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Doctoral Thesis

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Microbiota in Human Diseases

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Department of Biomedical Engineering

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Ulsan National Institute of Science and Technology

⁶

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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.

A handwritten signature in black ink that reads "Bobby Henderson".

Representative,
Church of the Flying Spaghetti Monster
Bobby Henderson



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13

Abstract

14 (Microbiome)

15 (PTB) Section 2 introduces...

16 (Periodontitis) Section 3 describes...

17 (Colon) Setion 4...

18 (Conclusion)

19

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

- 22 • Hong, Y. M., **Lee, Jaewoong**, Cho, D. H., Jeon, J. H., Kang, J., Kim, M. G., ... & Kim, J. K. (2023).
23 Predicting preterm birth using machine learning techniques in oral microbiome. *Scientific Reports*,
24 13(1), 21105.

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List of Abbreviations

- 94 **ACC** Accuracy
95 **ASV** Amplicon sequence variant
96 **AUC** Area-under-curve
97 **BA** Balanced accuracy
98 **C-section** Cesarean section
99 **DAT** Differentially abundant taxa
100 **F1** F1 score
101 **Faith PD** Faith's phylogenetic diversity
102 **FTB** Full-term birth
103 **GA** Gestational age
104 **MWU test** Mann-Whitney U-test
105 **PRE** Precision
106 **PROM** Prelabor rupture of membrane
107 **PTB** Preterm birth
108 **ROC curve** Receiver-operating characteristics curve
109 **rRNA** Ribosomal RNA
110 **SD** Standard deviation
111 **SEN** Sensitivity
112 **SPE** Specificity
113 **t-SNE** t-distributed stochastic neighbor embedding

¹¹⁴ 1 Introduction

¹¹⁵ The microbiome refers to the complex community of microorganisms, including bacteria, viruses, fungi,
¹¹⁶ and other microbes, that inhabit various environment within living organisms (Ursell, Metcalf, Parfrey,
¹¹⁷ & Knight, 2012; Gilbert et al., 2018). In humans, the microbiome plays a crucial role in maintaining
¹¹⁸ health (Lloyd-Price, Abu-Ali, & Huttenhower, 2016), influencing processes such as digestion (Lim, Park,
¹¹⁹ Tong, & Yu, 2020), immune response (Thaiss, Zmora, Levy, & Elinav, 2016; Kogut, Lee, & Santin, 2020;
¹²⁰ C. H. Kim, 2018), and even mental health (Mayer, Tillisch, Gupta, et al., 2015; X. Zhu et al., 2017;
¹²¹ X. Chen, D'Souza, & Hong, 2013). These microbial communities are not static nor constant, but rather
¹²² dynamic ecosystem that interacts with their host and respond to environmental changes. Recent studies
¹²³ have revealed that imbalances in the microbiome, known as dysbiosis, can contribute to a wide range of
¹²⁴ diseases, including obesity (John & Mullin, 2016; Tilg, Kaser, et al., 2011; Castaner et al., 2018), diabetes
¹²⁵ (Barlow, Yu, & Mathur, 2015; Hartstra, Bouter, Bäckhed, & Nieuwdorp, 2015; Sharma & Tripathi, 2019),
¹²⁶ infections (Whiteside, Razvi, Dave, Reid, & Burton, 2015; Alverdy, Hyoju, Weigerinck, & Gilbert, 2017),
¹²⁷ inflammatory conditions (Francescone, Hou, & Grivennikov, 2014; Peirce & Alviña, 2019; Honda &
¹²⁸ Littman, 2012), and cancers (Helmink, Khan, Hermann, Gopalakrishnan, & Wargo, 2019; Cullin, Antunes,
¹²⁹ Straussman, Stein-Thoeringer, & Elinav, 2021; Sepich-Poore et al., 2021; Schwabe & Jobin, 2013). Thus,
¹³⁰ understanding the composition of the human microbiomes is essential for developing new therapeutic
¹³¹ approaches that target these microbial populations to promote health and prevent diseases.

¹³² The microbiome participates a crucial role in overall health, influencing not only digestion and immune
¹³³ function but also systemic and neurological processes through the brain-gut axis (Martin, Osadchiy,
¹³⁴ Kalani, & Mayer, 2018; Aziz & Thompson, 1998; R. Li et al., 2024). The gut microbiota interact with
¹³⁵ the host through metabolic byproducts, immune signaling, and the production of neurotransmitters, *e.g.*
¹³⁶ serotonin and dopamine, which are essential for brain function and cognition. Disruptions in microbial
¹³⁷ composition, known as dysbiosis, have been linked to various diseases, including inflammatory bowel
¹³⁸ disease (Sultan et al., 2021; Baldelli, Scaldaferrri, Putignani, & Del Chierico, 2021), obesity (Kang et al.,
¹³⁹ 2022; Hamjane, Mechita, Nourouti, & Barakat, 2024; Pezzino et al., 2023), diabetes (Cai et al., 2024;
¹⁴⁰ X. Li et al., 2021; Y. Li et al., 2023), and cardiovascular diseases (Manolis, Manolis, Melita, & Manolis,
¹⁴¹ 2022; Tian et al., 2021). Furthermore, the brain-gut axis, a bidirectional communication system between
¹⁴² the gut microbiome composition and the central nervous system, has been implicated in mental disorders,
¹⁴³ *e.g.* anxiety disorder, depressive disorder, and neurodegenerative diseases. Emerging evidence suggested
¹⁴⁴ that alterations in the host microbiome can influence mood, cognitive function, and even behavior through
¹⁴⁵ immune modulation, vagus nerve signaling, and microbial metabolites. These findings highlight the
¹⁴⁶ microbiome as a critical factor in maintaining host health and suggest that targeted interventions, namely
¹⁴⁷ probiotics, antibiotics, dietary modification, and microbiome-based therapies, may hold promise for
¹⁴⁸ improving both physical and mental comfort. Hence, understanding the microbial effects could lead to
¹⁴⁹ novel therapeutic strategies for a wide range of health conditions.

¹⁵⁰ 16S ribosomal RNA (rRNA) gene sequencing is one of the most extensively applied methods for
¹⁵¹ characterizing microbial communities by targeting the conserved 16S rRNA gene, which contains both

152 highly conserved and variable regions in bacteria (Tringe & Hugenholtz, 2008; Janda & Abbott, 2007).
153 The conserved regions enable universal primer binding, while the variable regions provide the specificity
154 needed to differentiate microbial taxa. Among these regions, the V3-V4 region is frequently selected for
155 sequencing due to its balance between phylogenetic resolution and sequencing efficiency (Johnson et al.,
156 2019; López-Aladid et al., 2023). Therefore, the V3-V4 region offers sufficient variability to classify a
157 wide range of bacteria taxa while maintaining compatibility with widely used sequencing platforms.

158 On the other hand, PathSeq is a computational pipeline designed for the identification and analysis
159 of microbial sequences within short-read human sequencing data, such as next-generation sequencing
160 (Kostic et al., 2011; Walker et al., 2018). PathSeq's scalable and effective processing of massive amounts
161 of sequencing data allows large-scale microbial profiling possible. PathSeq workflow consists of two
162 main phases: a subtractive phase and an analytic phase. The subtractive phase is removing human-derived
163 reads by aligning them to a human reference genome; and, the analytic phase is mapping remaining reads
164 to microbial reference databases, not only bacterial reference genome, but also archaeal, fungal, and viral
165 reference genomes. This approach allows for the comprehensive detection of microbiome compositions,
166 without a requirement for targeted amplification. PathSeq presents a more comprehensive and objective
167 evaluation of microbiome compositions than conventional microbiome profiling techniques including 16S
168 rRNA gene sequencing, capturing an assortment of microbial species beyond bacteria. Therefore, PathSeq
169 is an effective instrument for metagenomic research, infectious disease study, and microbiome analysis in
170 environmental and clinical contexts because of its capacity to operate with complex sequencing datasets
171 (Ojesina et al., 2013; Park et al., 2024; Tejeda et al., 2021).

172 Diversity indices are essential techniques for evaluating the complexity and variety of microbial
173 communities, in ecological and microbiological research (Tucker et al., 2017; Hill, 1973). Alpha-diversity
174 index attributes to the heterogeneity within a specific community, obtaining the number of different taxa
175 and the distribution of taxa among the individuals, *i.e.*, richness and evenness. On the other hand, beta-
176 diversity index measures the variations in microbiome compositions between the individuals, highlighting
177 differences among the microbiome compositions of the study participants (B.-R. Kim et al., 2017).
178 Altogether, by providing a thorough understanding of microbiome compositions, diversity indices, *e.g.*
179 alpha-diversity and beta-diversity, allow us to investigate factors that affecting community variability and
180 structure.

181 Differentially abundant taxa (DAT) detection is a key analytical approach in microbiome study to
182 identify microbial taxa that significantly differ in abundance between distinct study participant groups.
183 This DAT detection method is particularly valuable for understanding how microbial communities vary
184 across different conditions, such as disease states, environmental factors, and/or experimental treatments.
185 Various statistical and computational techniques, *e.g.* LEfSe (Segata et al., 2011), DESeq2 (Love, Huber,
186 & Anders, 2014), ANCOM (Lin & Peddada, 2020), and ANCOM-BC (Lin, Eggesbø, & Peddada,
187 2022; Lin & Peddada, 2024), are commonly used to assess differential abundance while accounting for
188 compositional and sparsity-related challenges in microbiome composition data (Swift, Cresswell, Johnson,
189 Stilianoudakis, & Wei, 2023; Cappellato, Baruzzo, & Di Camillo, 2022). Thus, identifying DAT can
190 provide insights into microbial biomarkers associated with specific health conditions or disease statuses,

enabling potential applications in diagnostics and therapeutics. However, due to the nature of microbiome composition data and the influence of sequencing depth, appropriate normalization and statistically adjustments are necessary to ensure reliable and stable detection of differentially abundant microbes (Xia, 2023; Pan, 2021). Integrating DAT detection analysis with functional profiling further enhances our understanding of the biological significance of microbial shifts or dysbiosis. As microbiome research advances, improving methodologies for DAT selection remains essential for uncovering meaningful microbial association and their potential roles in human diseases.

Classification is one of the supervised machine learning techniques used to categorized data into predefined classes based on features within the data (Kotsiantis, Zaharakis, & Pintelas, 2006; Sen, Hajra, & Ghosh, 2020). In other words, the method learns the relationship between input features and their corresponding output classes through the process of training a classification model using labeled data. Classification models are essential for advising choices in a wide range of applications, including medical diagnostics (Omondiagbe, Veeramani, & Sidhu, 2019). Thus, researchers could uncover sophisticated connections in input features and corresponding classes and produce reliable prediction by utilizing machine learning classification.

Random forest classification is one of the ensemble machine learning methods that constructs several decision trees during training and aggregates their results to provide classification predictions (Breiman, 2001). A portion of the features and classes—known as bootstrapping (Jiang & Simon, 2007; Champagne, McNairn, Daneshfar, & Shang, 2014; J.-H. Kim, 2009) and feature bagging (Bryll, Gutierrez-Osuna, & Quek, 2003; Alelyani, 2021; Yaman & Subasi, 2019)—are utilized to construct each tree in the forest. The majority vote from each tree determines the final classification, which lowers the possibility of overfitting in comparison to a single decision tree. Furthermore, random forest classifier offers several advantages, including its robustness to outliers and its ability to calculate the feature importance.

Evaluating the performance of a machine learning classification model is essential to ensure its reliability and effectiveness in real-world solutions and applications (Novaković, Veljović, Ilić, Papić, & Tomović, 2017; Hossin & Sulaiman, 2015; Hand, 2012). A confusion matrix is a tabular representation of predictions of classification, showing the counts of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN) (Table 1). From this matrix, evaluations can be derived: accuracy (ACC; Equation 1), balanced accuracy (BA; Equation 2), F1 score (F1; Equation 3), sensitivity (SEN; Equation 4), specificity (SPE; Equation 5), and precision (PRE; Equation 6). These metrics are in [0, 1] range and high metrics are good metrics. The confusion matrix also helps in identifying specific types of errors, such as a tendency to produce false positive or false negatives, offering valuable insights for improving the classification model. By combining the confusion matrix with other evaluation metrics, researchers can comprehensively assess the classification metrics and refine it for real-world solutions and applications.

The receiver-operating characteristics (ROC) curve is a graphical representation used to evaluate the performance of a classification model by plotting the sensitivity against (1-specificity) at multiple threshold setting (Gonçalves, Subtil, Oliveira, & de Zea Bermudez, 2014; Obuchowski & Bullen, 2018; Centor, 1991). The ROC curve illustrates the trade-off between detecting true positives while minimizing false positives, suggesting determining the optimal decision threshold for classification. A key metric

230 derived from the ROC curve is the area-under-curve (AUC), which quantifies overall ability of the
231 classification model to discriminate between positive and negative predictions. An AUC value of 0.5
232 indicates a model performing no better than random chance, while value closer to 1.0 suggests high
233 predictive accuracy. Thus, by analyzing the AUC value of the ROC curve, researchers can compare
234 different models and select the better classification model that offers the best balance between sensitivity
235 and specificity for a given application.

236 (Limitation & Novelty)

Table 1: Confusion matrix

		Predicted	
		Positive	Negative
Actual	Positive	True positive (TP)	False negative (FN)
	Negative	False positive (FP)	True negative (TN)

237

$$\text{ACC} = \frac{\text{TP} + \text{TN}}{\text{TP} + \text{FN} + \text{FP} + \text{TN}} \quad (1)$$

238

$$\text{BA} = \frac{1}{2} \times \left(\frac{\text{TP}}{\text{TP} + \text{FP}} + \frac{\text{TN}}{\text{TN} + \text{FN}} \right) \quad (2)$$

239

$$\text{F1} = \frac{2 \times \text{TP}}{2 \times \text{TP} + \text{FP} + \text{FN}} \quad (3)$$

240

$$\text{SEN} = \frac{\text{TP}}{\text{TP} + \text{FP}} \quad (4)$$

241

$$\text{SPE} = \frac{\text{TN}}{\text{TN} + \text{FN}} \quad (5)$$

$$\text{PRE} = \frac{\text{TP}}{\text{TP} + \text{FP}} \quad (6)$$

242 **2 Predicting preterm birth using random forest classifier in salivary mi-**
243 **crobiome**

244 This section includes the published contents:

245 Hong, Y. M., **Lee, Jaewoong**, Cho, D. H., Jeon, J. H., Kang, J., Kim, M. G., ... & Kim, J. K. (2023).
246 Predicting preterm birth using machine learning techniques in oral microbiome. *Scientific Reports*, 13(1),
247 21105.

