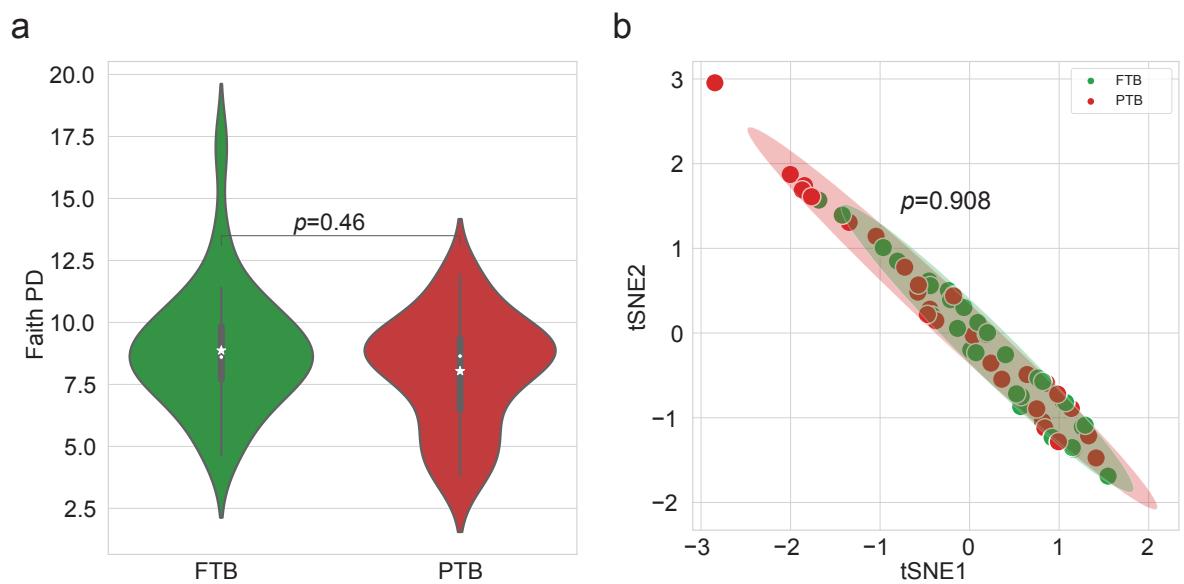


Figure 4: **Diversity indices.**

**(a)** Alpha diversity index (Faith PD). There is no statistically significant difference between the PTB and FTB group (MWU test  $p = 0.46$ ). **(b)** t-SNE plot with beta diversity index (Hamming distance). There is no statistically significant difference between the PTB and FTB group (PERMANOVA test  $p = 0.908$ )

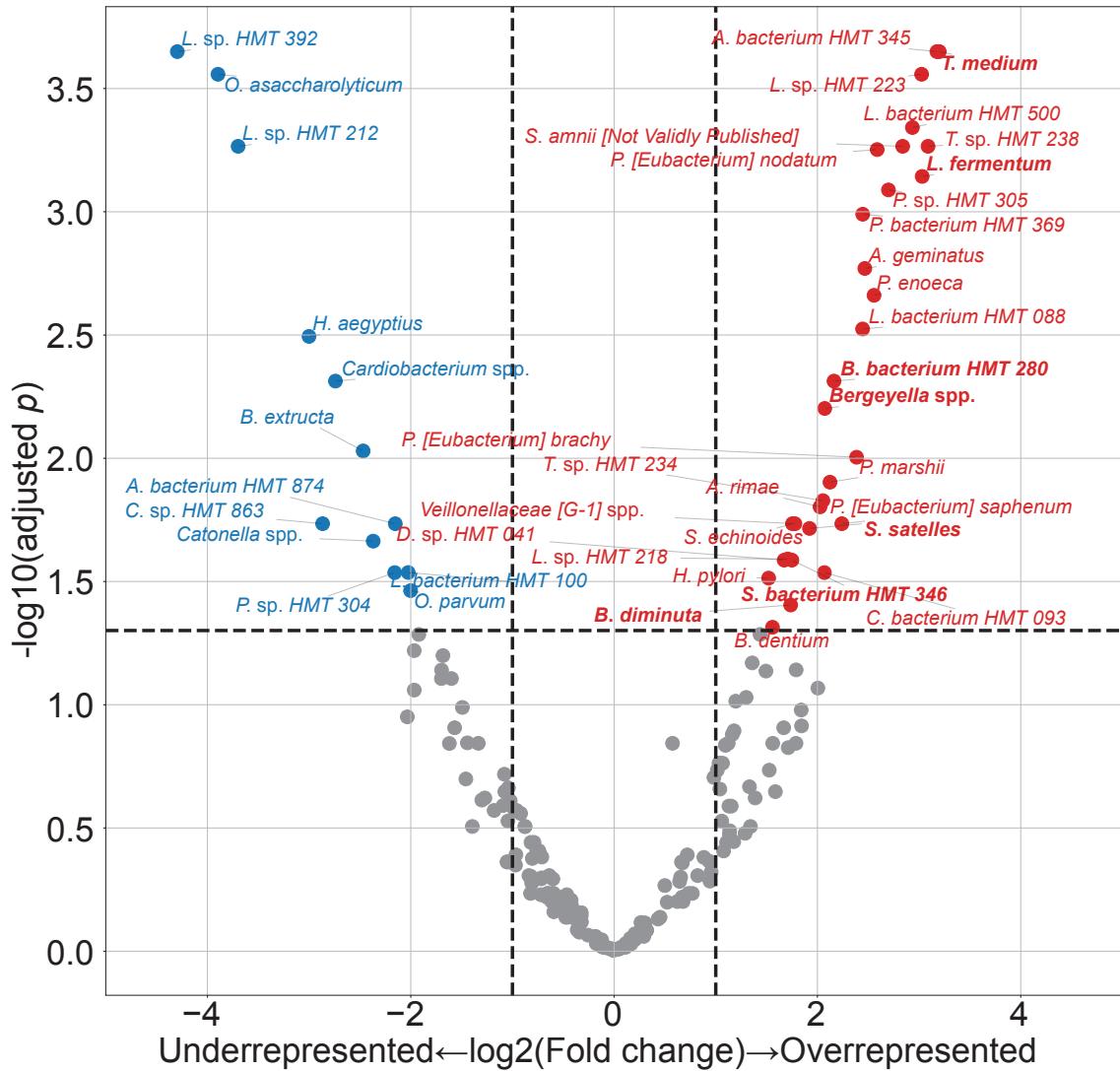
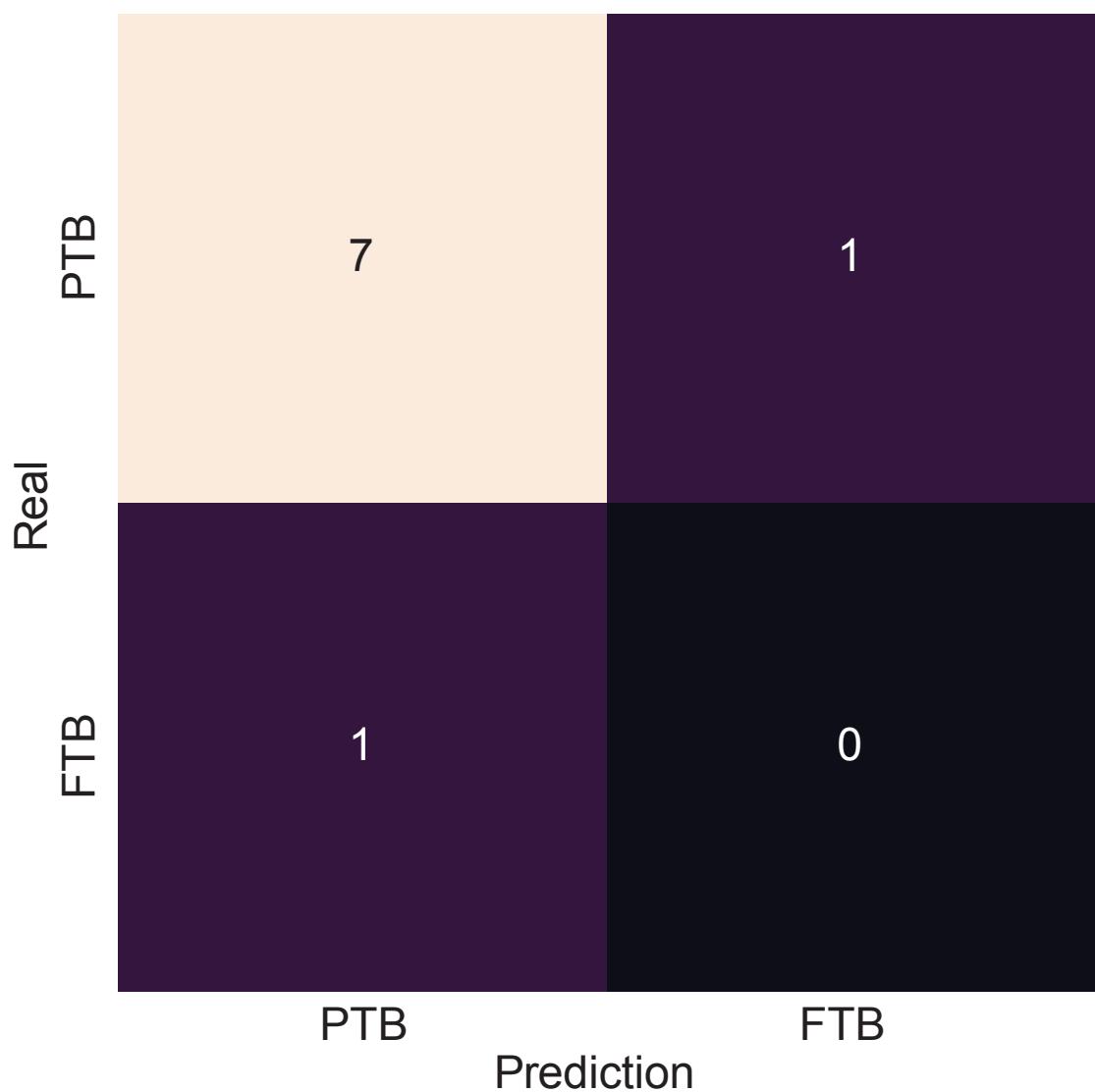


Figure 5: PROM-related DAT.

Only seven of these 42 PROM-related DAT overlapped with PTB-related DAT (bold text). Blue dots represented PROM-underrepresented DAT, while red dots represented PROM-overrepresented DAT.



**Figure 6: Validation of random forest-based PTB prediction model.**

Nine twin pregnancies (eight PTB subjects and a FTB subject) that were excluded in the initial study subjects were subjected to a validation procedure. The random forest-based PTB prediction model shows 87.5% accuracy, comparable to the PTB classification evaluations on the singleton study subjects ( $0.714 \pm 0.061$ . Mean  $\pm$  SD)

## 2.4 Discussion

In this study, we employed salivary microbiome compositions to develop the random forest-based PTB prediction models to estimate PTB risks. Previous reports have indicated bidirectional associations between pregnancy outcomes and salivary microbiome compositions (Han & Wang, 2013). Nevertheless, the salivary microbiome composition is not yet elucidated. Salivary microbial dysbiosis, including gingival inflammation and periodontitis, have been connected to unfavorable pregnancy outcomes, such as PTB (Ide & Papapanou, 2013). However, the techniques utilized in recent research that primarily focus on recognized infections have led to inconsistent outcomes.

One of the most common salivary taxa that has been examined is *Fusobacterium nucleatum* (Han, 2015; Brennan & Garrett, 2019; Bolstad, Jensen, & Bakken, 1996), that is a Gram-negative, anaerobic, and filamentous bacteria. *Fusobacterium nucleatum* can be separated from not only the salivary microbiome but also the vaginal microbiome (Vander Haar, So, Gyamfi-Bannerman, & Han, 2018; Witkin, 2019). In both animal and human investigation, *Fusobacterium nucleatum* infection has been linked to risk of PTB (Doyle et al., 2014). According to recent researches, the placenta women who give birth prematurely may include additional salivary microbiome dysbiosis, such as *Bergeyella* spp. and *Porphyromonas gingivalis* (León et al., 2007; Katz, Chegini, Shiverick, & Lamont, 2009). Although *Bergeyella* spp. were one of the PROM-overrepresented DAT (Figure 5), it was excluded in the final 25 PTB-related DAT. Furthermore, *Porphyromonas gingivalis* and *Campylobacter gracilis* were pathogens of periodontitis in sub-gingival microbiome (Yang et al., 2022). *Lactobacillus gasseri* was also one of the FTB-enriched DAT (Figure 1), and it is well established that early PTB risk can be reduced by *Lactobacillus gasseri* in the vaginal microbiome (Basavaprabhu, Sonu, & Prabha, 2020; Payne et al., 2021).

With DAT comprising 22 FTB-enriched DAT and three PTB-enriched DAT (Figure 1), we discovered that the FTB study participants had the majority of the essential DAT that distinguished between the PTB and FTB groups. Thus, we hypothesize that the pathogenesis and pathophysiology of PTB may have been triggered by an absence of species with protective characteristics. The association between unfavorable pregnancy outcomes and a dysfunctional microbiome has been explained through two distinct processes. According to the first hypothesis, periodontal pathogens originating in the gingival biofilm might spread from the infected salivary microbiome over the placenta microbiome, invade the intra-amniotic fluid and fetal circulation, and then have a direct impact on the fetoplacental unit, leading to bacteremia (Hajishengallis, 2015). Based on the second hypothesis, inflammatory mediators and endotoxins that generated by the sub-gingival inflammation and derived from dental plaque of periodontitis may spread throughout the body and reach the fetoplacental unit (Stout et al., 2013; Aagaard et al., 2014). Despite belonging to the same species, some subgroups of the salivary microbiome may influence pregnancy outcomes in both favorable and adverse manners. Following this line of argumentation, the salivary microbiome composition or their dysbiosis are more significant than the existence of particular bacteria.

Notably, microbial alteration that take place throughout pregnancy may be expected results of a healthy pregnancy. Those pregnancy-related vulnerabilities to dental problem like periodontitis can be explained by three factors. Because of hormone-driven gingival hyper-reactivity to the salivary microbiome in the

oral biofilm including sub-gingival biofilm, these conditions are prevalent in pregnant women. For insight at the relationship between the salivary microbiome composition and PTB, further studies with pathway analysis are warranted.

Our study confirmed that salivary microbiome composition could provide potential biomarkers for predicting pregnancy complications including PTB risks using random forest-based classification models, despite a limited number of study participants and a tiny validation sample size. Another limitation of our study was 16S rRNA sequencing. In other words, unlike the shotgun sequencing, 16S rRNA sequencing only focused on bacteria, not viruses nor fungi. We did not delve into other variables like nutrition status and socioeconomic statuses of study participants that might affect the salivary microbiome composition.

Notwithstanding these limitations, this prospective examination showed the promise of the random forest-based PTB prediction models based on mouthwash-derived salivary microbiome composition. Before applying the methods developed in this study in a clinical context, more multi-center and extensive research is warranted to validate our findings.

### **3 Random forest prediction model for periodontitis statuses based on the salivary microbiomes**

**This section includes the published contents:**

#### **3.1 Introduction**

Saliva microbial dysbiosis brought on by the accumulation of plaque results in periodontitis, a chronic inflammatory disease of the tissue that surrounds the tooth (Kinane, Stathopoulou, & Papapanou, 2017). Loss of periodontal attachment is a consequence of periodontitis, which may lead to irreversible bone loss and, eventually, permanent tooth loss if left untreated. A new classification criterion of periodontal diseases was created in 2018, about 20 years after the 1999 statements of the previous one. Even with this evolution, radiographic and clinical markers of periodontitis progression remain the primary methods for diagnosing periodontitis (Papapanou et al., 2018). Such tools, nevertheless, frequently demonstrate the prior damage from periodontitis rather than its present condition. Certain individuals have a higher risk of periodontitis, a higher chance of developing severe generalized periodontitis, and a worse response to common salivary bacteria control techniques utilized to prevent and treat periodontitis. As a result, the 2017 framework for diagnosing periodontitis additionally allows for the potential development of biomarkers to enhance diagnosis and treatment of periodontitis (Tonetti, Greenwell, & Kornman, 2018). Instead of only depending on the progression of periodontitis, a new etiological indication based on the current state must be introduced in order to enable appropriate intervention through early detection of periodontitis. Thus, the current clinical diagnostic techniques that rely on periodontal probing can be uncomfortable for patients with periodontitis (Canakci & Canakci, 2007).

Due to the development of salivaomics, in this manner, the examination of saliva has emerged as a significant alternative to the conventional ways of identifying periodontitis (Altingöz et al., 2021; Melguizo-Rodríguez, Costela-Ruiz, Manzano-Moreno, Ruiz, & Illescas-Montes, 2020). Given that saliva sampling is non-invasive, painless, and accessible to non-specialists, it may be a valuable instrument for diagnosing periodontitis (Zhang et al., 2016). Furthermore, much research has suggested that periodontitis could be a trigger in the development and exacerbation of metabolic syndrome (Morita et al., 2010; Nesbitt et al., 2010). Consequently, alteration in these levels of salivary microbiome markers may serve as high effective diagnostic, prognostic, and therapeutic indicators for periodontitis and other systemic diseases (Miller, Ding, Dawson III, & Ebersole, 2021; Čižmárová et al., 2022). The pathogenesis of periodontitis typically comprises qualitative as well as quantitative alterations in the salivary microbial community, despite that it is a complex disease impacted by a number of contributing factors including age, smoking status, stress, and nourishment (Abusleme, Hoare, Hong, & Diaz, 2021; Lafaurie et al., 2022). Depending on the severity of periodontitis, the salivary microbial community's diversity and characteristics vary (Abusleme et al., 2021), indicating that a new etiological diagnostic standards might be microbial community profiling based on clinical diagnostic criteria. As a consequence, salivary microbiome

compositions have been characterized in numerous research in connection with periodontitis. High-throughput sequencing, including 16S rRNA gene sequencing, has recently used in multiple studies to identify variations in the bacterial composition of sub-gingival plaque collections from periodontal healthy individuals and patients with periodontitis (Altabtbaei et al., 2021; Iniesta et al., 2023; Nemoto et al., 2021). This realization has rendered clear that alterations in the salivary microbial community—especially, shifts to dysbiosis—are significant contributors to the pathogenesis and development of periodontitis (Lamont, Koo, & Hajishengallis, 2018). Yet most of these research either focused only on the microbiome alterations in sub-gingival plaque collection, comprised a limited number of periodontitis study participants, or did not account for the impact of multiple severities of periodontitis.