248 **2.1 Introduction**

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

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

266 Preterm prelabor rupture of membranes (PROM) brought on by gestational inflammation and infection
267 contribute to about 70% of PTB cases (Romero, Dey, & Fisher, 2014). Nevertheless, as antibiotics and
268 anti-inflammatory therapeutic strategies were ineffective to decrease PTB occurrence rates, the pathology
269 of PTB has not been entirely elucidated by inflammatory and infectious pathways (Romero, Hassan, et al.,
270 2014). Recent researches on maternal microbiomes were beginning to examine unidentified connections
271 of PTB as a consequence of developmental processes in molecular biological technology (Fettweis et al.,
272 2019).

273 However, as anti-inflammatory and antibiotic therapies were insufficient to lower PTB occurrence
274 rates, infectious and inflammatory processes are insufficient to exhaustively clarify the pathogenesis and
275 pathophysiology of PTB. It has been hypothesized that the microbiota linked to PTB originate from either
276 a hematogenous pathway or the female genitourinary tract increasing through the vagina and/or cervix.
277 (Han & Wang, 2013). Vaginal microbiome compositions have been found in women who eventually

278 acquire PTB, and recent studies have tried to predict PTB risk using cervico-vaginal fluid (Kindinger et
279 al., 2017). Even though previous investigation have confirmed the potential relationships between the
280 vaginal microbiome compositions and PTB, these studies are only able to clarify an upward trajectory.

281 Multiple unfavorable birth outcomes, including PROM and PTB, have been linked to periodontitis
282 as an independence risk factor, according to numerous epidemiological researches (Offenbacher et al.,
283 1996). It is expected that the oral microbiome will be able to explain additional hematogenous pathways
284 in light of these precedents; however, the oral microbiome composition of fetuses is limited understood.

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

288 **2.2 Materials and methods**

289 **2.2.1 Study design and study participants**

290 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
291 Declaration of Helsinki (Goodyear, Krleza-Jeric, & Lemmens, 2007). The Institutional Review Board
292 authorized this study (IRB file No. 2019-01-024). Participants who were admitted for elective cesarean
293 sections (C-sections) or induction births, as well as those who had written informed consent obtained
294 with premature labor or PROM, were eligible.
295

296 **2.2.2 Clinical data collection and grouping**

297 Questionnaires and electronic medical records were implemented to gather information on both previous
298 and current pregnancy outcomes. The following clinical data were analyzed:

- 299 • maternal age at delivery
- 300 • diabetes mellitus
- 301 • hypertension
- 302 • overweight and obesity
- 303 • C-section
- 304 • history PROM or PTB
- 305 • gestational week on delivery
- 306 • birth weight
- 307 • sex

308 **2.2.3 Salivary microbiome sample collection**

309 Salivary microbiome samples were collected 24 hours before to delivery using mouthwash. The standard
310 methods of sterilizing were performed. Medical experts oversaw each stage of the sample collecting
311 procedure. Participants received instruction not to eat, drink, or brush their teeth for 30 minutes before
312 sampling salivary microbiome. Saliva samples were gathered by washing the mouth for 30 seconds with
313 12 mL of a mouthwash solution (E-zen Gargle, JN Pharm, Pyeongtaek, Gyeonggi, Korea). The samples
314 were tagged with the anonymous ID for each participant and kept at 4 °C until they underwent further
315 processing. Genomic DNA was extracted using an ExgeneTM Clinic SV kit (GeneAll Biotechnology,
316 Seoul, Korea) following with the manufacturer instructions and store at -20 °C.

317 **2.2.4 16s rRNA gene sequencing**

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

324 **2.2.5 Bioinformatics analysis**

325 The independent *t*-test was utilized to evaluate the differences of continuous values between from the
326 PTB participants than the FTB participants; χ^2 -square test was applied to decide statistical differences of
327 categorical values. Clinical measurement comparisons were conducted using SPSS (version 20.0) (Spss
328 et al., 2011). At $p < 0.05$, statistical significance was taken into consideration.

329 QIIME2 (version 2022.2) was implemented to import 16S rRNA gene sequences from salivary
330 microbiome samples of study participants for additional bioinformatics processing (Bolyen et al., 2019).
331 DADA2 was used to verify the qualities of raw sequences (Callahan et al., 2016). The remain sequences
332 were clustered into amplicon sequence variants (ASVs). Diversity indices, namely Faith PD for alpha
333 diversity index (Faith, 1992) and Hamming distance for beta diversity index (Hamming, 1950), were
334 calculated. MWU test (Mann & Whitney, 1947), and PERMANOVA multivariate test were evaluated for
335 measuring statistical significance (Anderson, 2014; Kelly et al., 2015).

336 Taxonomic assignment were implemented with HOMD (version 15.22) (T. Chen et al., 2010).
337 Afterward, DESeq2 was implemented to identify differentially abundant taxa (DAT) that could dis-
338 tinguish between salivary microbiome from PTB and FTB participants (Love et al., 2014). Taxa with
339 $|\log_2 \text{FoldChange}| > 1$ and $p < 0.05$ were considered as statistically significant.

340 The taxa for predicting PTB using salivary microbiome data were determined using a random forest
341 classifier (Breiman, 2001). Through stratified *k*-fold cross-validation (*k* = 5) that preserves the existence
342 rate of PTB and FTB participants, consistency and trustworthy classification were ensured (Wong & Yeh,
343 2019).

344 **2.2.6 Data and code availability**

345 All sequences from the 59 study participants have been added to the Sequence Read Archives (project ID
346 PRJNA985119): <https://dataview.ncbi.nlm.nih.gov/object/PRJNA985119>. Docker image that
347 employed throughout this study is available in the DockerHub: https://hub.docker.com/r/fumire/helixco_premature. Every code used in this study can be found on GitHub: https://github.com/CompbioLabUnist/Helixco_Premature.

350 **2.3 Results**

351 **2.3.1 Overview of clinical information**

352 In the beginning, 69 volunteer mothers were recruited for this study. However, due to insufficient clinical
353 information or twin pregnancies, 10 participants were excluded from the study participants. Demographic
354 and clinical information of the study participants are displayed in Table 2. Because PROM is one of the
355 leading factors of PTB, it was prevalent in the PTB group than the FTB group. Other maternal clinical
356 factors did not significantly differ between the FTB and PTB groups. There were no cases in both groups
357 that had a history of simultaneous periodontal disease or cigarette smoking.

358 **2.3.2 Comparison of salivary microbiomes composition**

359 The salivary microbiome composition was composed of 13953804 sequences from 59 study participants,
360 with 102305.95 ± 19095.60 and 64823.41 ± 15841.65 (mean \pm SD) reads/sample before and following
361 the quality-check stage, accordingly. There was not a significant distinction between the PTB and FTB
362 groups with regard to on alpha diversity nor beta diversity metrics (Figure 4).

363 DESeq2 was used to select 32 DAT that distinguish between the PTB and FTB groups out of the 465
364 species that were examined (Love et al., 2014): 26 FTB-enriched DAT and six PTB-enriched DAT. Seven
365 PROM-related DAT were removed from these 32 PTB-related DAT to lessen the confounding effect of
366 PROM (Figure 5). Therefore, there were a total of 25 PTB-related DAT: 22 FTB-enriched DAT and three
367 PTB-enriched DAT (Figure 1).

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

370 **2.3.3 Random forest classification to predict PTB risk**

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

Table 2: Standard clinical information of study participants.

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

	PTB (n=30)	FTB (n=29)	p-value
Maternal age (years)	31.8 \pm 5.2	33.7 \pm 4.5	0.687
C-section	20 (66.7%)	24 (82.7%)	0.233
Previous PTB history	4 (13.3%)	1 (3.4%)	0.353
PROM	12 (40.0%)	1 (3.4%)	0.001
Pre-pregnant overweight	8 (26.7%)	7 (24.1%)	1.000
Gestational weight gain (kg)	9.0 \pm 5.9	11.5 \pm 4.6	0.262
Diabetes	2 (6.7%)	2 (6.9%)	1.000
Hypertension	11 (36.7%)	4 (13.8%)	0.072
Gestational age (weeks)	32.5 \pm 3.4	38.3 \pm 1.1	\leq 0.001
Birth weight (g)	1973.4 \pm 686.6	3283.4 \pm 402.7	\leq 0.001
Male	14 (46.7%)	13 (44.8%)	1.000

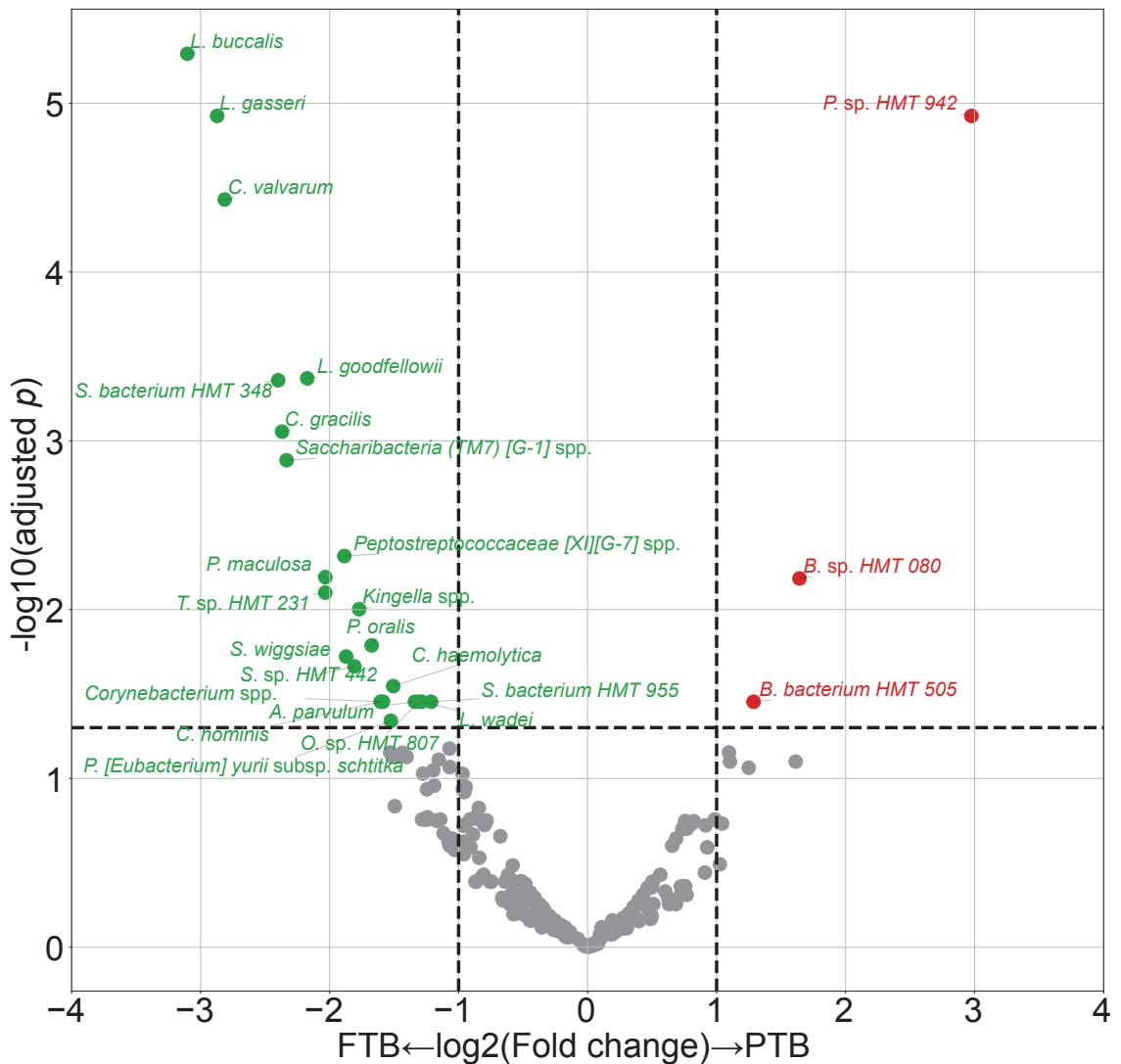


Figure 1: DAT volcano plot.

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

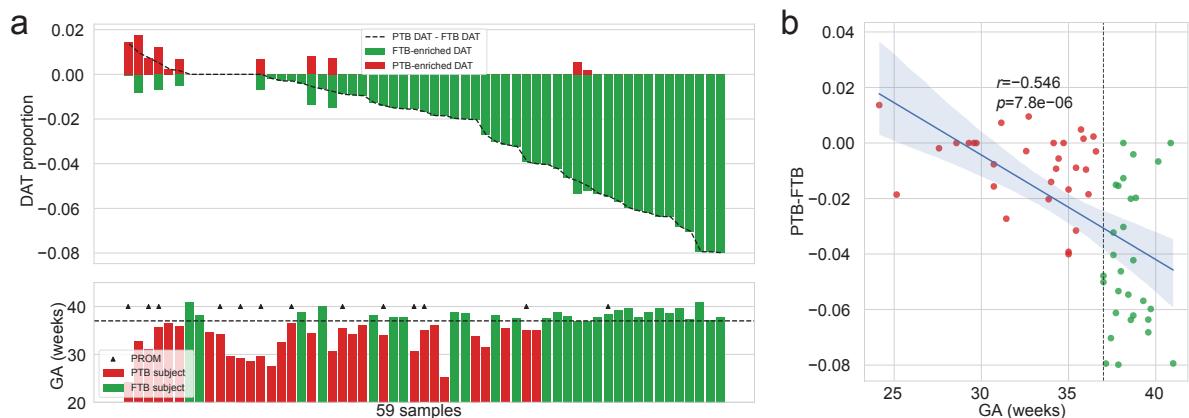


Figure 2: **Salivary microbiome compositions over DAT.**

(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.