For the objective of diagnosing periodontitis, previous research has developed machine learning-based prediction models based on oral microbiome compositions, such as the sub-gingival microbial dysbiosis index (T. Chen, Marsh, & Al-Hebshi, 2022; Chew, Tan, Chen, Al-Hebshi, & Goh, 2024), which have demonstrated good diagnostic evaluation and could be applied to individual saliva collection. Despite offering valuable details, these indicators are frequently restricted by their limited emphasis on classifying the multiple severities of periodontitis. Furthermore, many of these machine learning models currently in practice are trained solely upon the existence of periodontitis rather than on the multiple severities of periodontitis.

Recently, we employed multiplex quantitative-PCR and machine learning-based classification model to predict the severity of periodontitis based on the amount of nine pathogens of periodontitis from saliva collections (E.-H. Kim et al., 2020). On the other hand, the fact that we focused merely at nine pathogens for periodontitis and neglected the variety bacterial species associated to the various severities of periodontitis constrained the breadth of our investigation. By developing a machine learning model that could classify multiple severities of periodontitis based on the salivary microbiome composition, this study aims to fill these knowledge gaps and produce more accurate and therapeutically useful guidance to evaluate progression of periodontitis. Hence, in order to examine the salivary microbiome composition of both healthy controls and patient with periodontitis, we applied 16S rRNA gene sequencing. Furthermore, employing the 2018 classification criteria, we sought to find biomarkers (species) for the precise prediction of periodontitis severities (Papapanou et al., 2018; Chapple et al., 2018).

## **3.2 Materials and methods**

### **3.2.1 Study participants enrollment**

Between 2018-08 and 2019-03, 250 study participants—100 healthy controls, 50 patients with stage I periodontitis, 50 patients with stage II periodontitis, and 50 patients with stage III periodontitis—visited the Department of Periodontics at Pusan National University Dental Hospital. The Institutional Review Board of the Pusan National University Dental Hospital accepted this study protocol and design (IRB No. PNUDH-2016-019). Every study participants provided their written informed authorization after being fully informed about this study's objectives and methodologies. Exclusion criteria for the study participants are followings:

1. People who, throughout the previous six months, underwent periodontal therapy, including root planing and scaling.
2. People who struggle with systemic conditions that may affect periodontitis developments, such as diabetes.
3. People who, throughout the previous three months, were prescribed anti-inflammatory medications or antibiotics.
4. Women who were pregnant or breastfeeding.
5. People who have persistent mucosal lesions, e.g. pemphigus or pemphigoid, or acute infection, e.g. herpetic gingivostomatitis.
6. Patient with grade C periodontitis or localized periodontitis (< 30% of teeth involved).

### **3.2.2 Periodontal clinical parameter diagnosis**

A skilled periodontist conducted each clinical procedure. Six sites per tooth were used to quantify gingival recession and probing depth: mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual, and distolingual (Huang et al., 2007). A periodontal probe (Hu-Friedy, IL, USA) was placed parallel to the major axis of the tooth at each tooth location in order to gather measurements. The cementoenamel junction of the tooth was analyzed to determine the clinical attachment level, and the deepest point of probing was taken to determine the periodontal pocket depth from the marginal gingival level of the tooth. Plaque index was measured by probing four surfaces per tooth: mesial, distal, buccal, and palatal or lingual. Plaque index was scored by the following criteria:

0. No plaque present.
1. A thin layer of plaque that adheres to the surrounding tissue of the tooth and free gingival margin.  
Only through the use of a periodontal probe on the tooth surface can the plaque be existed.
2. Significant development of soft deposits that are visible within the gingival pocket, which is a region between the tooth and gingival margin.

3. Considerable amount of soft matter on the tooth, the gingival margin, and the gingival pocket.

The arithmetic average of the plaque indices collected from every tooth was determined to calculate plaque index of each study participant. By probing four surfaces per tooth, mesial, distal, buccal, and palatal or lingual, to assess gingival bleeding, the gingival index was scored by the following criteria:

0. Normal gingiva: without inflammation nor discoloration.
1. Mild inflammation: minimal edema and slight color changes, but no bleeding on probing.
2. Moderate inflammation: edema, glazing, redness, and bleeding on probing.
3. Severe inflammation: significant edema, ulceration, redness, and spontaneous bleeding.

The arithmetic average of the gingival indices collected from every tooth was determined to calculate gingival index of each study participant. The relevant data was not displayed, despite that furcation involvement and bleeding on probing were thoroughly utilized into account during the diagnosis process.

Periodontitis was diagnosed in respect to the 2018 classification criteria (Papapanou et al., 2018; Chapple et al., 2018). An experienced periodontist diagnosed the periodontitis severity by considering complexity, depending on clinical examinations including radiographic images and periodontal probing. Periodontitis is categorized into Healthy, stage I, stage II, and stage III with the following criteria:

- Healthy:
  1. Bleeding sites < 10%
  2. Probing depth:  $\leq$  3 mm
- Stage I:
  1. No tooth loss because of periodontitis.
  2. Inter-dental clinical attachment level at the site of the greatest loss: 1-2 mm
  3. Radiographic bone loss: < 15%
- Stage II:
  1. No tooth loss because of periodontitis.
  2. Inter-dental clinical attachment level at the site of the greatest loss: 3-4 mm
  3. Radiographic bone loss: 15-33%
- Stage III:
  1. Teeth loss because of periodontitis:  $\leq$  teeth
  2. Inter-dental clinical attachment level at the site of the greatest loss:  $\geq$  5 mm
  3. Radiographic bone loss: > 33%

### **3.2.3 Saliva sampling and DNA extraction procedure**

All study participants received instructions to avoid eating, drinking, brushing, and using mouthwash for at least an hour prior to the saliva sample collection process. These collections were conducted between 09:00 and 11:00. Mouth rinse was collected by rinsing the mouth for 30 seconds with 12 mL of a solution (E-zen Gargle, JN Pharm, Korea). All saliva samples were tagged with anonymous ID and stored at -4 °C.

Bacteria DNA was extracted from saliva samples using an Exgene™Clinic SV DNA extraction kit (GeneAll, Seoul, Korea), and quality and quantity of bacterial DNA was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Hyper-variable regions (V3-V4) of the 16S rRNA gene were amplified using the following primer:

- Forward: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNNGCWGCAG-3'
- Reverse: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'

The standard protocols of the Illumina 16S Metagenomic Sequencing Library Preparation were followed in the preparation of the libraries. The PCR conditions were as follows:

1. Heat activation at 95 °C for 30 seconds.
2. 25 cycles of 95 °C for 30 seconds.
3. 55 °C for 30 seconds.
4. 72 °C for 30 seconds.

NexteraXT Indexed Primer was applied to amplification 10 μL of the purified initial PCR products for the final library creation. The second PCR used the same conditions as the first PCR conditions but with 10 cycles. 16S rRNA gene sequencing was performed via 2×300 bp paired-end sequencing at Macrogen Inc. (Macrogen, Seoul, Korea) using Illumina MiSeq platform (Illumina, San Diego, CA, USA).

### **3.2.4 Bioinformatics analysis**

We computed alpha-diversity and beta-diversity indices to quantify the divergence of phylogenetic information. Following alpha-diversity indices were calculated using the scikit-bio Python package (version 0.5.5) (Rideout et al., 2018): Abundance-based Coverage Estimator (ACE) (Chao & Lee, 1992), Chao1 (Chao, 1984), Fisher (Fisher, Corbet, & Williams, 1943), Margalef (Magurran, 2021), and Observed ASVs (DeSantis et al., 2006). These alpha-diversity indices were compared using the MWU test.

Aitchison index for a beta-diversity index was calculated using QIIME2 (version 2020.8) (Aitchison, Barceló-Vidal, Martín-Fernández, & Pawlowsky-Glahn, 2000; Bolyen et al., 2019). We employed the t-SNE algorithm to illustrate multi-dimensional data from the beta-diversity index computation (Van der Maaten & Hinton, 2008). The beta-diversity index was compared using PERMANOVA test (Anderson, 2014; Kelly et al., 2015) and MWU test.

DAT between multiple periodontitis stages were identified by ANCOM (Lin & Peddada, 2020). The log-transformed absolute abundances of DAT were analyzed by hierarchical clustering in order to identify sub-groups with similar abundance patterns on periodontitis severities. Additionally, we examined the relative proportions among the 20 DAT in order to reduce the effect of salivary bacteria that differ insignificantly across the multiple severities of periodontitis.

### **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.

**Table 3: Feature combinations and their evaluations**

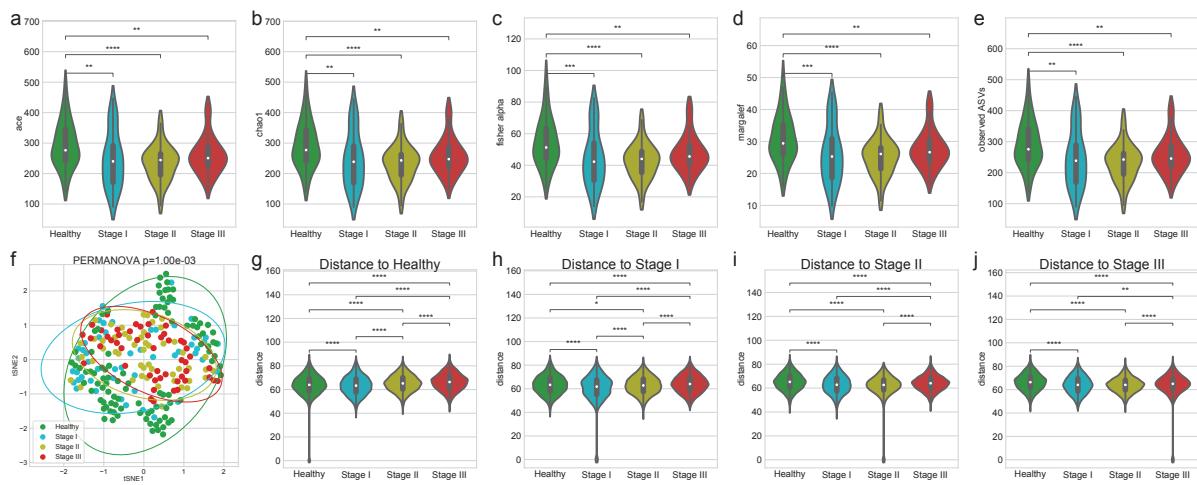
Classification performance with the most important taxon, the two most important taxa, and taxa with the best-balanced accuracy. *P.gingivalis* and *Act.* are *Porphyromonas gingivalis* and *Actinomyces* spp., respectively.

**Table 4: List of DAT among healthy status and periodontitis stages**

**Table 5: Feature the importance of taxa in the classification of different periodontal statuses**  
Taxa are ranked in descending order of importance; from most important to least important.

**Table 6: Beta-diversity pairwise comparisons on the periodontitis statuses**

Statistically significant (p-value) was determined by the PERMANOVA test.



**Figure 7: Diversity indices.**

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 MWU test and the PERMANOVA test:  $p \leq 0.01$  (\*\*) and  $p \leq 0.0001$  (\*\*\*\*).

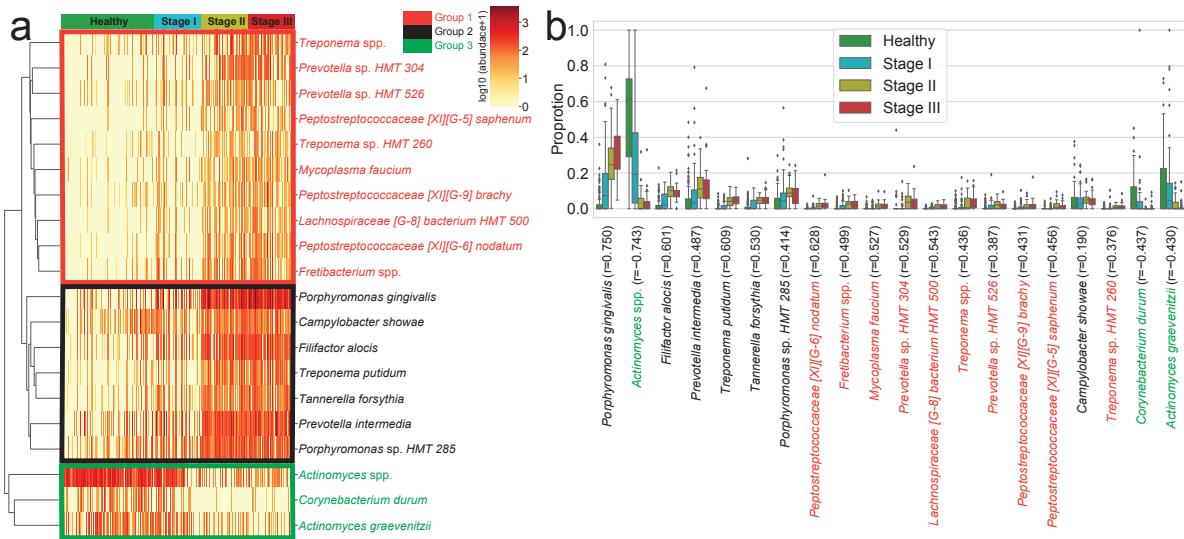


Figure 8: **Differentially abundant taxa (DAT).**

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.

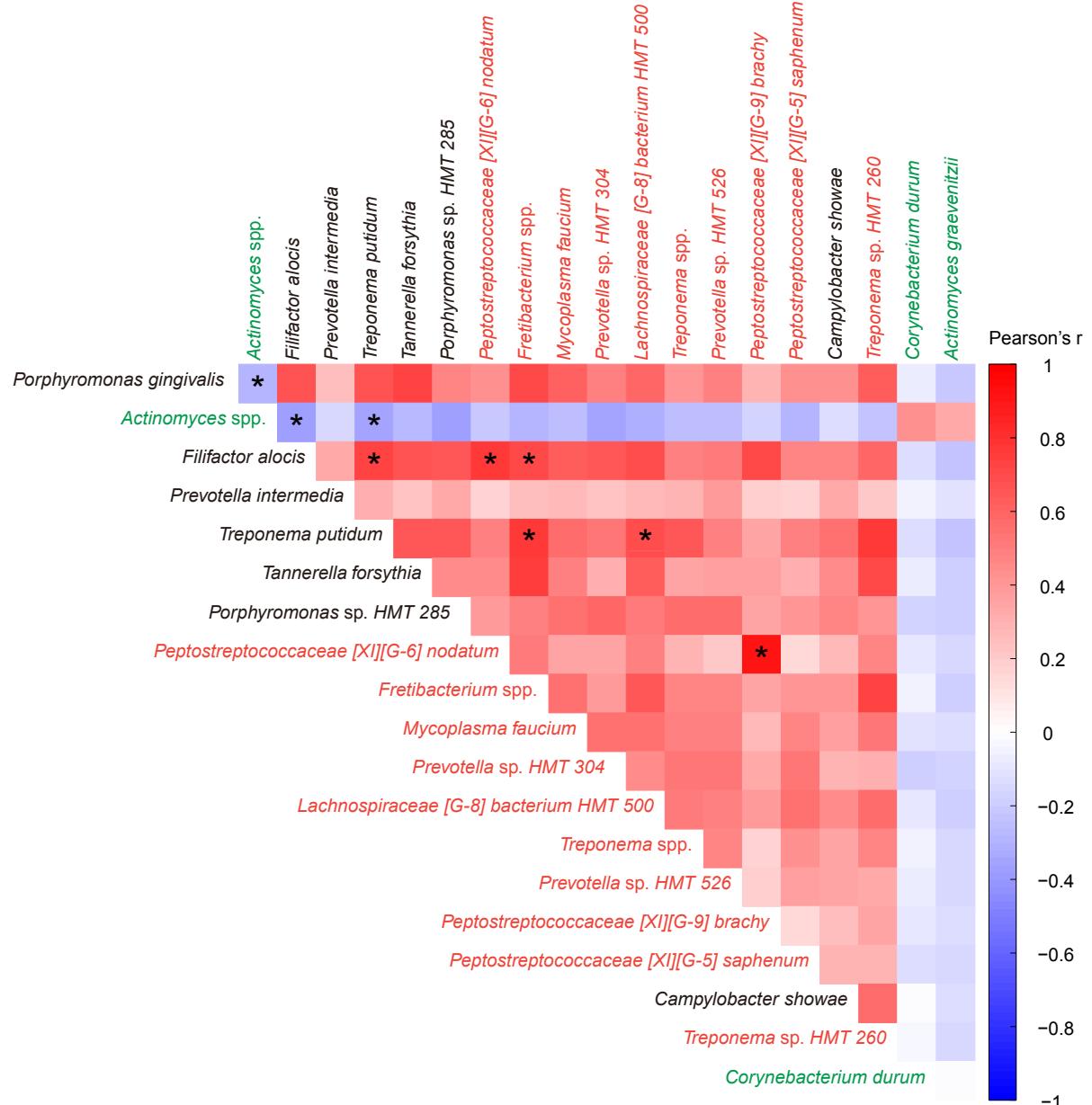
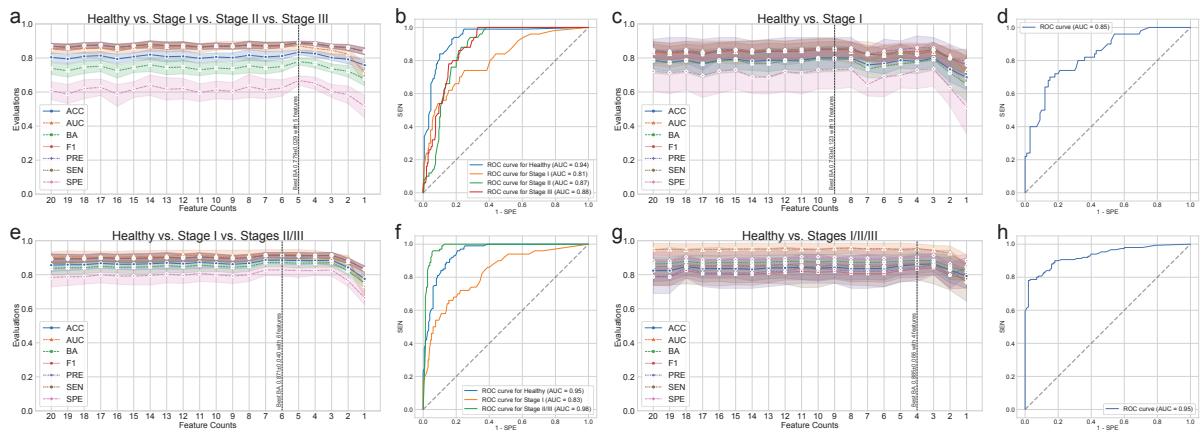


Figure 9: Correlation heatmap.