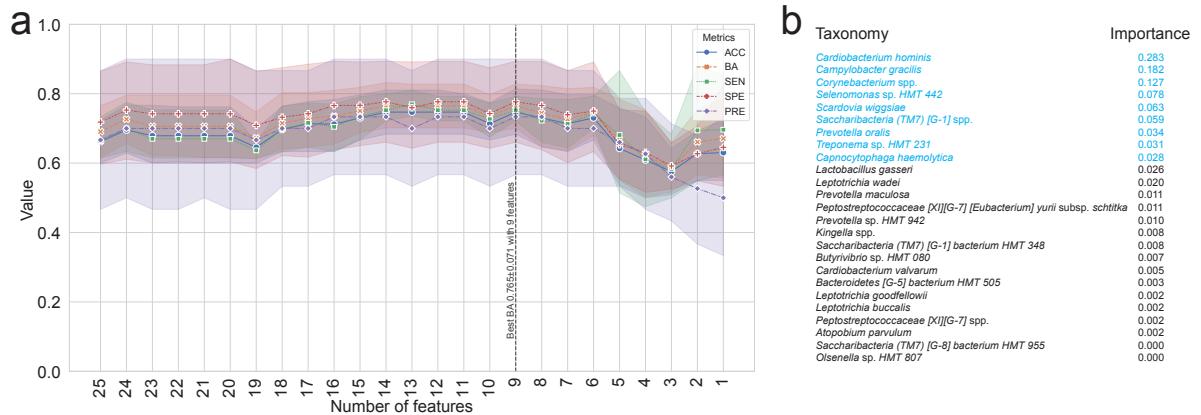


Figure 3: **Random forest-based PTB prediction model.**

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

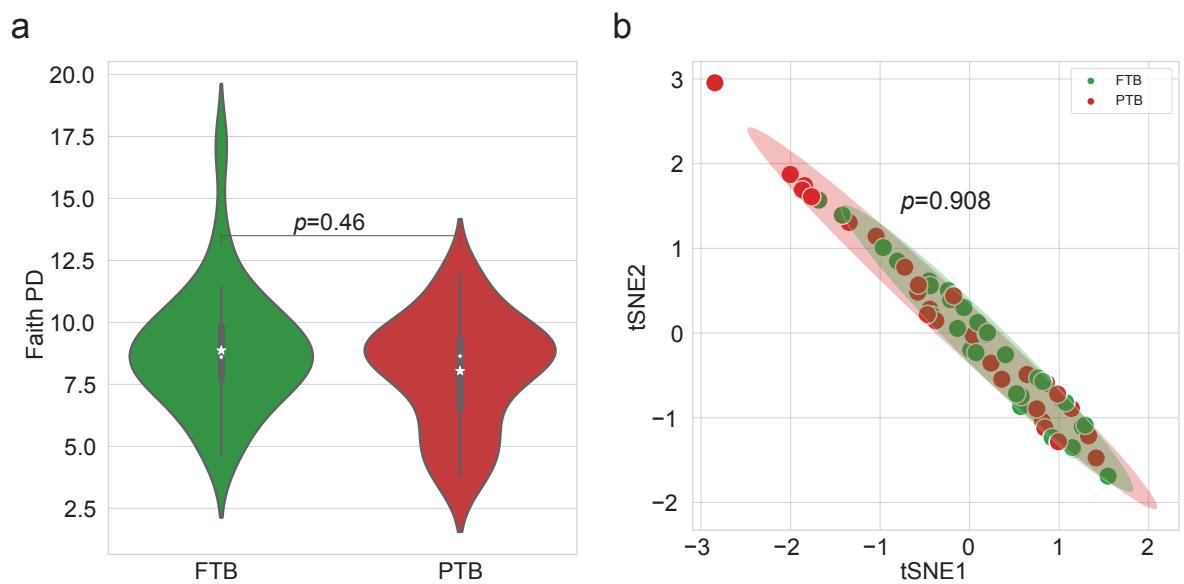


Figure 4: **Diversity indices.**

(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 $p = 0.908$)

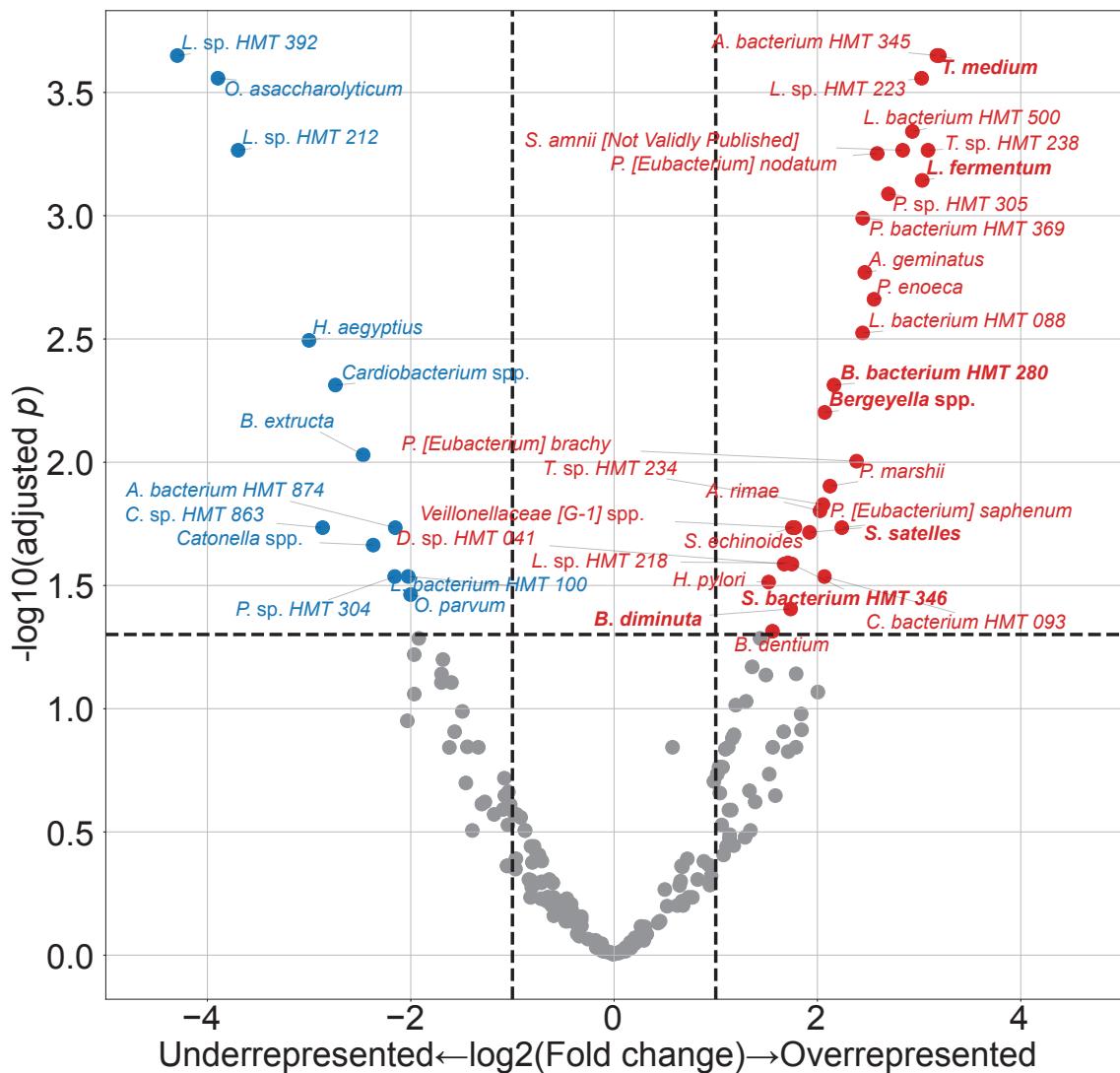


Figure 5: PROM-related DAT.

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.

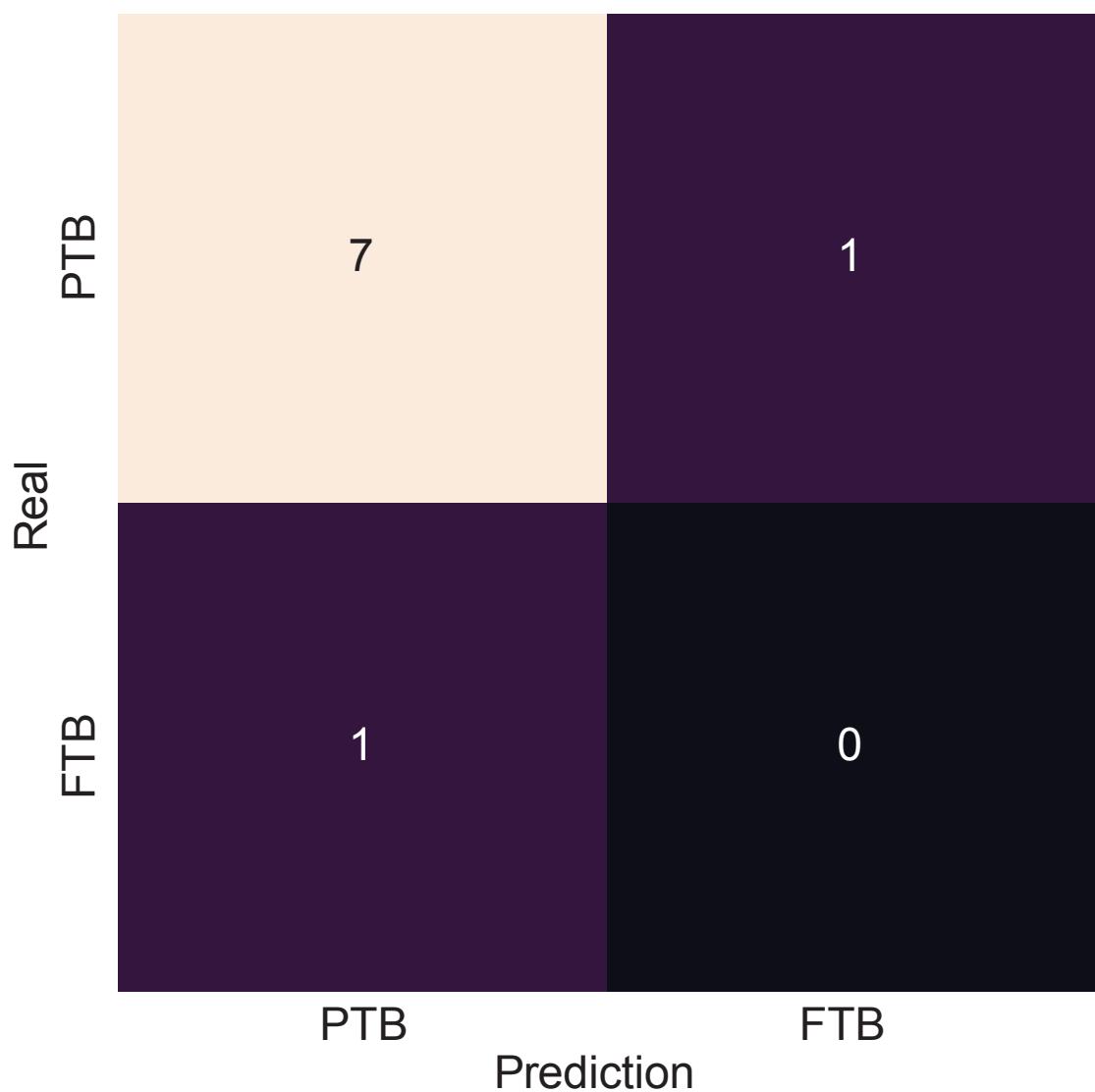


Figure 6: Validation of random forest-based PTB prediction model.

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 ± 0.061 . Mean \pm SD)

378 **2.4 Discussion**

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

386 One of the most common salivary taxa that has been examined is *Fusobacterium nucleatum* (Han,
387 2015; Brennan & Garrett, 2019; Bolstad, Jensen, & Bakken, 1996), that is a Gram-negative, anaerobic, and
388 filamentous bacteria. *Fusobacterium nucleatum* can be separated from not only the salivary microbiome
389 but also the vaginal microbiome (Vander Haar, So, Gyamfi-Bannerman, & Han, 2018; Witkin, 2019). In
390 both animal and human investigation, *Fusobacterium nucleatum* infection has been linked to risk of PTB
391 (Doyle et al., 2014). According to recent researches, the placenta women who give birth prematurely may
392 include additional salivary microbiome dysbiosis, such as *Bergeyella* spp. and *Porphyromonas gingivalis*
393 (León et al., 2007; Katz, Chegini, Shiverick, & Lamont, 2009). Although *Bergeyella* spp. were one of the
394 PROM-overrepresented DAT (Figure 5), it was excluded in the final 25 PTB-related DAT. Furthermore,
395 *Porphyromonas gingivalis* and *Campylobacter gracilis* were pathogens of periodontitis in sub-gingival
396 microbiome (Yang et al., 2022). *Lactobacillus gasseri* was also one of the FTB-enriched DAT (Figure
397 1), and it is well established that early PTB risk can be reduced by *Lactobacillus gasseri* in the vaginal
398 microbiome (Basavaprabhu, Sonu, & Prabha, 2020; Payne et al., 2021).

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

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

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

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

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

430 **3 Random forest prediction model for periodontitis statuses based on the**
431 **salivary microbiomes**

432 **This section includes the published contents:**

433

434 **3.1 Introduction**

435 Saliva microbial dysbiosis brought on by the accumulation of plaque results in periodontitis, a chronic
436 inflammatory disease of the tissue that surrounds the tooth (Kinane, Stathopoulou, & Papapanou, 2017).
437 Loss of periodontal attachment is a consequence of periodontitis, which may lead to irreversible bone loss
438 and, eventually, permanent tooth loss if left untreated. A new classification criterion of periodontal diseases
439 was created in 2018, about 20 years after the 1999 statements of the previous one (Papapanou et al.,
440 2018). Even with this evolution, radiographic and clinical markers of periodontitis progression remain the
441 primary methods for diagnosing periodontitis (Papapanou et al., 2018). Such tools, nevertheless, frequently
442 demonstrate the prior damage from periodontitis rather than its present condition. Certain individuals have
443 a higher risk of periodontitis, a higher chance of developing severe generalized periodontitis, and a worse
444 response to common salivary bacteria control techniques utilized to prevent and treat periodontitis. As a
445 result, the 2017 framework for diagnosing periodontitis additionally allows for the potential development
446 of biomarkers to enhance diagnosis and treatment of periodontitis (Tonetti, Greenwell, & Kornman, 2018).
447 Instead of only depending on the progression of periodontitis, a new etiological indication based on the
448 current state must be introduced in order to enable appropriate intervention through early detection of
449 periodontitis. Thus, the current clinical diagnostic techniques that rely on periodontal probing can be
450 uncomfortable for patients with periodontitis (Canakci & Canakci, 2007).