Pearson's correlations between DAT 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: ACC, AUC, BA, F1, PRE, SEN, and SPE. **(a)** Classification performance for healthy vs. stage I vs. stage II vs. stage III. **(b)** 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).

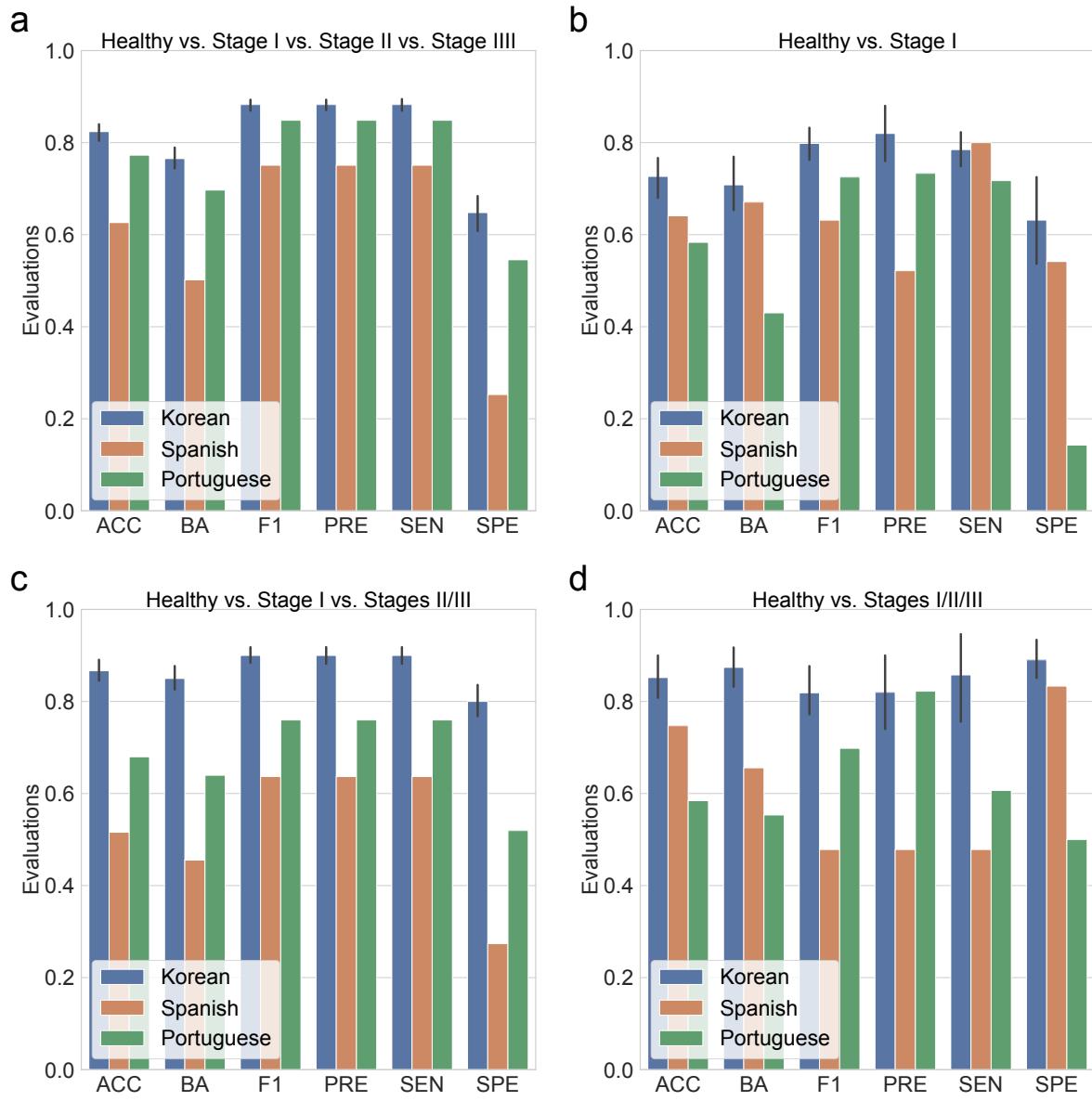


Figure 11: **Random forest classification metrics from external datasets.**

The classification metrics in the random forest classifications were as follows: ACC, AUC, BA, F1, PRE, SEN, and SPE. **(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.

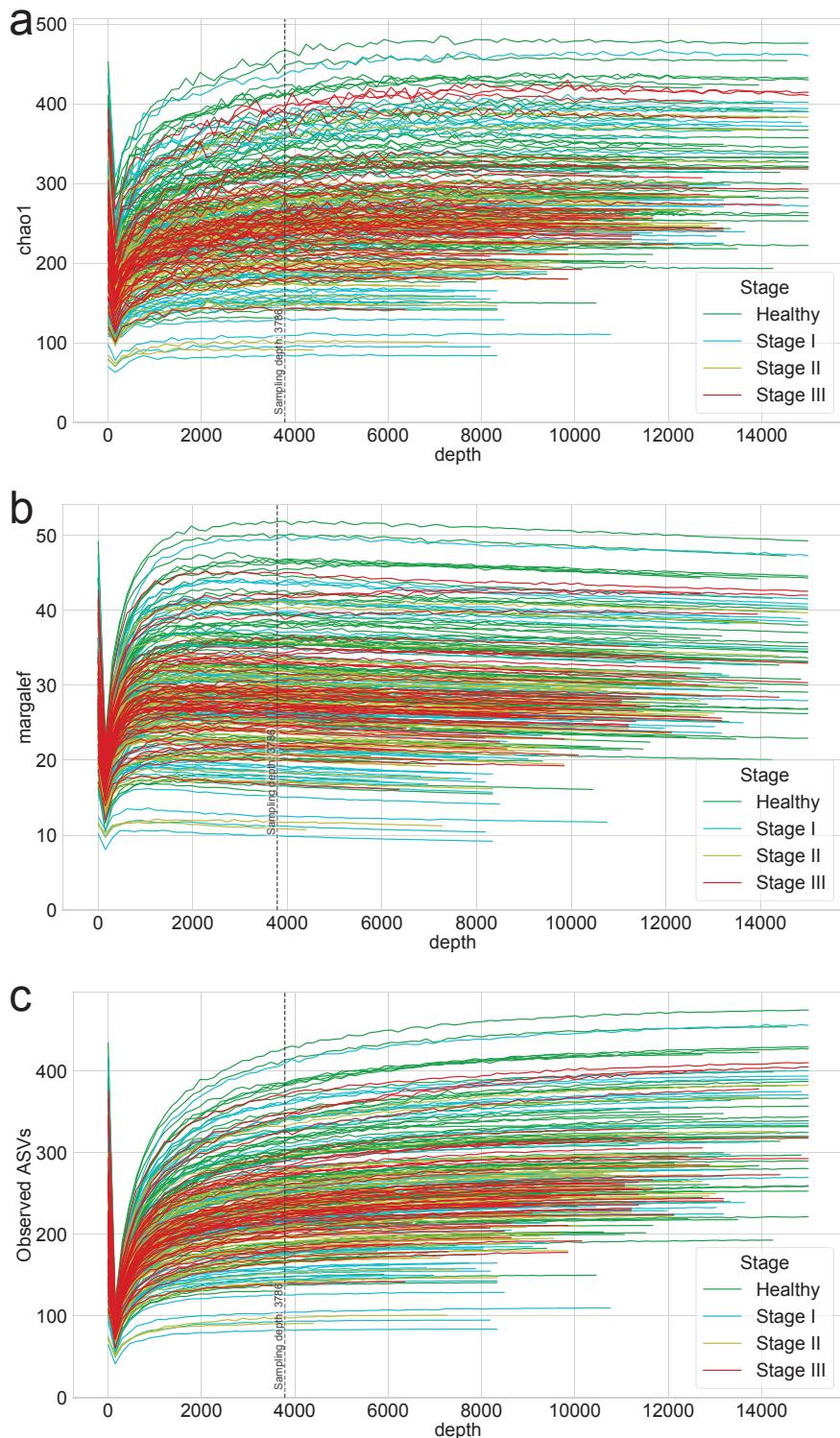
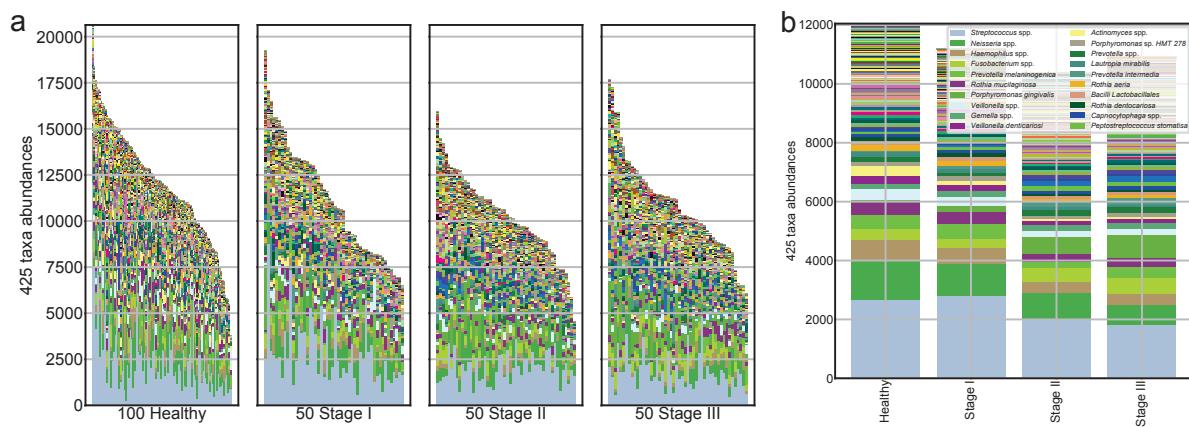


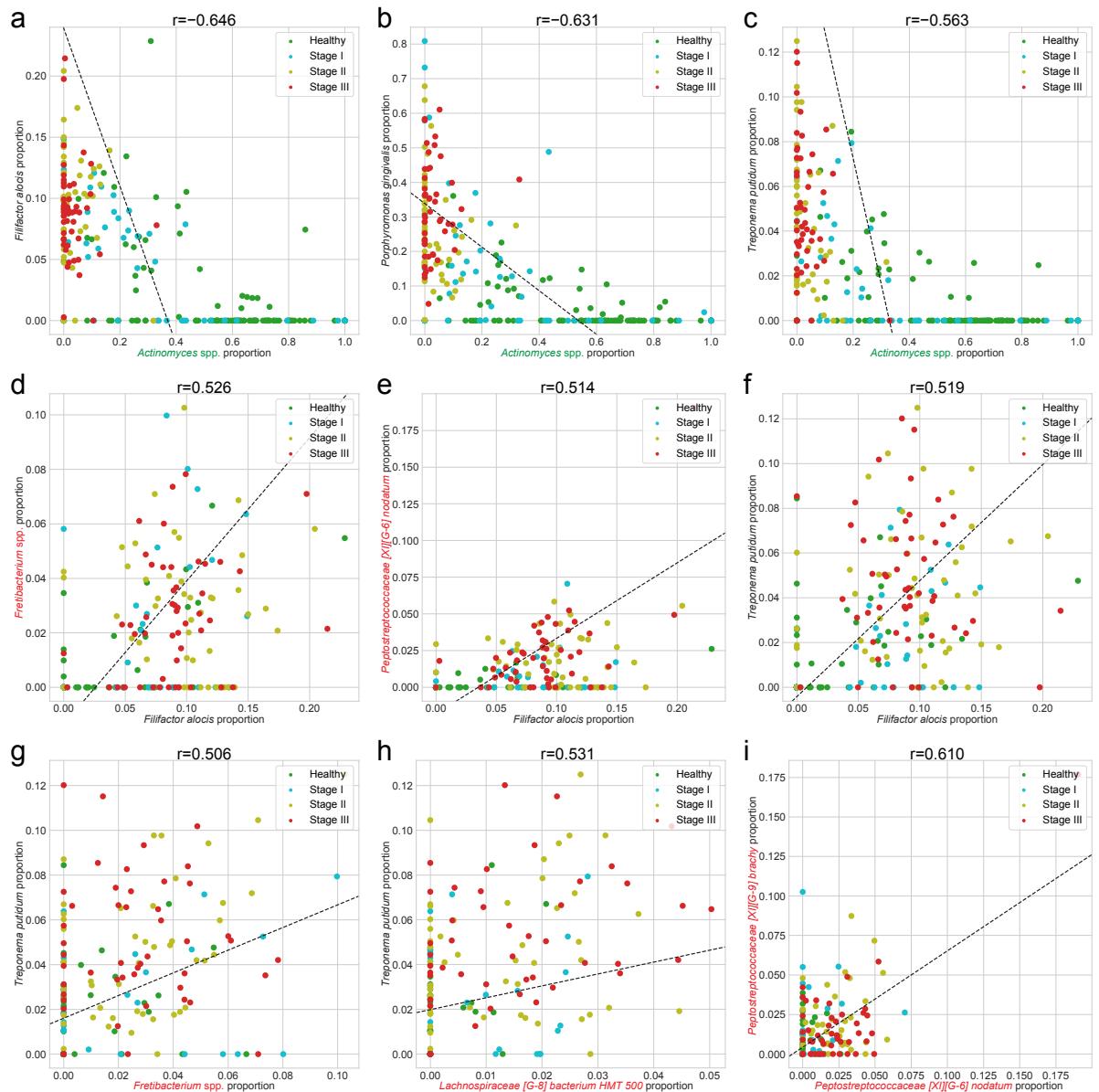
Figure 12: Rarefaction curves for alpha-diversity indices.

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 13: Salivary microbiome compositions in the different periodontal statuses.**

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 14: 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).

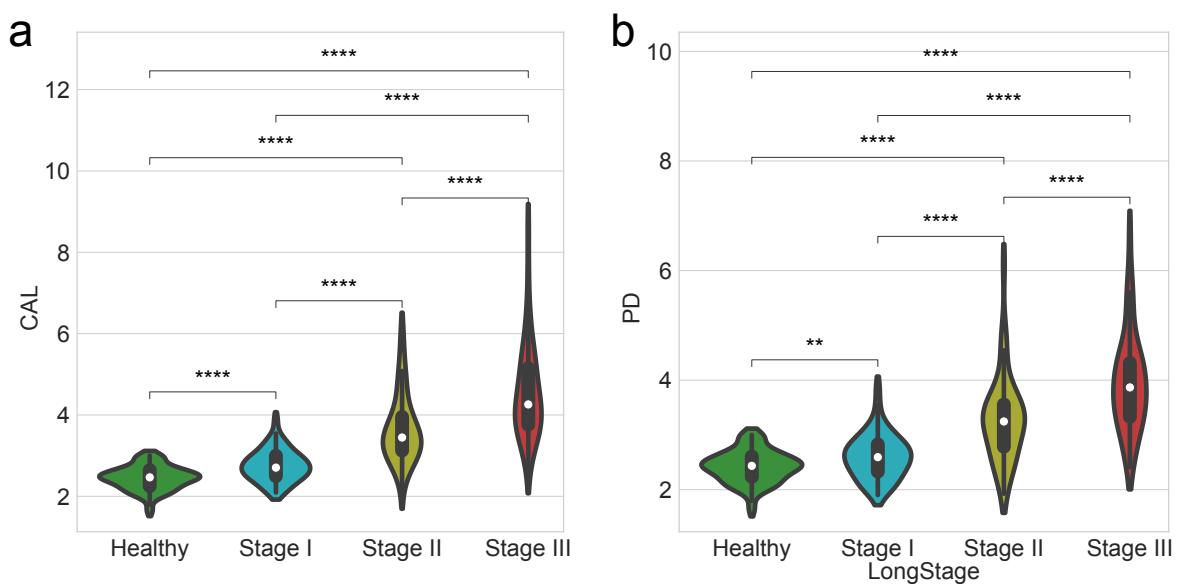


Figure 15: **Clinical measurements by the periodontitis statuses.**

Comparisons of clinical measurement among healthy controls and patients with various periodontitis stages. **(a)** Clinical attachment level **(b)** Probing depth. Statistical significance determined by the MWU test:  $p \leq 0.01$  (\*\*) and  $p \leq 0.0001$  (\*\*\*\*).

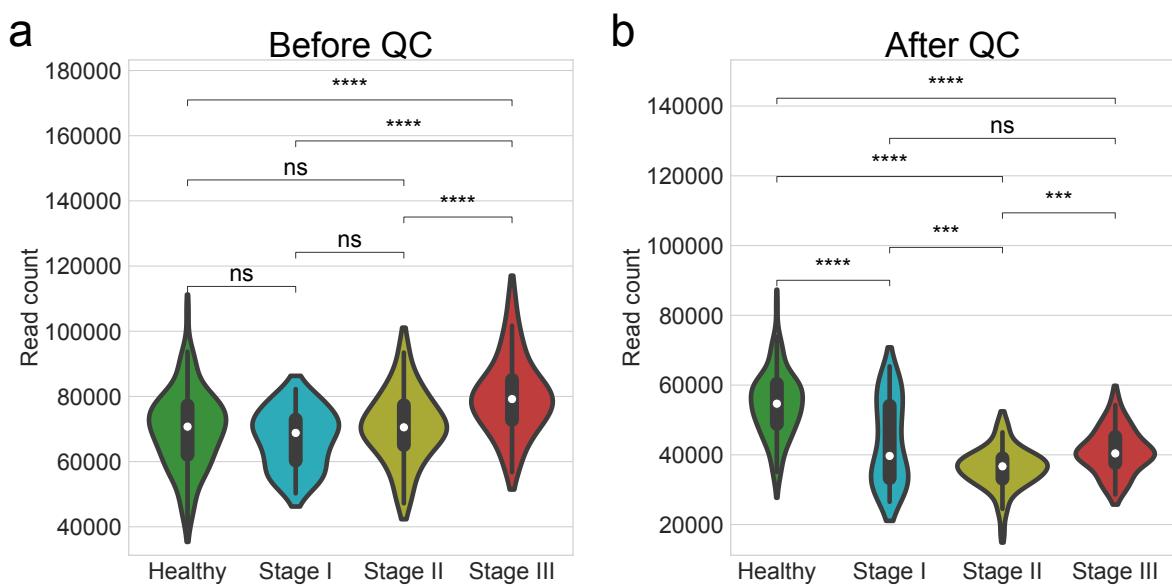


Figure 16: **Number of read counts by the periodontitis statuses.**

Comparisons of the number of read counts among healthy controls and patients with various periodontitis stages. **(a)** Before quality check **(b)** After quality check. Statistical significance determined by the Mann-Whitney U-test:  $p > 0.05$  (ns),  $p \leq 0.001$  (\*\*\*) $,$  and  $p \leq 0.0001$  (\*\*\*\*).