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

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

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

483 Recently, we employed multiplex quantitative-PCR and machine learning-based classification model
484 to predict the severity of periodontitis based on the amount of nine pathogens of periodontitis from
485 saliva collections (E.-H. Kim et al., 2020). On the other hand, the fact that we focused merely at nine
486 pathogens for periodontitis and neglected the variety bacterial species associated to the various severities
487 of periodontitis constrained the breadth of our investigation. By developing a machine learning model
488 that could classify multiple severities of periodontitis based on the salivary microbiome composition,
489 this study aims to fill these knowledge gaps and produce more accurate and therapeutically useful
490 guidance to evaluate progression of periodontitis. Hence, in order to examine the salivary microbiome
491 composition of both healthy controls and patients with periodontitis in multiple stages, we applied
492 16S rRNA gene sequencing. Furthermore, employing the 2018 classification criteria, we sought to find
493 biomarkers (species) for the precise prediction of periodontitis severities (Papapanou et al., 2018; Chapple
494 et al., 2018).

495 **3.2 Materials and methods**

496 **3.2.1 Study participants enrollment**

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

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

514 **3.2.2 Periodontal clinical parameter diagnosis**

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

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

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

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

532 0. Normal gingiva: without inflammation nor discoloration.

533 1. Mild inflammation: minimal edema and slight color changes, but no bleeding on probing.

534 2. Moderate inflammation: edema, glazing, redness, and bleeding on probing.

535 3. Severe inflammation: significant edema, ulceration, redness, and spontaneous bleeding.

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

539 Periodontitis was diagnosed in respect to the 2018 classification criteria (Papapanou et al., 2018;
540 Chapple et al., 2018). An experienced periodontist diagnosed the periodontitis severity by considering
541 complexity, depending on clinical examinations including radiographic images and periodontal probing.

542 Periodontitis is categorized into healthy, stage I, stage II, and stage III with the following criteria:

543 • Healthy:

544 1. Bleeding sites < 10%

545 2. Probing depth: \leq 3 mm

546 • Stage I:

547 1. No tooth loss because of periodontitis.

548 2. Inter-dental clinical attachment level at the site of the greatest loss: 1-2 mm

549 3. Radiographic bone loss: < 15%

550 • Stage II:

551 1. No tooth loss because of periodontitis.

552 2. Inter-dental clinical attachment level at the site of the greatest loss: 3-4 mm

553 3. Radiographic bone loss: 15-33%

554 • Stage III:

555 1. Teeth loss because of periodontitis: \leq teeth

556 2. Inter-dental clinical attachment level at the site of the greatest loss: \geq 5 mm

557 3. Radiographic bone loss: > 33%

558 **3.2.3 Saliva sampling and DNA extraction procedure**

559 All study participants received instructions to avoid eating, drinking, brushing, and using mouthwash for
560 at least an hour prior to the saliva sample collection process. These collections were conducted between
561 09:00 and 11:00. Mouth rinse was collected by rinsing the mouth for 30 seconds with 12 mL of a solution
562 (E-zen Gargle, JN Pharm, Korea). All saliva samples were tagged with anonymous ID and stored at -4 °C.

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

- 567 • Forward: 5' -TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNNGCWGCAG-3'
568 • Reverse: 5' -GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'

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

- 571 1. Heat activation for 30 seconds at 95 °C.
572 2. 25 cycles for 30 seconds at 95 °C.
573 3. 30 seconds at 55 °C.
574 4. 30 seconds at 72 °C.

575 NexteraXT Indexed Primer was applied to amplification 10 µL of the purified initial PCR products for
576 the final library creation. The second PCR used the same conditions as the first PCR conditions but with
577 10 cycles. 16S rRNA gene sequencing was performed via 2×300 bp paired-end sequencing at Macrogen
578 Inc. (Macrogen, Seoul, Korea) using Illumina MiSeq platform (Illumina, San Diego, CA, USA).

579 **3.2.4 Bioinformatics analysis**

580 We computed alpha-diversity and beta-diversity indices to quantify the divergence of phylogenetic
581 information. Following alpha-diversity indices were calculated using the scikit-bio Python package
582 (version 0.5.5) (Rideout et al., 2018), and these alpha-diversity indices were compared using the MWU
583 test:

- 584 • Abundance-based Coverage Estimator (ACE) (Chao & Lee, 1992)
585 • Chao1 (Chao, 1984)
586 • Fisher (Fisher, Corbet, & Williams, 1943)
587 • Margalef (Magurran, 2021)
588 • Observed ASVs (DeSantis et al., 2006)
589 • Berger-Parker *d* (Berger & Parker, 1970)
590 • Gini index (Gini, 1912)

- Shannon (Weaver, 1963)
- Simpson (Simpson, 1949)

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 the 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.

Differentially abundant taxa (DAT) among multiple periodontitis severities were selected from the salivary microbiome compositions by ANCOM (Lin & Peddada, 2020). In contrast to conventional techniques that examine raw abundance counts, ANCOM applies log-ratio between taxa to account for the salivary microbiome composition data. The log-transformed abundances of DAT were subjected to hierarchical clustering to discover subgroups of DAT with similar patterns on periodontitis severities. Furthermore, we examined the relative proportion among the DAT in order to reduce the effects of other salivary bacteria that differ non-significantly across the multiple periodontitis severities.

As previously stated (E.-H. Kim et al., 2020), we used stratified k -fold cross-validation ($k = 10$) by severity of periodontitis to achieve consistent and trustworthy classification results (Wong & Yeh, 2019). Additionally, we utilized various features with confusion matrices and their derivations to evaluate the classification outcomes in order to identify which features optimize classification evaluations and decrease sequencing efforts. Using the DAT discovered by ANCOM, we iteratively removed the least significant taxa from the input features (taxa) of the random forest classification models using the backward elimination method.

We investigated external datasets from Spanish individuals (Iniesta et al., 2023) and Portuguese individuals (Relvas et al., 2021) to confirm that our random forest classification was consistent. To ascertain repeatability and dependability, the external datasets were processed using the same pipeline and parameters as those used for our study participants.

3.2.5 Data and code availability

All sequences from the 250 study participants have been added to the Sequence Read Archives (project ID PRJNA976179): <https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA976179>. Docker image that employed throughout this study is available in the DockerHub: https://hub.docker.com/repository/docker/fumire/periodontitis_16s. Every code used in this study can be found on GitHub: https://github.com/CompbioLabUnist/Periodontitis_16S.

627 **3.3 Results**

628 **3.3.1 Summary of clinical information and sequencing data**

629 Among clinical information of the study participants, clinical attachment level, probing depth, plaque
630 index, and gingival index, were significantly increased with periodontitis severity (Kruskal-Wallis test
631 $p < 0.001$), while sex were observed no significant difference (Table 2). Notably, clinical attachment level
632 and probing depth have significant differences among the periodontitis severities (MWU test $p < 0.01$;
633 Figure 15). Additionally, 71461.00 ± 11792.30 and 45909.78 ± 11404.65 reads per sample were obtained
634 before and after filtering low-quality reads and trimming extra-long tails, respectively (Figure 16).

635 **3.3.2 Diversity indices reveal differences among the periodontitis severities**

636 Rarefaction curves showed that the sequencing depth was sufficient (Figure 12). Alpha-diversity in-
637 dices indicated significant differences between the healthy and the periodontitis stages (MWU test
638 $p < 0.01$; Figure 7a-e); however, there were no significant differences between the periodontitis stages.
639 This emphasizes how essential it is to classify the salivary microbiome compositions and distinguish
640 between the stages of periodontitis using machine learning approaches.

641 The confidence ellipses of the tSNE-transformed beta-diversity index (Aitchison index) indicated
642 distinct distributions among the periodontitis severities (PERMANOVA $p \leq 0.001$; Figure 7f). Aitchison
643 index demonstrated significant differences every pairwise of the periodontitis severities (PERMANOVA
644 test $p \leq 0.001$; Table 7). Significant differences in the distances between periodontitis severities further
645 demonstrated the uniqueness of each severity of periodontitis (MWU test $p \leq 0.05$; Figure 7g-j).

646 **3.3.3 DAT among multiple periodontitis severities and their correlation**

647 Of the 425 total taxa that identified in the salivary microbiome composition (Figure 13), 20 DAT were
648 identified (Table 5). Three separate subgroups were formed from the participants-level abundances of the
649 DAT using a hierarchical clustering methodology (Figure 8a):

- 650 • Group 1
- 651 1. *Treponema* spp.
- 652 2. *Prevotella* sp. HMT 304
- 653 3. *Prevotella* sp. HMT 526
- 654 4. *Peptostreptococcaceae [XI][G-5] saphenum*
- 655 5. *Treponema* sp. HMT 260
- 656 6. *Mycoplasma faecium*
- 657 7. *Peptostreptococcaceae [XI][G-9] brachy*
- 658 8. *Lachnospiraceae [G-8] bacterium* HMT 500
- 659 9. *Peptostreptococcaceae [XI][G-6] nodatum*
- 660 10. *Fretibacterium* spp.

- 661 • Group 2
- 662 1. *Porphyromonas gingivalis*
- 663 2. *Campylobacter showae*
- 664 3. *Filifactor alocis*
- 665 4. *Treponema putidum*
- 666 5. *Tannerella forsythia*
- 667 6. *Prevotella intermedia*
- 668 7. *Porphyromonas* sp. HMT 285

- 669 • Group 3
- 670 1. *Actinomyces* spp.
- 671 2. *Corynebacterium durum*
- 672 3. *Actinomyces graevenitzii*

673 Ten DAT that were significant enriched in stage II and stage III, but deficient in healthy formed Group
674 1. Furthermore, in comparison to the healthy, the seven DAT of Group 2 were significantly enriched in
675 each of the stages of periodontitis. On the other hand, three DAT in Group 3 were deficient in stage II
676 and stage III, but significantly enriched in healthy. The relative proportions of the DAT further supported
677 these findings (Figure 8b), suggesting that the DAT is primarily linked to periodontitis rather than other
678 salivary bacteria.

679 Correlation analysis from the DAT showed that DAT from Group 3 was negatively correlated with
680 Group 1 and Group 2 (Figure 9), and strong correlations were observed the nine pairs of DAT (Figure 14).

681 3.3.4 Classification of periodontitis severities by random forest models

682 Based on the proportion of DAT, random forest classifier were trained to classify the periodontitis
683 severities (Table 6). First of all, we conducted multi-label classification for the multiple periodontitis
684 severities, namely healthy, stage I, stage II, and stage III. In this setting, we classified multiple periodontitis
685 severities with the highest BA of 0.779 ± 0.029 (Table 4). AUC ranged between 0.81 and 0.94 (Figure
686 10b).

687 Second, since timely detection in dentistry is demanding (Tonetti et al., 2018), we implemented a
688 random forest classification for both healthy and stage I. Remarkably, the random forest classifier had
689 the highest BA at 0.793 ± 0.123 (Table 4). In this setting, this model showed high AUC value for the
690 classifying of stage I from healthy (AUC=0.85; Figure 10d).

691 Third, based on the findings that the salivary microbiome composition in stage II is more comparable
692 to those in stage III than to other severities (Figure 7f and Figure 7j), we combined stage II and stage III
693 to perform a multi-label classification.

Table 3: Clinical characteristics of the study subjects.

Significant differences were assessed using the Kruskal-Wallis test. NA: Not applicable.

Index	Healthy	Stage I	Stage II	Stage III	p-value
Age (year)	33.83±13.04	43.30±14.28	50.26±11.94	51.08±11.13	6.18E-17
Gender (Male)	44 (44.0%)	22 (44.0%)	25 (50.0%)	25 (50.0%)	NA
Smoking (Never)	83 (83.0%)	36 (72.0%)	34 (68.0%)	29 (58.0%)	NA
Smoking (Ex)	12 (12.0%)	7 (14.0%)	9 (18.0%)	10 (20.0%)	NA
Smoking (Current)	2 (2.0%)	7 (14.0%)	7 (14.0%)	10 (20.0%)	NA
Number of teeth	28.03±2.23	27.36±1.80	26.72±2.89	25.74±4.34	8.07E-05
Attachment level (mm)	2.45±0.29	2.75±0.38	3.64±0.83	4.54±1.14	1.82E-35
Probing depth (mm)	2.42±0.29	2.61±0.40	3.27±0.76	3.95±0.88	6.43E-28
Plaque index	17.66±16.21	35.46±23.75	54.40±23.79	58.30±25.25	3.23E-22
Gingival index	0.09±0.16	0.44±0.46	0.85±0.52	1.06±0.52	2.59E-32

Table 4: 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.

Classification	Features	ACC	AUC	BA	F1	PRE	SEN	SPE
Healthy vs. Stage I vs. Stage II vs. Stage III	<i>P.gingivalis</i>	0.758±0.051	0.716±0.177	0.677±0.068	0.839±0.034	0.839±0.034	0.516±0.102	
	<i>P.gingivalis+Act.</i>	0.792±0.043	0.822±0.105	0.723±0.057	0.861±0.029	0.861±0.029	0.584±0.086	
Top 5 taxa		0.834±0.022	0.870±0.079	0.779±0.029	0.889±0.015	0.889±0.015	0.668±0.033	
Healthy vs. Stage I	<i>Act.</i>	0.687±0.116	0.725±0.145	0.647±0.159	0.762±0.092	0.760±0.128	0.781±0.116	0.513±0.224
	<i>Act.+P.gingivalis</i>	0.733±0.119	0.831±0.081	0.713±0.122	0.797±0.097	0.797±0.126	0.798±0.082	0.627±0.191
Top 9 taxa		0.800±0.103	0.852±0.103	0.793±0.123	0.849±0.080	0.850±0.112	0.857±0.090	0.730±0.193
Healthy vs. Stage I vs. Stages II/III	<i>P.gingivalis</i>	0.776±0.042	0.736±0.196	0.748±0.047	0.832±0.031	0.832±0.031	0.664±0.062	
	<i>P.gingivalis+Act.</i>	0.843±0.035	0.876±0.109	0.823±0.039	0.882±0.026	0.882±0.026	0.764±0.052	
Top 6 taxa		0.885±0.036	0.914±0.027	0.871±0.038	0.914±0.027	0.914±0.025	0.828±0.051	
Healthy vs. Stages I/II/III	<i>P.gingivalis</i>	0.792±0.114	0.856±0.105	0.819±0.088	0.776±0.089	0.840±0.092	0.756±0.175	0.883±0.054
	<i>P.gingivalis+Act.</i>	0.828±0.121	0.926±0.074	0.847±0.116	0.797±0.123	0.800±0.126	0.830±0.191	0.864±0.074
Top 4 taxa		0.860±0.078	0.953±0.049	0.885±0.066	0.832±0.079	0.840±0.128	0.864±0.157	0.905±0.070

Table 5: List of DAT among healthy status and periodontitis stages

No.	Taxonomy	ANCOM W score
1	<i>Porphyromonas gingivalis</i>	424
2	<i>Actinomyces</i> spp.	424
3	<i>Filifactor alocis</i>	421
4	<i>Prevotella intermedia</i>	419
5	<i>Treponema putidum</i>	418
6	<i>Tannerella forsythia</i>	415
7	<i>Porphyromonas</i> sp. HMT 285	412
8	<i>Peptostreptococcaceae [XI][G-6] nodatum</i>	412
9	<i>Fretibacterium</i> spp.	411
10	<i>Mycoplasma faecium</i>	411
11	<i>Prevotella</i> sp. HMT 304	411
12	<i>Lachnospiraceae [G-8] bacterium</i> HMT 500	409
13	<i>Treponema</i> spp.	408
14	<i>Prevotella</i> sp. HMT 526	401
15	<i>Peptostreptococcaceae [XI][G-9] brachy</i>	400
16	<i>Peptostreptococcaceae [XI][G-5] saphenum</i>	398
17	<i>Campylobacter showae</i>	395
18	<i>Treponema</i> sp. HMT 260	393
19	<i>Corynebacterium durum</i>	393
20	<i>Actinomyces graevenitzii</i>	387

Table 6: 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.