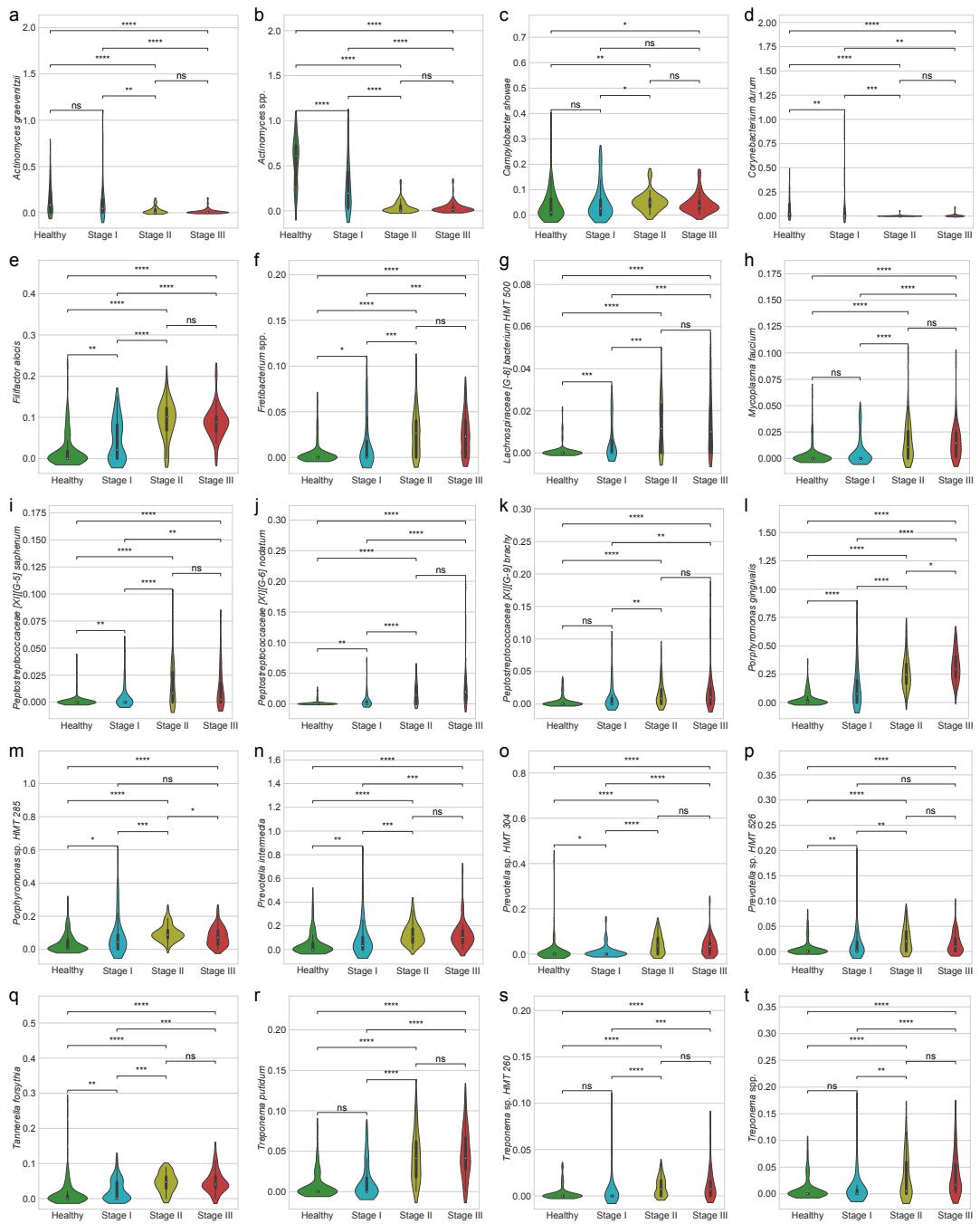
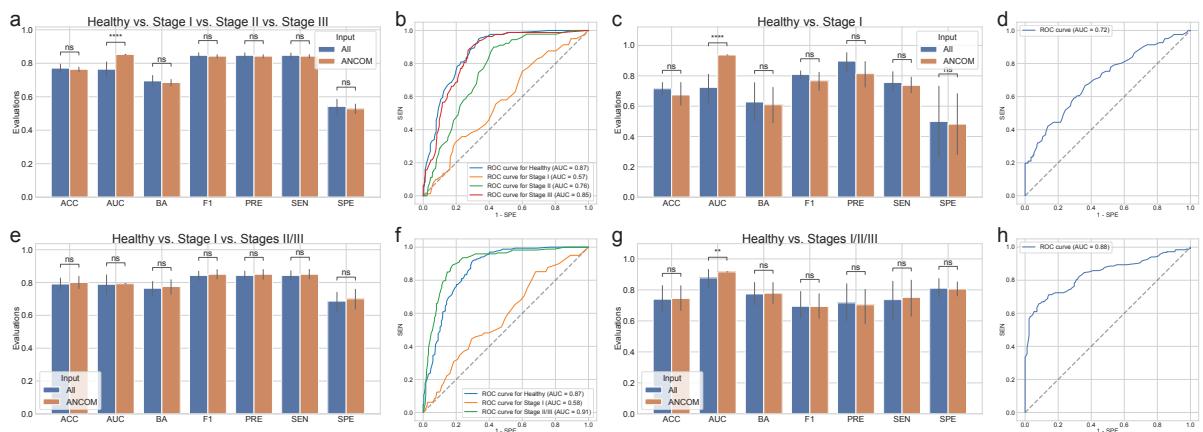


Figure 17: Proportion of DAT.

(a) *Actinomyces graevenitzii* (b) *Actinomyces* spp. (c) *Campylobacter showae* (d) *Corynebacterium durum* (e) *Filifactor alocis* (f) *Fretibacterium* spp. (g) *Lachnospiraceae [G-8] bacterium HMT 500* (h) *Mycoplasma faecium* (i) *Peptostreptococcaceae [XI][G-5] saphenum* (j) *Peptostreptococcaceae [XI][G-6] nodatum* (k) *Peptostreptococcaceae [XI][G-9] brachy* (l) *Porphyromonas gingivalis* (m) *Porphyromonas* sp. HMT 285 (n) *Prevotella intermedia* (o) *Prevotella* sp. HMT 304 (p) *Prevotella* sp. HMT 526 (q) *Tannerella forsythia* (r) *Treponema putidum* (s) *Treponema* sp. HMT 260 (t) *Treponema* spp. Statistical significance determined by the MWU test:  $p > 0.05$  (ns),  $p \leq 0.05$  (\*),  $p \leq 0.01$  (\*\*),  $p \leq 0.001$  (\*\*\*), and  $p \leq 0.0001$  (\*\*\*\*).



**Figure 18: Random forest classification metrics with the full microbiome compositions and ANCOM-selected DAT compositions.**

The classification metrics in the random forest classifications were as follows: ACC, AUC, BA, F1, PRE, SEN, and SPE. **(a)** Classification performance for healthy vs. stage I vs. stage II vs. stage III. **(b)** 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 (g). Statistical significance determined by the MWU test:  $p > 0.05$  (ns),  $p \leq 0.01$  (\*\*), and  $p \leq 0.0001$  (\*\*\*\*).