Condition	Healthy vs. Stage I vs. Stage II vs. Stage III			Healthy vs. Stage I			Healthy vs. Stage I vs. Stage II/III			Healthy vs. Stage VII/III		
	Rank	Taxa	Importance	Taxa	Importance	Taxa	Importance	Taxa	Importance	Taxa	Importance	
1	<i>Porphyromonas gingivalis</i>	0.297	<i>Actinomyces spp.</i>	0.195	<i>Porphyromonas gingivalis</i>	0.360	<i>Porphyromonas gingivalis</i>	0.426	<i>Porphyromonas gingivalis</i>	0.461		
2	<i>Actinomyces spp.</i>	0.195	<i>Actinomyces graevenitzii</i>	0.054	<i>Actinomyces spp.</i>	0.125	<i>Actinomyces spp.</i>	0.244	<i>Actinomyces spp.</i>	0.257		
3	<i>Prevotella intermedia</i>	0.054	<i>Actinomyces graevenitzii</i>	0.052	<i>Porphyromonas sp. HMT 285</i>	0.055	<i>Actinomyces graevenitzii</i>	0.049	<i>Actinomyces spp.</i>	0.059		
4	<i>Actinomyces graevenitzii</i>	0.052	<i>Lachnospiraceae (G-8) bacterium HMT 500</i>	0.050	<i>Lachnospiraceae (G-8) bacterium HMT 500</i>	0.052	<i>Corynebacterium durum</i>	0.046	<i>Corynebacterium durum</i>	0.035		
5	<i>Filifactor alocis</i>	0.050	<i>Campylobacter showae</i>	0.042	<i>Campylobacter showae</i>	0.050	<i>Prevotella intermedia</i>	0.036	<i>Filifactor alocis</i>	0.032		
6	<i>Campylobacter showae</i>	0.042	<i>Filifactor alocis</i>	0.040	<i>Corynebacterium durum</i>	0.052	<i>Tannerella forsythia</i>	0.039	<i>Campylobacter showae</i>	0.023		
7	<i>Porphyromonas sp. HMT 285</i>	0.040	<i>Treponema spp.</i>	0.032	<i>Treponema spp.</i>	0.037	<i>Treponema spp.</i>	0.023	<i>Prevotella intermedia</i>	0.022		
8	<i>Corynebacterium durum</i>	0.032	<i>Tannerella forsythia</i>	0.026	<i>Tannerella forsythia</i>	0.029	<i>Treponema spp.</i>	0.021	<i>Prevotella intermedia</i>	0.022		
9	<i>Treponema spp.</i>	0.032	<i>Prevotella intermedia</i>	0.025	<i>Prevotella intermedia</i>	0.026	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.018	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.015		
10	<i>Tannerella forsythia</i>	0.026	<i>Freibacterium spp.</i>	0.023	<i>Freibacterium spp.</i>	0.018	<i>Lachnospiraceae (G-8) bacterium HMT 500</i>	0.014	<i>Lachnospiraceae (G-8) bacterium HMT 500</i>	0.010		
11	<i>Treponema putidum</i>	0.025	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.021	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.018	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.011	<i>Tannerella forsythia</i>	0.009		
12	<i>Freibacterium spp.</i>	0.023	<i>Treponema putidum</i>	0.019	<i>Treponema putidum</i>	0.014	<i>Treponema putidum</i>	0.010	<i>Freibacterium spp.</i>	0.009		
13	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.021	<i>Prevotella sp. HMT 526</i>	0.018	<i>Prevotella sp. HMT 526</i>	0.011	<i>Prevotella sp. HMT 526</i>	0.009	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.006		
14	<i>Treponema sp. HMT 260</i>	0.019	<i>Prevotella sp. HMT 526</i>	0.018	<i>Prevotella sp. HMT 260</i>	0.008	<i>Freibacterium spp.</i>	0.008	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.004		
15	<i>Prevotella sp. HMT 526</i>	0.018	<i>Prevotella sp. HMT 260</i>	0.017	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.008	<i>Treponema sp. HMT 260</i>	0.008	<i>Treponema sp. HMT 260</i>	0.004		
16	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.018	<i>Mycoplasma faecium</i>	0.014	<i>Mycoplasma faecium</i>	0.004	<i>Prevotella sp. HMT 304</i>	0.005	<i>Mycoplasma faecium</i>	0.004		
17	<i>Prevotella sp. HMT 304</i>	0.017	<i>Prevotella sp. HMT 304</i>	0.014	<i>Prevotella sp. HMT 304</i>	0.003	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.003	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.002		
18	<i>Mycoplasma faecium</i>	0.014	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.013	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.003	<i>Prevotella sp. HMT 304</i>	0.004	<i>Prevotella sp. HMT 304</i>	0.001		
19	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.013	<i>Lachnospiraceae (G-8) bacterium HMT 500</i>									
20	<i>Lachnospiraceae (G-8) bacterium HMT 500</i>											

Table 7: Beta-diversity pairwise comparisons on the periodontitis statuses

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

Group 1	Group 2	p-value
Healthy	Stage I	0.001
Healthy	Stage II	0.001
Healthy	Stage III	0.001
Stage I	Stage II	0.001
Stage I	Stage III	0.001
Stage II	Stage III	0.737

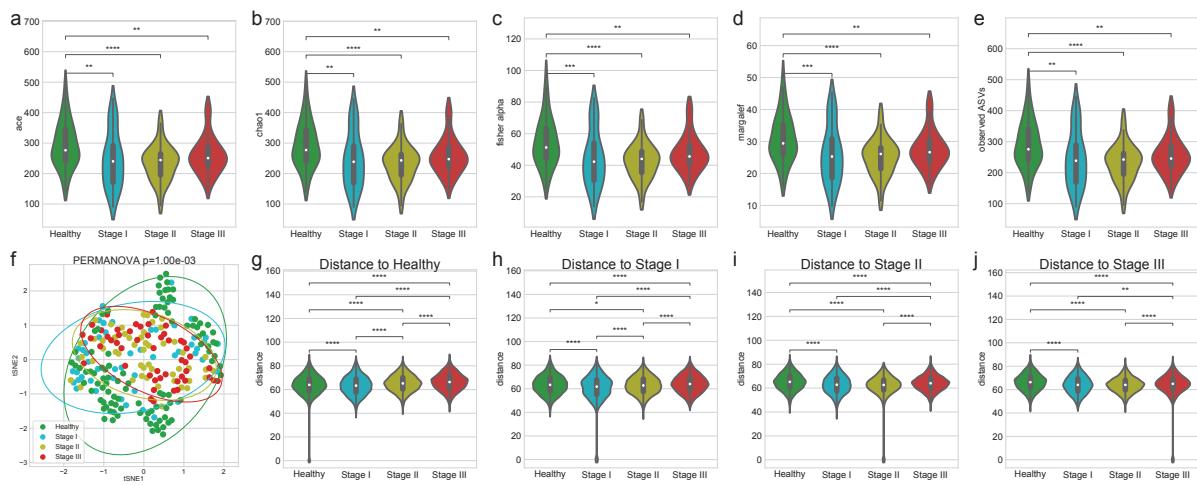


Figure 7: Diversity indices.

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 $p \leq 0.0001$ (****).

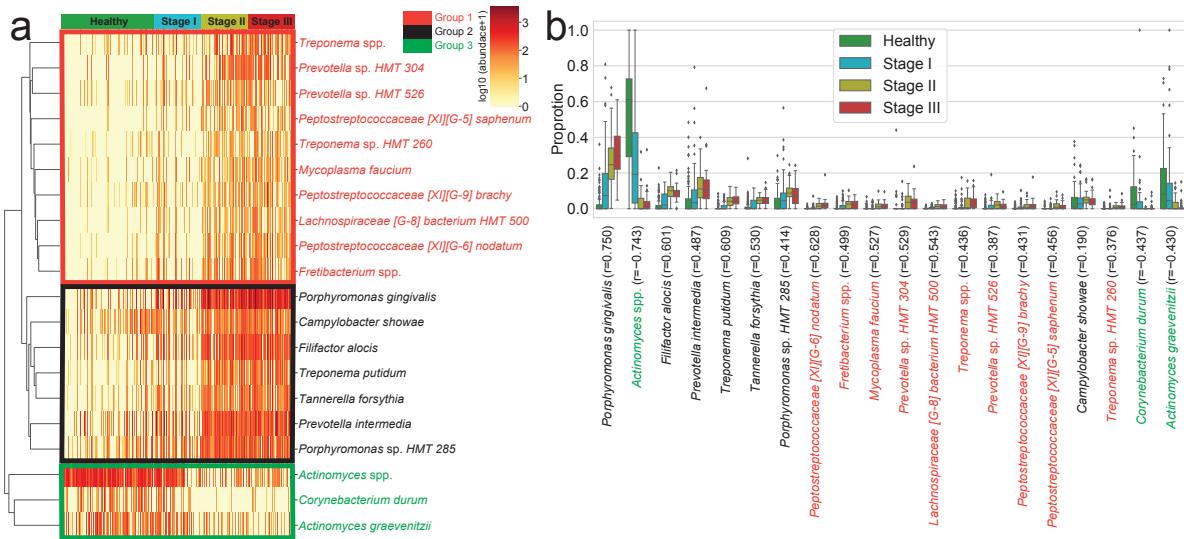


Figure 8: **Differentially abundant taxa (DAT).**

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.

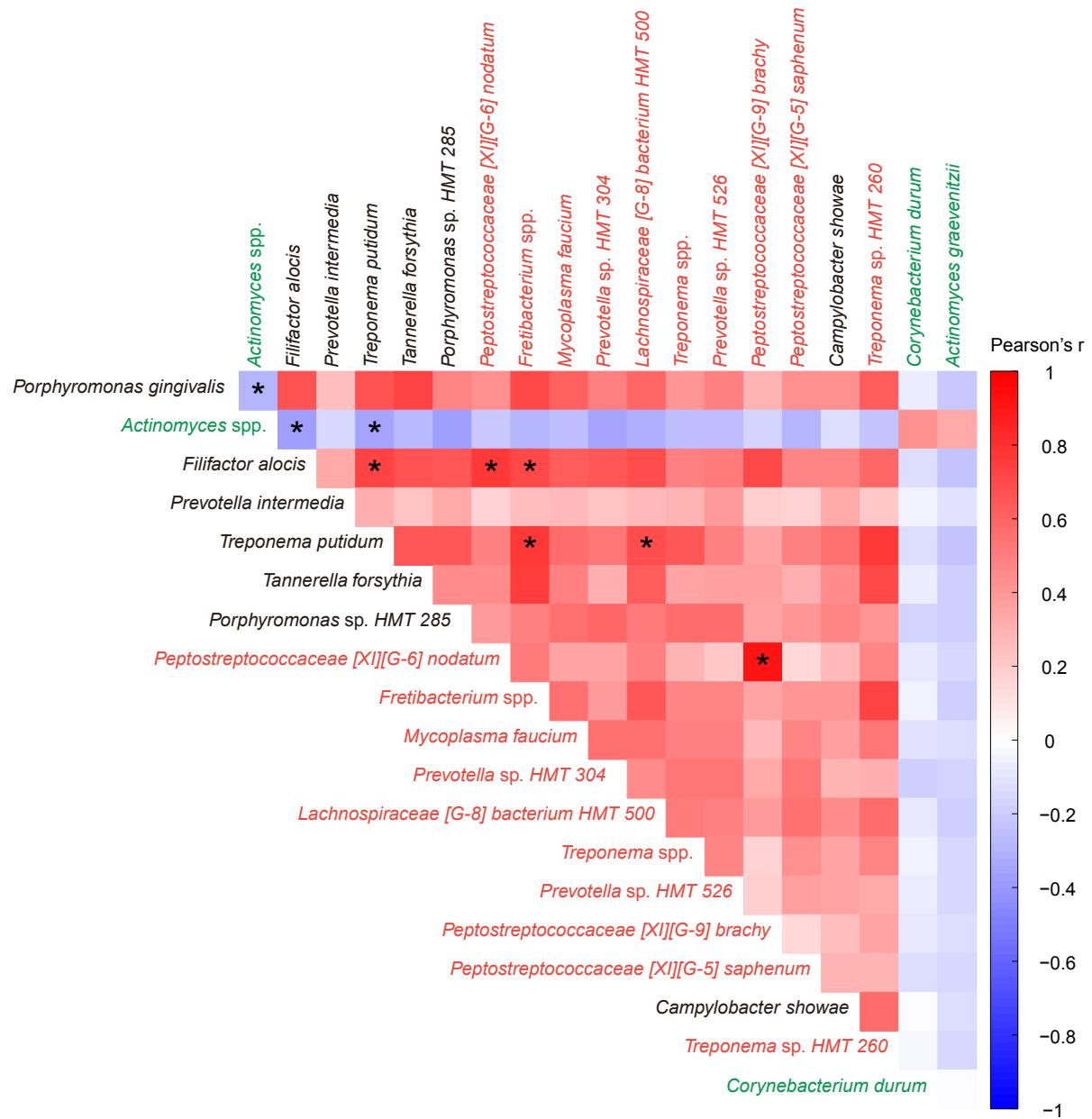


Figure 9: Correlation heatmap.

Pearson's correlations between DAT in healthy status and periodontitis stages. Statistical significance was determined by strong correlation, i.e., $| \text{coefficient} | \geq 0.5$ (*).

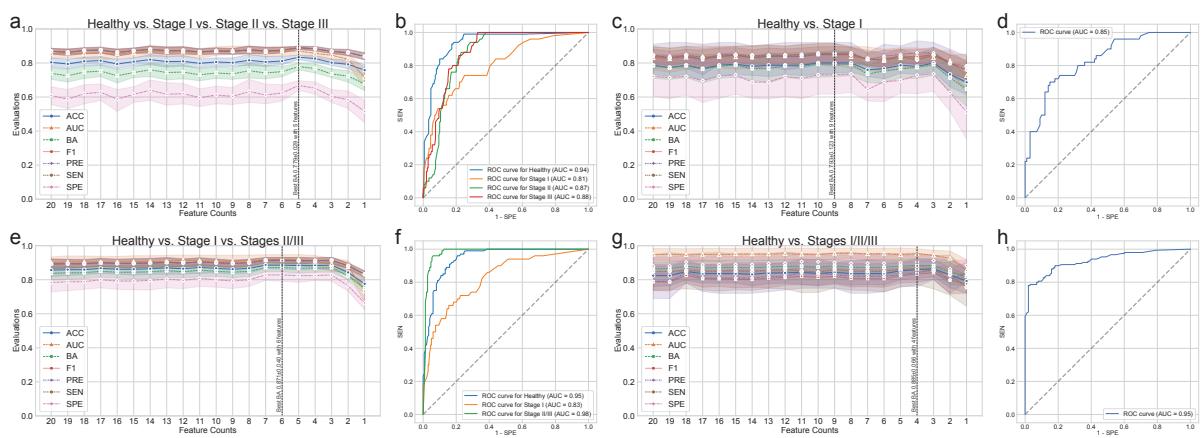


Figure 10: Random forest classification metrics.

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).

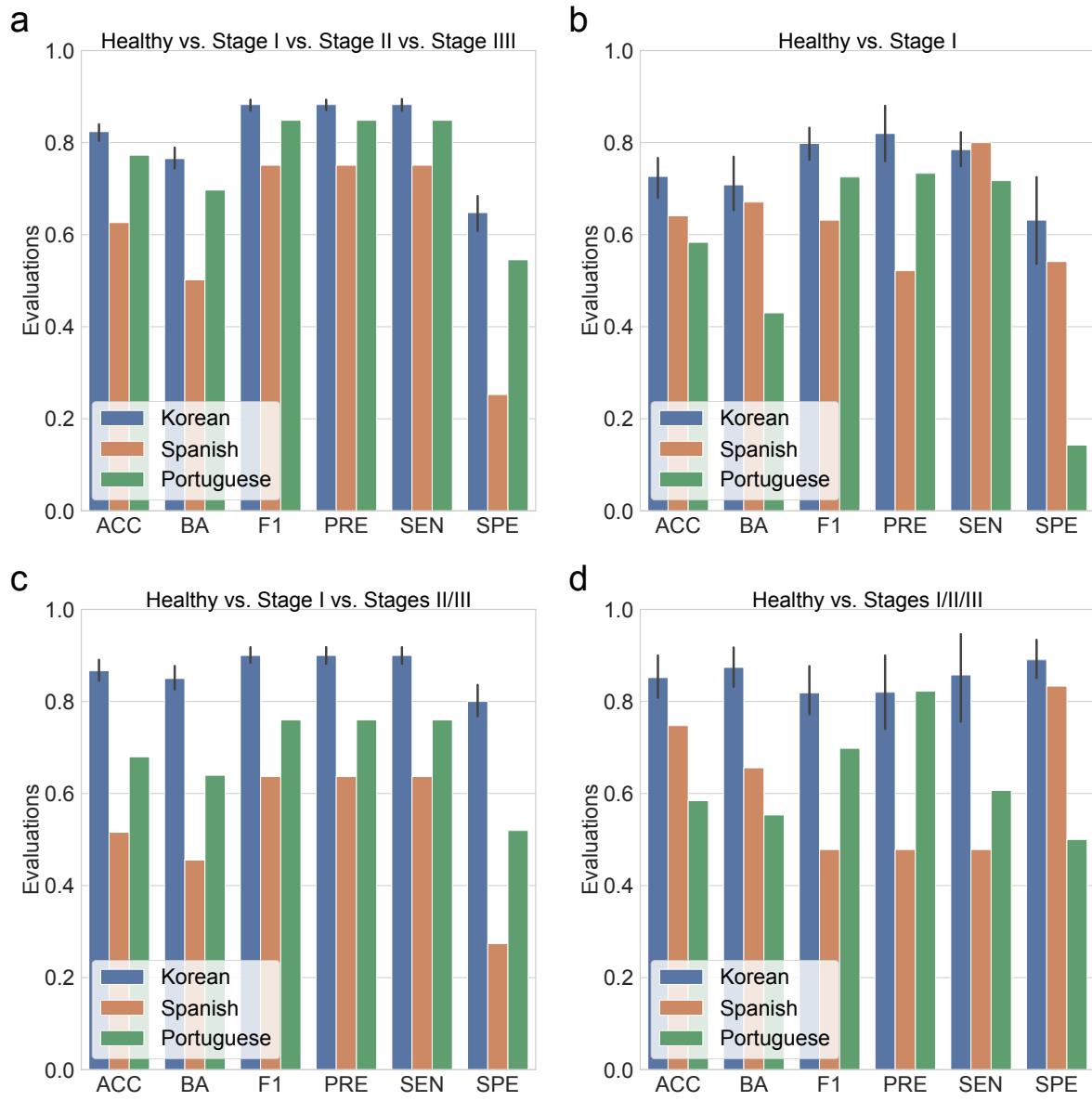


Figure 11: **Random forest classification metrics from external datasets.**

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.

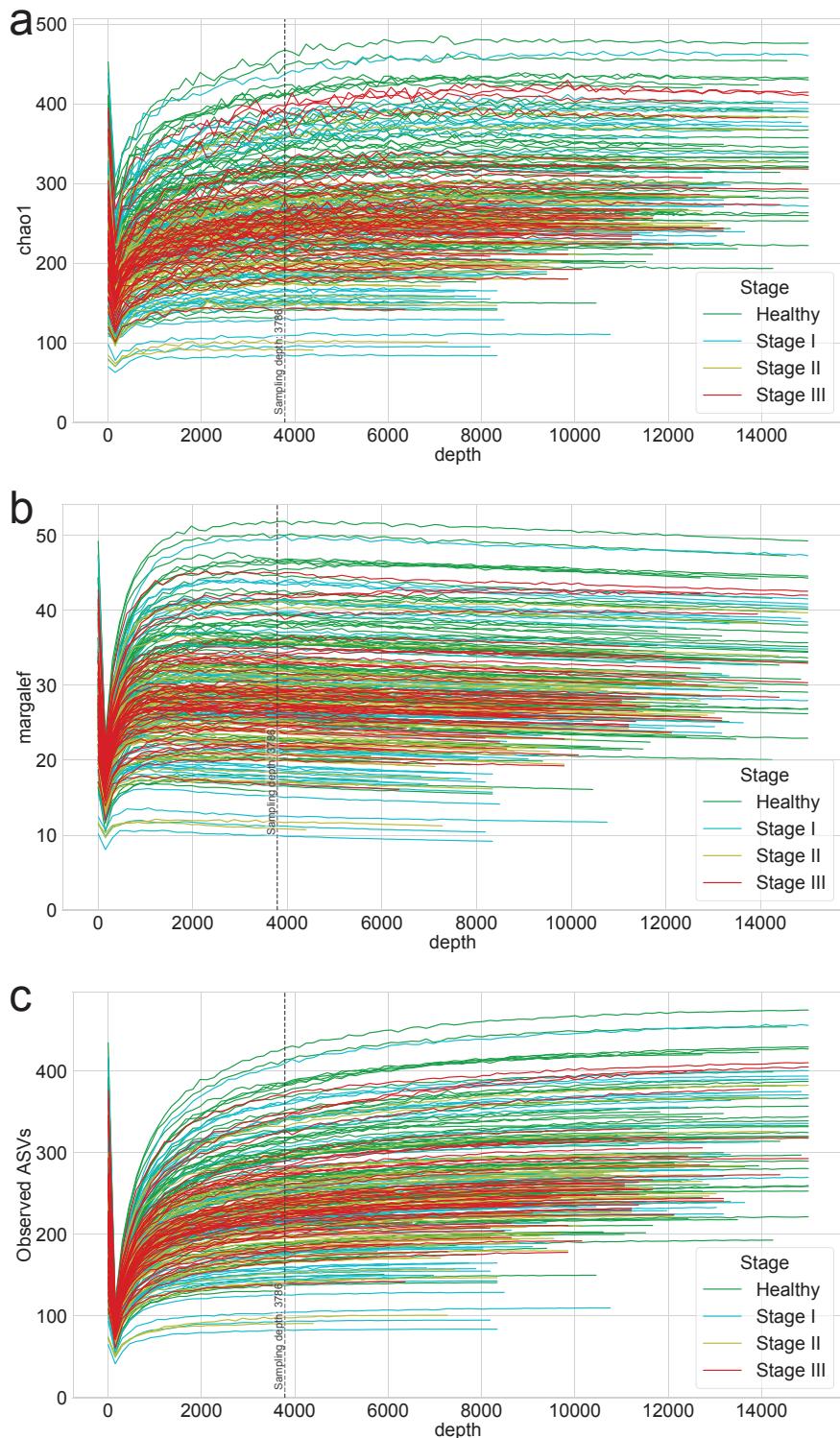


Figure 12: Rarefaction curves for alpha-diversity indices.

Rarefaction of (a) chao1 (b) margalef, and (c) observed ASVs were generated to measure species richness and determine the sampling depth of each sample.

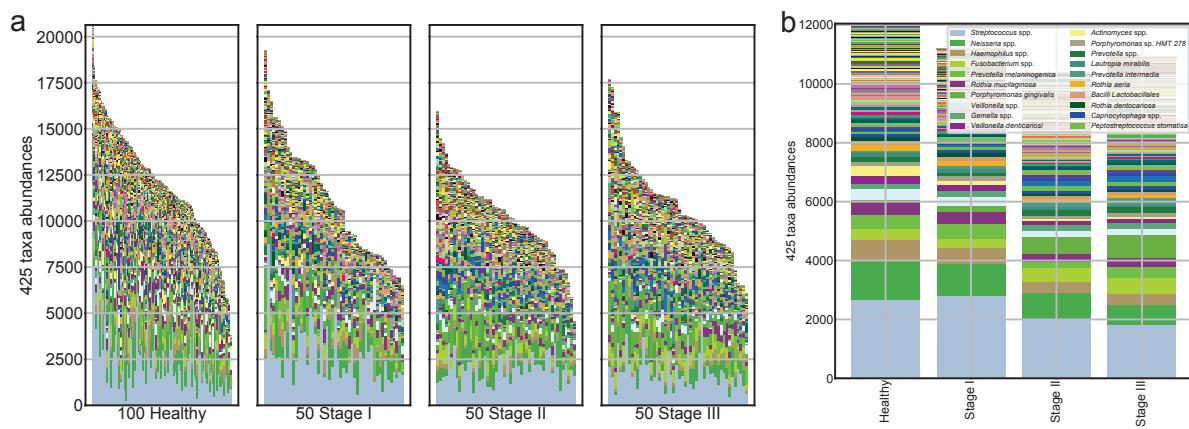


Figure 13: Salivary microbiome compositions in the different periodontal statuses.

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).

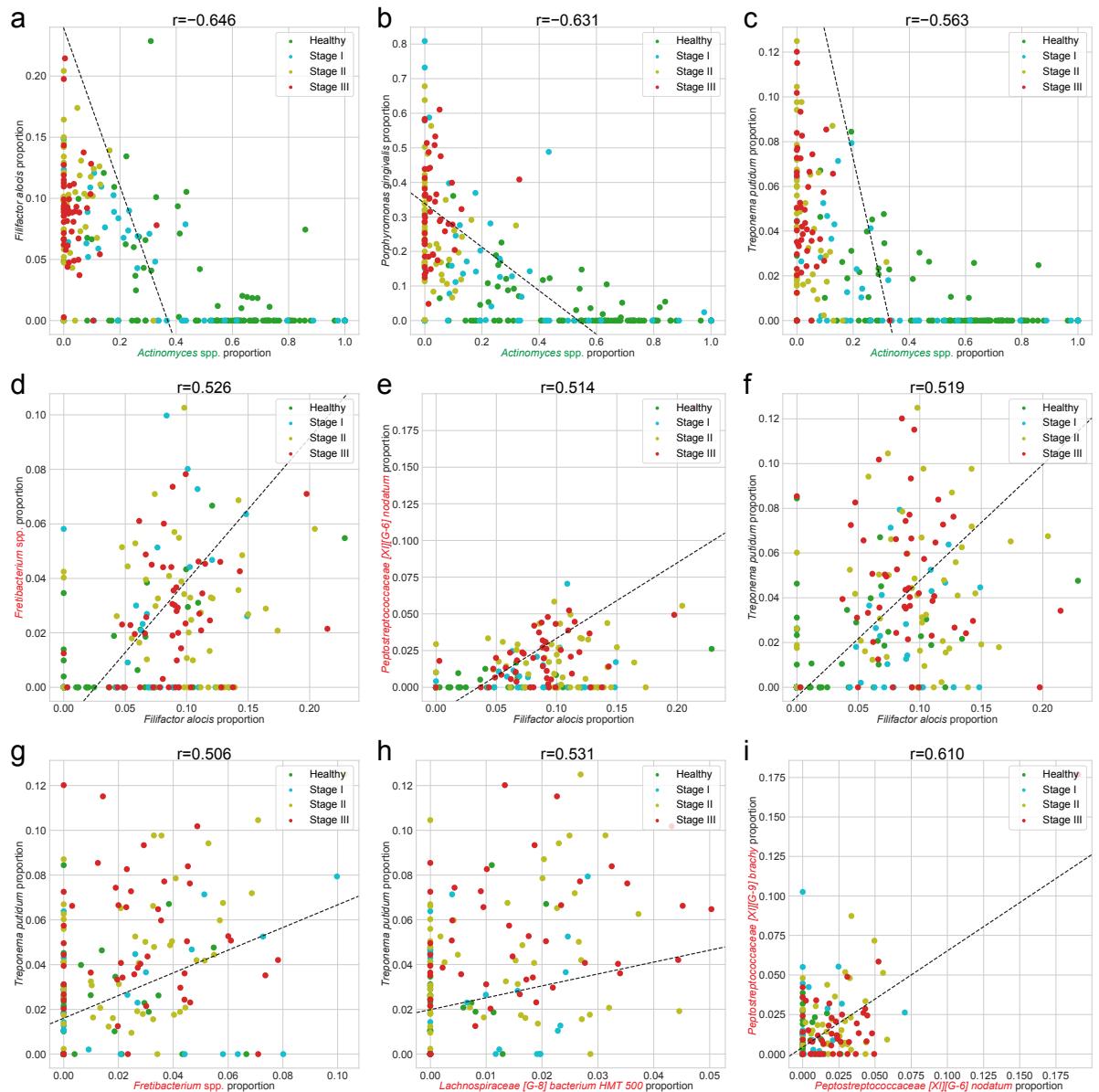


Figure 14: Correlation plots for differentially abundant taxa.