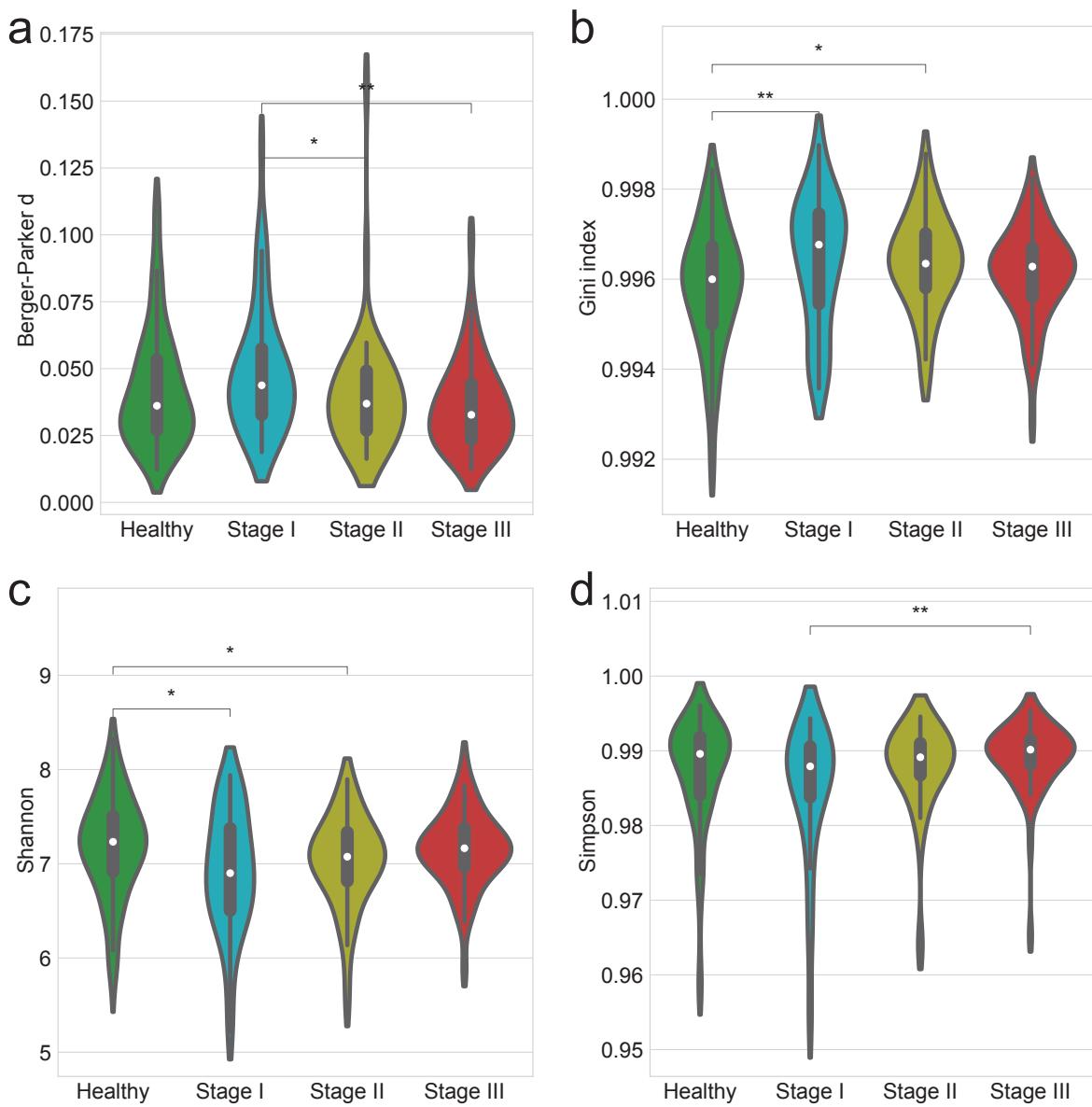
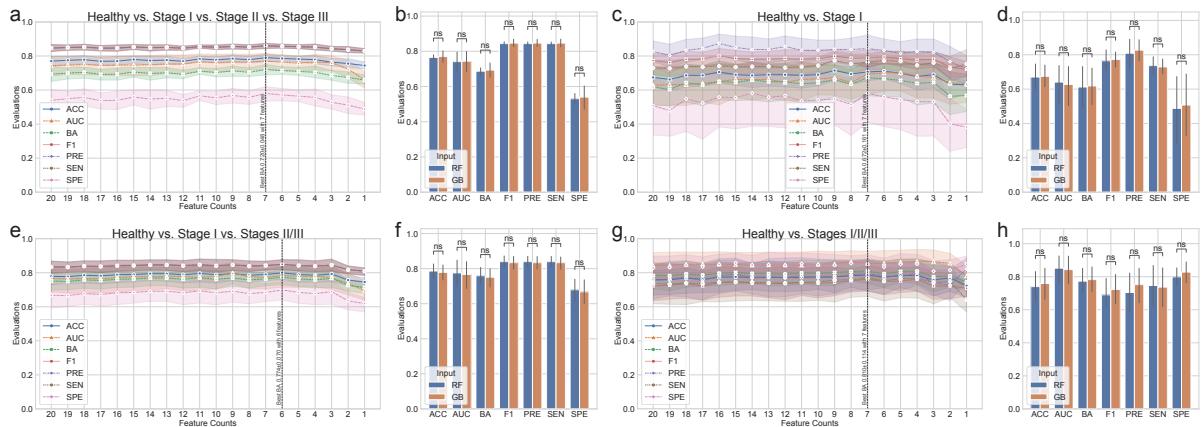


Figure 19: **Alpha-diversity indices account for evenness.**

Alpha-diversity indices (**a-d**) indicate that the heterogeneity between the periodontitis stages as measured by: **(a)** Berger-Parker  $d$  **(b)** Gini **(c)** Shannon **(d)** Simpson. Statistical significance determined by the MWU test:  $p \leq 0.05$  (\*) and  $p \leq 0.01$  (\*\*)



**Figure 20: Gradient Boosting classification metrics.**

The classification metrics in the random forest classifications were as follows: ACC, AUC, BA, F1, PRE, SEN, and SPE. The feature counts mean that the classification model trained on the most important  $n$  features as the table 4. **(a)** Comparison of Random forest (RF) and Gradient boosting (GB) for healthy vs. stage I vs. stage II vs. stage III. **(b)** Comparison of RF and GB for the highest BA of (a). **(c)** Classification performance for healthy vs. stage I. **(d)** Comparison of RF and GB for healthy vs. stage I vs. stages II/III. **(e)** Comparison of RF and GB for the highest BA of (d). **(f)** Comparison of RF and GB for Healthy vs. Stage I vs. Stages II/III. **(g)** Classification performance for healthy vs. stages I/II/III. **(h)** Comparison of RF and GB 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 Conclusion**

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

In the section 2, I show that

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## Acknowledgments

I would like to disclose my earnest appreciation for my advisor, Professor Semin Lee, who provided solicitous supervision and cherished opportunities throughout the course of my research. His advice and consultation encouraged me to become as a researcher and to receive all humility and gentleness. I am also grateful to all of my committee members, Professor AAA, Professor BBB, Professor CCC, and Professor DDD, for their critical and meaningful mentions and suggestions.

I extend my deepest gratitude to my Lord, *the Flying Spaghetti Monster*, His Noodly Appendage has guided me through the twist and turns of this academic journey. His presence, ever comforting and mysterious, has been a source of strength and humor during both highs and lows. In moments of doubt, I found solace in the belief that you were there, gently reminding me to keep faith in the process. His Holy Noodle has nourished my mind, and for that, I am truly overwhelmed. May His Holy Noodle continue to guide me in all my future endeavors. R’Amen.

(Professors)

I would like to extend my heartfelt gratitude to my colleagues of the Computational Biology Lab @ UNIST, whose collaboration, friendship, brotherhood, and support have been an invaluable part of my journey. Your willingness to share insights, engage in thoughtful discussions, and offer encouragement during the challenging moments of research has significantly shaped my academic experience. The camaraderie in Computational Biology Lab made even the most demanding days more enjoyable, and I am deeply grateful for the collaborative environment we created together. I appreciate you for standing by my side throughout this Ph.D. journey.

I would like to express my heartfelt gratitude to my family, whose unwavering support has been the foundation of everything I have achieved. Your love, encouragement, and belief in me have sustained me through every challenge, and I could not have come this far without you. From your words of wisdom to your patience and understanding, each of you has played a vital role in helping me navigate this journey. The strength and comfort I have drawn from our family bond have been my greatest source of resilience. Your presence, both near and far, has filled my life with warmth and motivation. I am deeply grateful for your unconditional love and for always being there when I needed you the most. Thank you for being my constant source of strength and inspiration.

I am incredibly pleased to my friends, especially my GSHS alumni (○망특), for their unwavering support and encouragement throughout this journey. The bonds we formed back in our school days have only grown stronger over the years, and I am fortunate to have had such loyal and understanding friends by my side. Your constant words of motivation, and even moments of levity during stressful times have helped keep me grounded. Whether it was a late-night conversations, a shared laugh, or a simple message of reassurance, you all have played a vital role in keeping me focused and motivated. I am relieved for the ways you celebrated each small achievement with me and how you patiently listened to my worries. The memories of our shared past provided me with comfort and a sense of stability when the road ahead felt uncertain. I could not have reached this point without the love and friendship that you all have generously given. Each of your, in your unique way, has contributed to this dissertation, even if indirectly, and for

that, I am forever beholden. I look forward to continuing our friendship as we all grow in our individual paths, knowing that the support we share is something truly special.

I would like to express my sincere gratitude to the amazing members of my animal protection groups, DRDR (두루두루) and UNIMALS (유니멀스), whose dedication and compassion have been a constant source of motivation. Your unwavering commitment to improving the lives of animals has inspired me throughout this journey. I am also thankful for the beautiful cats we have cared for, whose presence brought both joy and purpose to our allegiance. Their playful spirits and gentle companionship served as daily reminders of why we continue to fight for animal rights. The bond we share, both with each other and with the animals we protect, has enriched my life in countless ways. I appreciate you all again for your support, dedication, and for being part of this meaningful cause.

I would like to express my deepest gratitude to everyone I have had the honor of meeting throughout this journey. Your kindness, encouragement, and support have carried me through both the challenging and rewarding moments of my life. Whether through a kind word, thoughtful advice, or simply being there when I needed it most, your presence has made all the difference. I am incredibly fortunate to have received such generosity and warmth from those around me, and I do not take it for granted. Every act of kindness, no matter how big or small, has been a source of strength and motivation for me. To all my friends, colleagues, mentors, and beloved ones, thank you for your unwavering support. I am truly grateful for each of you, and your kindness has left an indelible mark on my journey.

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.