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).

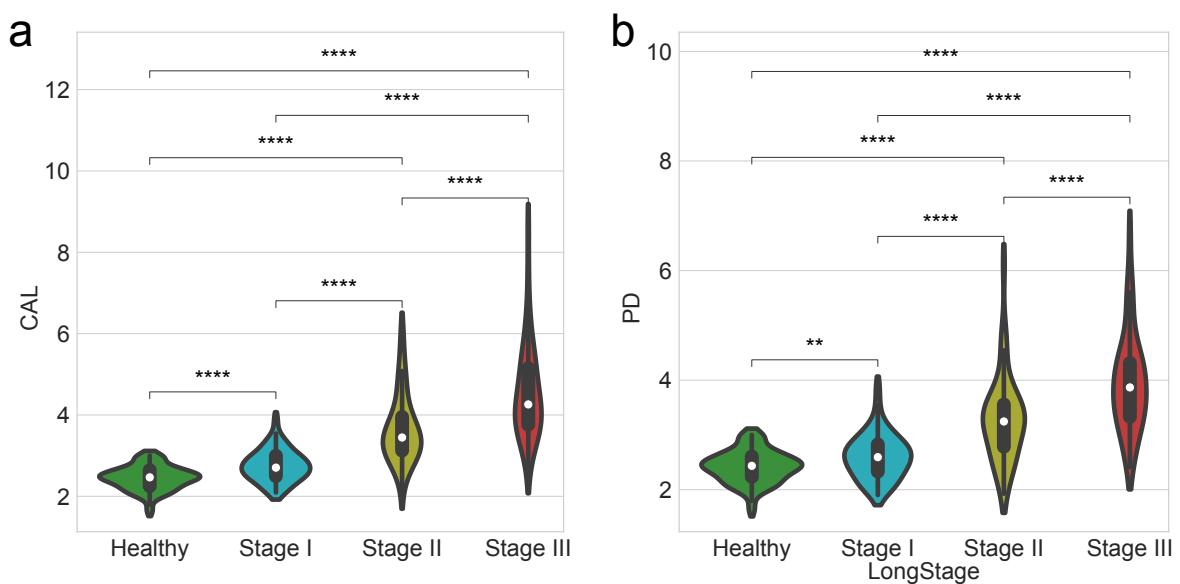


Figure 15: **Clinical measurements by the periodontitis statuses.**

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 $p \leq 0.0001$ (****).

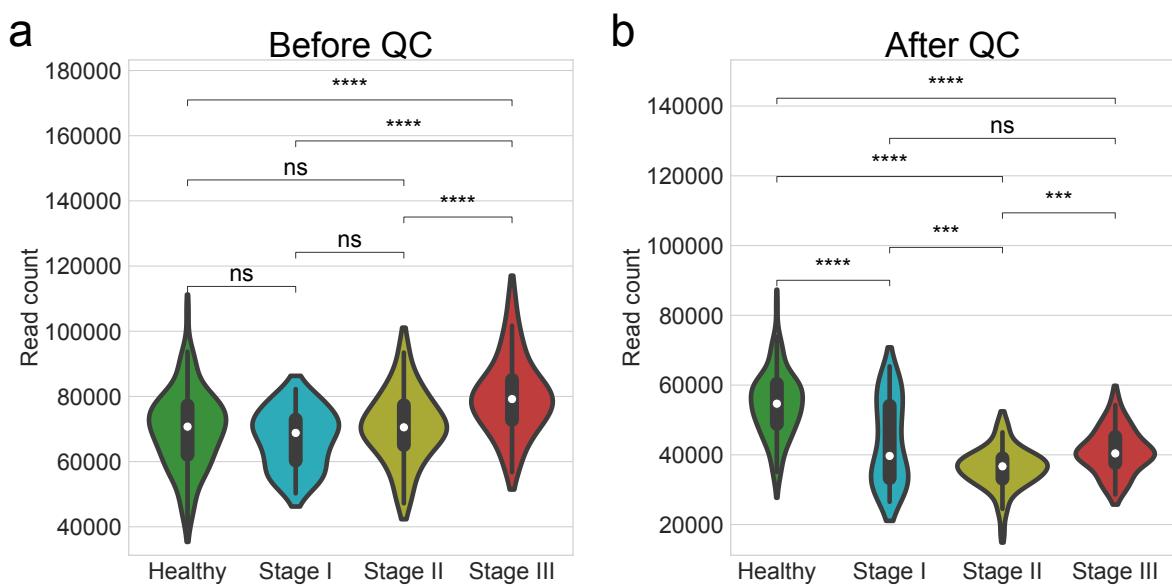


Figure 16: **Number of read counts by the periodontitis statuses.**

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 MWU test: $p > 0.05$ (ns), $p \leq 0.001$ (***) , and $p \leq 0.0001$ (****).

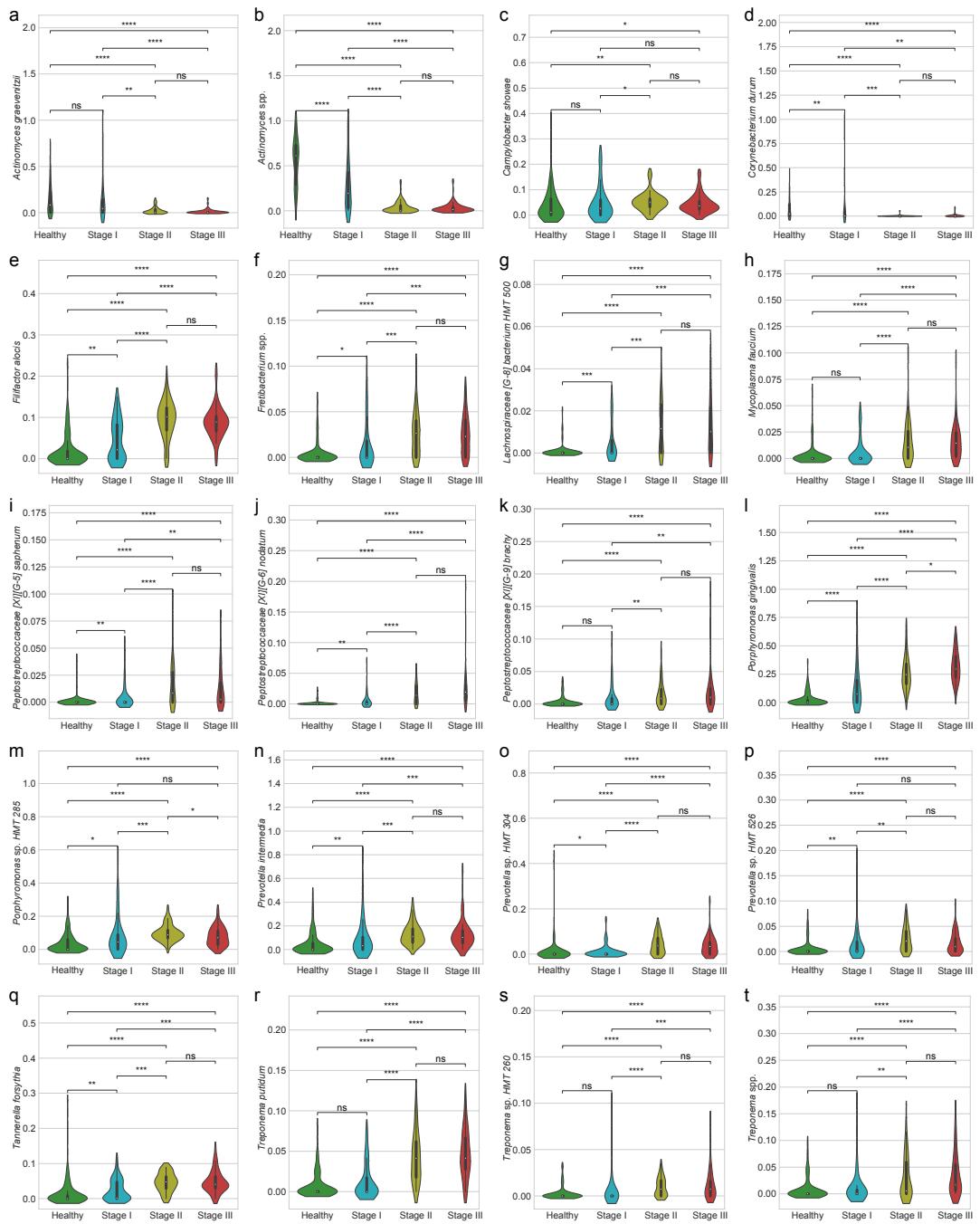


Figure 17: Proportion of DAT.

(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 $p \leq 0.0001$ (****).

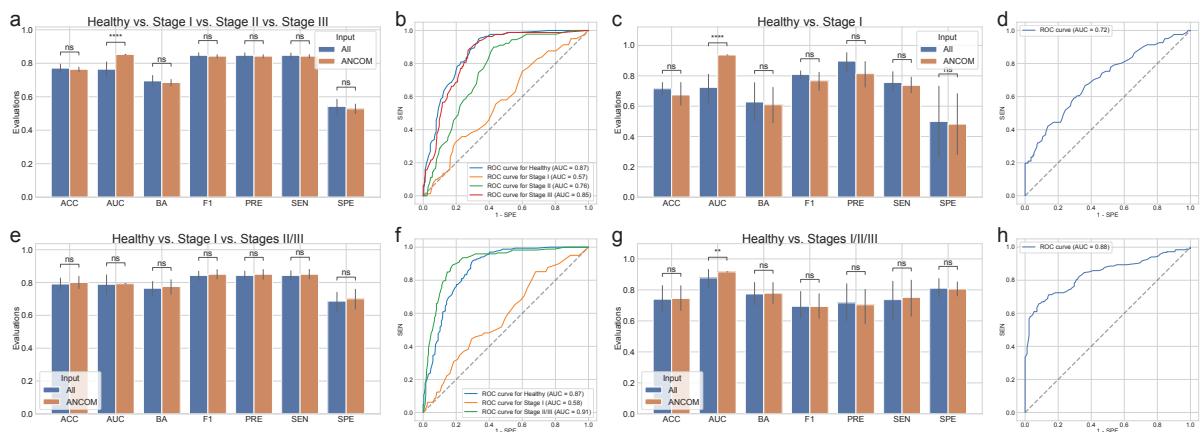


Figure 18: Random forest classification metrics with the full microbiome compositions and ANCOM-selected DAT compositions.

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 $p \leq 0.0001$ (****).

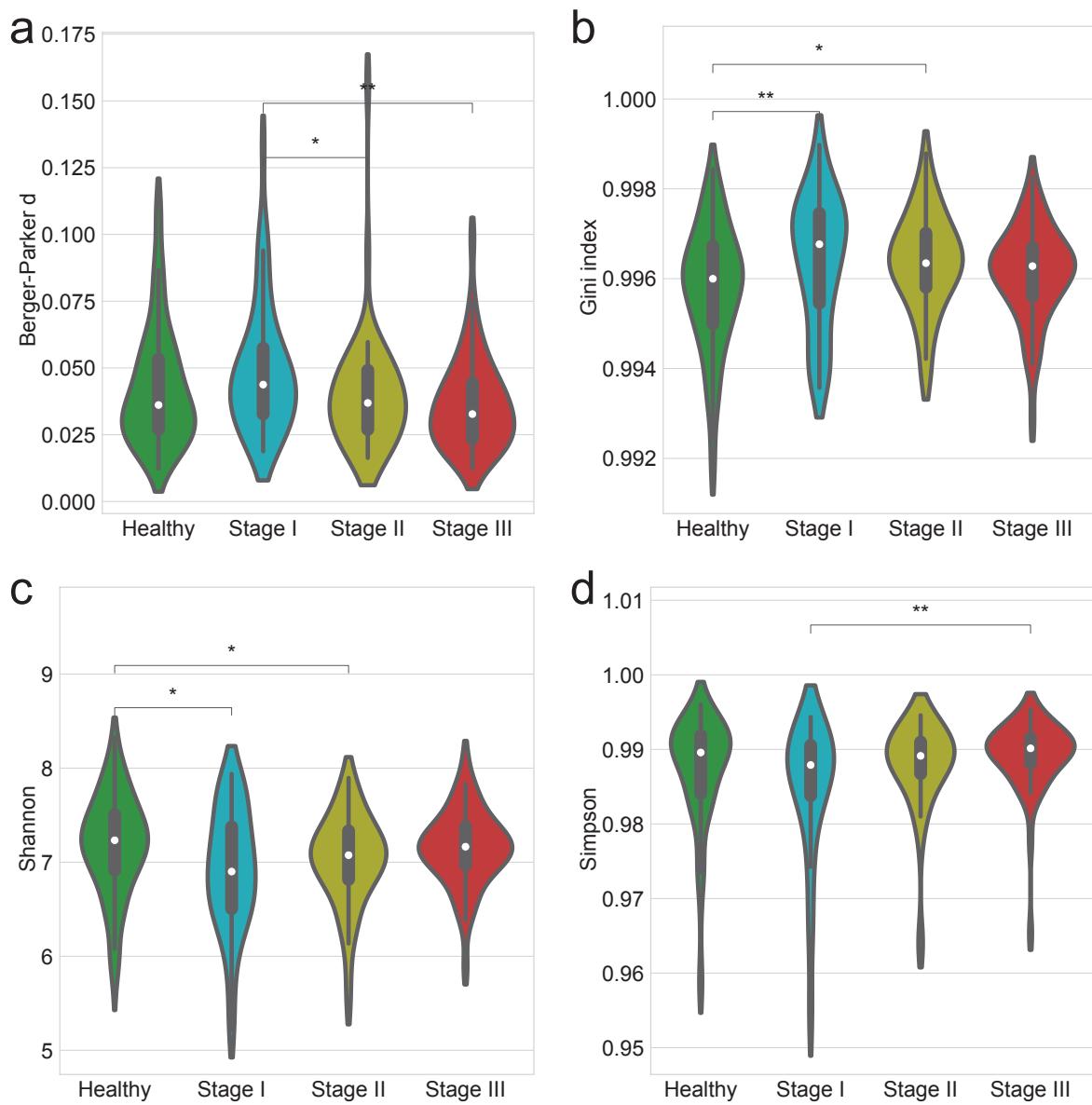


Figure 19: **Alpha-diversity indices account for evenness.**

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 $p \leq 0.01$ (**)

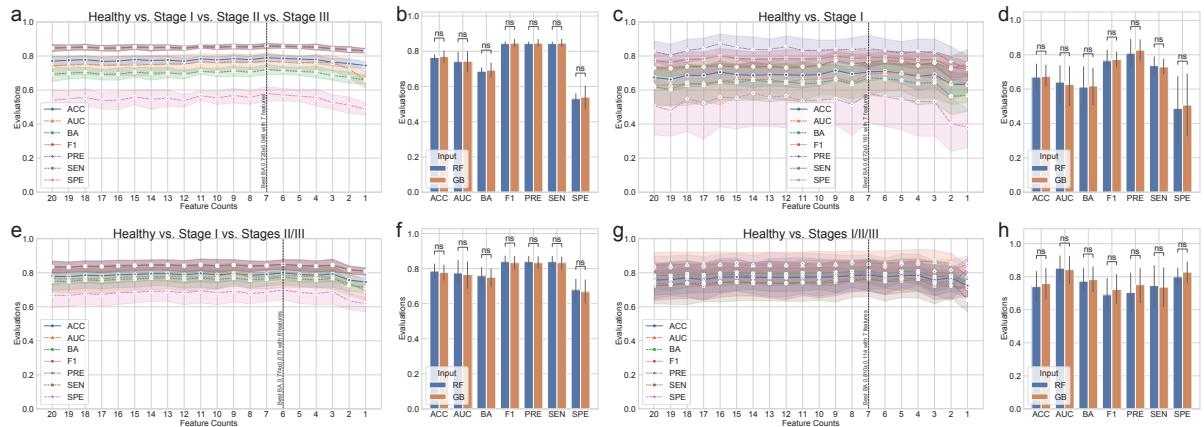


Figure 20: Gradient Boosting classification metrics.

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 5. **(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.

694 **3.4 Discussion**

695 In order to investigate at potential alterations in the salivary microbiome compositions based on periodontal
696 statuses, including healthy, stage I, stage II, and stage III, we employed 16S rRNA gene sequencing to
697 perform a cross-sectional periodontitis analysis. In this study, the 2018 periodontitis classification served
698 as the basis for the classification of periodontitis severities (Papapanou et al., 2018). There were notable
699 variations in the salivary microbiome composition among the multiple severities of periodontitis (Figure
700 13). Furthermore, our random forest classification model based on the proportions of DAT in the salivary
701 microbiome compositions across study participants to predict multiple periodontitis statuses with high
702 AUC of 0.870 ± 0.079 (Table 4).

703 Previous research identified the red complex as the primary pathogens of periodontitis (Listgarten,
704 1986): *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. Other studies, however,
705 have shown that periodontal pathogens communicate with other bacteria in the salivary microbiome
706 networks to generate dental plaque prior to the pathogenesis and development of periodontitis (Lamont &
707 Jenkinson, 2000; Rosan & Lamont, 2000; Yoshimura, Murakami, Nishikawa, Hasegawa, & Kawaminami,
708 2009).

709 Using subgingival plaque collections, recent researches have suggested a connection between the
710 periodontitis severity and the salivary microbiome compositions (Altabtbaei et al., 2021; Iniesta et al.,
711 2023; Nemoto et al., 2021). Therefore, we have examined the salivary microbiome compositions of
712 patients with multiple severities of periodontitis and periodontally healthy controls, extending on earlier
713 studies.

714 According to our findings, the salivary microbiome compositions have 425 taxa (Figure 13). We
715 computed the alpha-diversity indices to determine the variability within each salivary microbiome
716 composition, including ace (Chao & Lee, 1992), chao1 (Chao, 1984), fisher alpha (Fisher et al., 1943),
717 margalef (Magurran, 2021), observed ASVs (DeSantis et al., 2006), Berger-Parker *d* (Berger & Parker,
718 1970), Gini index (Gini, 1912), Shannon (Weaver, 1963), and Simpson (Simpson, 1949) (Figure 7 and
719 Figure 19). Alpha-diversity indices suggested that the microbial richness of periodontally healthy controls
720 was higher than that of patients with periodontitis (Figure 7a-e and Figure 19). These results are in line with
721 findings with that patients with advanced periodontitis, namely stage II and stage III, have less diversified
722 communities than periodontally healthy controls (Jorth et al., 2014). Recognizing that the periodontitis
723 severity increases the amount of *Porphyromonas gingivalis*, the salivary microbiome compositions from
724 periodontally healthy controls conserved microbial networks dominated by *Streptococcus* spp. (Figure
725 13). *Porphyromonas gingivalis* is one of the known periodontal pathogen that could cause dysbiosys
726 in the salivary microbiomes, suggesting in the pathophysiology of periodontitis. Despite this finding,
727 earlier research found that subgingival microbiome of patients with periodontitis had a greater alpha-
728 diversity index (observed ASVs) than that of healthy controls (Iniesta et al., 2023), might due to the
729 different sampling sites between saliva and subgingival plaque. On the other hand, another research
730 has addressed significant discrepancies in alpha-diversity indices from subgingival plaque, saliva, and
731 tongue biofilms from healthy controls and periodontitis patients, resulting the highest alpha-diversity

732 index in saliva collections (Belstrøm et al., 2021). Moreover, early-stage periodontitis, namely stage I,
733 did not determine statisticall ysiginificant differences in alpha-diversity indices compared to advanced
734 periodontitis, including stage II and stage III (Figure 7a-e). Accordingly, saliva collection of stage I
735 periodontitis may exhibit heterogeneity, indicating a midpoint condition between a healthy state and
736 advanced periodontitis (stage II and stage III). Likewise, gingivitis is often associated with low abundances
737 of the majority of periodontal pathogens, including *Porphyromonas gingivalis*, *Tannerella forsythia*, and
738 *Treponema denticola* (Abusleme et al., 2021). Compared to healthy controls, patients with stage I
739 periodontitis have higher detection rates of *Porphyromonas gingivalis* and *Tannerella forsythia* (Tanner et
740 al., 2006, 2007).

741 Therefore, we calculated beta-diversity indices to analyze the differences between the study partici-
742 pants. The distances for the multiple stages of periodontitis, including stage I, stage II, and stage III, as
743 well as healthy controls (Figure 4g-j and Table 7), suggesting notable differences among the multiple
744 periodontitis severities. In other words, the composition of the salivary microbiome compositions varies
745 depending on the periodontitis stages, so that supporting the findings from a previous study (Iniesta et al.,
746 2023). Taken together that it is nearly impossible to fully restore the attachment level after it has been lost
747 due to the progression and development of periodontitis, the ability to rapidly screen for periodontitis in
748 its early phases using saliva collections would be highly beneficial for effective disease management and
749 treatment.

750 Of the total of 425 taxa in the salivary microbiome composition that have been identified (Figure 13),
751 ANCOM was applied to select 20 taxa as the DAT that indicated notable abundance variation among
752 the periodontitis severities (Figure 8 and Table 5). Three sub-groups were formed from the DAT using
753 hierarchical clustering (Figure 8a). Surprisingly, two of the red complex pathogens (Rôças, Siqueira Jr,
754 Santos, Coelho, & de Janeiro, 2001), *Porphyromonas gingivalis* and *Tannerella forsythia*, were classified
755 in Group 2 and were more prevalent in stage II and stage II periodontitis compared to healthy controls.
756 *Campylobacter showae* was additionally placed in Group 2 of the orange complex pathogens (Gambin et
757 al., 2021). Furthermoe, some of the DAT in Group 2 have reported their crucial roles in pathogenesis
758 and development of periodontitis: *Filifactor alocis* (Aruni et al., 2015), *Treponema putidum* (Wyss et
759 al., 2004), *Tannerella forsythia* (Stafford, Roy, Honma, & Sharma, 2012; W. Zhu & Lee, 2016), and
760 *Prevotella intermedia* (Karched, Bhardwaj, Qudeimat, Al-Khabbaz, & Ellepol, 2022). Taken together,
761 this indicates that DAT in Group 2 is essential to periodontitis. The portion of some Group 1 DAT,
762 including *Peptostreptococcaceae[XI][G-5] saphenum*, *Peptostreptococcaceae[XI][G-6] nodatum*, and
763 *Peptostreptococcaceae[XI][G-9] brachy*, in healthy controls and patients with periodontitis significantly
764 differed, according to earlier research (Lafaurie et al., 2022). These outcomes support our research,
765 implying that Group 1 DAT are also essential to the etiology and progression of periodontitis. However,
766 in contrast to patients with periodontitis, Group 3 DAT, namely *Corynebacterium durum* and *Actinomyces*
767 *graevenitzii*, were enriched in healthy controls, which is consistent with earlier research (Redanz et al.,
768 2021; Nibali et al., 2020).

769 In our correlation analysis (Figure 9), we have discovered strongly negative correlations (coefficient \leq
770 -0.5) between DAT of Group 3 and these of Group 1 and Group 2; we have also identified nine DAT

pairs with strong correlations (coefficient $\leq -0.5 \vee$ coefficient ≥ 0.5) (Figure 14). Interestingly, there were strongly negative correlations (coefficient ≤ -0.5) between Group 2 DAT and *Actinomyces* spp., taxa which belong to Group 3: *Filifactor alocis* (Figure 14a), *Porphyromonas gingivalis* (Figure 14b), and *Treponema putidum* (Figure 14c). Taken together that pathogens, including *Filifactor alocis* (Aja, Mangar, Fletcher, & Mishra, 2021; Hiranmayi, Sirisha, Rao, & Sudhakar, 2017), *Porphyromonas gingivalis* (Rôças et al., 2001), and *Treponema putidum* (Wyss et al., 2004), become dominant taxa in patients with stage III periodontitis. On the other hand, commensal salivary bacteria, such as *Actinomyces* spp., gradually declined. Additionally, several DAT from Group 1 and Group 2 exhibited strong positive correlations (coefficient ≥ 0.5) (Figure 14d-i). It has been established that all of these DAT from Group 1 and Group 2 are periodontal pathogens: *Filifactor alocis* (Aja et al., 2021; Hiranmayi et al., 2017), *Fretibacterium* spp. (Teles, Wang, Hajishengallis, Hasturk, & Marchesan, 2021), *Lachnospiraceae[G-8] bacterium HMT 500* (Lafaurie et al., 2022), *Peptostreptococcaceae[XI][G-6] nodatum* (Lafaurie et al., 2022; Haffajee, Teles, & Socransky, 2006), *Peptostreptococcaceae[XI][G-9] brachy* (Lafaurie et al., 2022), and *Treponema putidum* (Wyss et al., 2004). Thus, these fundamental roles of identified periodontal pathogens in the pathophysiology and progression of periodontitis are further supported by these strong positive correlations (coefficient ≥ 0.5), suggesting that advanced periodontitis, i.e., stage III, might arise from the additional DAT from Group 1 and Group 2.

Moreover, to predict periodontitis statuses from salivary microbiome composition, we have constructed machine-learning classification models based on random forest for four classification settings:

1. healthy vs. stage I vs. stage II vs. stage III
2. healthy vs. stage I
3. healthy vs. stage I vs. stages II/III
4. healthy vs. stages I/II/III

Porphyromonas gingivalis and *Actinomyces* spp. were the two most important taxa (feature) in all classification settings. This finding aligns with a recent study that identifies *Actinomyces* spp. as the most prevalent bacteria in both the healthy gingivitis controls, while *Porphyromonas gingivalis* is recognized as the most predominant taxon within the periodontitis subjects, based on analyses of subgingival plaque samples (Nemoto et al., 2021). We have previously developed machine learning models for the classification of periodontitis, with the objective of predicting the severities of chronic periodontitis by analyzing the copy numbers of nine known salivary bacteria species. We classified healthy controls and patients with periodontitis utilizing bacterial combinations in conjunction with a random forest model (E.-H. Kim et al., 2020):

- AUC: 94%
- BA: 84%
- SEN: 95%
- SPE: 72%

Another study established a machine-learning model for the classification of periodontitis, employing 266 species derived from the buccal microbiome (Na et al., 2020):

- AUC: 92%

- 810 • BA: 84%
811 • SEN: 94%
812 • SPE: 74%
- 813 By separating patients with periodontitis from healthy controls using only four DAT, *e.g.* *Actinomyces*
814 *graevenitzii*, *Actinomyces* spp., *Corynebacterium durum*, and *Porphyromonas gingivalis*, our machine
815 learning model performed better than previously published models (Figure 10, Table 4, and Table 6):
816 • AUC: $95.3\% \pm 4.9\%$
817 • BA: $88.5\% \pm 6.6\%$
818 • SEN: $86.4\% \pm 15.7\%$
819 • SPE: $90.5\% \pm 7.0\%$
- 820 This result showed that by detecting Group 3 bacteria that were substantially abundant in health
821 controls than patients with periodontitis, our study increased BA by at least 5% and SPE by at least 17%.
822 Furthermore, we have validated our machine-learning prediction model using openly accessible 16S
823 gene rRNA sequencing data from Portuguese (Iniesta et al., 2023) and Spanish participants (Relvas et
824 al., 2021) in order to ensure the consistency of our random forest classification model (Figure 11). Our
825 classification models employed in this study were primarily developed and assessed on Korean study par-
826 ticipants, which may limit their generalizability to other ethnic groups with different salivary microbiome
827 compositions (Premaraj et al., 2020; Renson et al., 2019). Therefore, the evaluations of this periodonti-
828 tis classification models can be affected by ethnic-specific variances and differences, highlighting the
829 necessity for additional validation and adjustment across a spectrum of ethnic backgrounds.
- 830 Regarding the clinical characteristics and potential confounders influencing the analysis of salivary
831 microbiome compositions connected with periodontitis severity, this study had a number of limitations
832 that were pointed out. We did not offer clinical information, such as the percentage of teeth, the percentage
833 of bleeding on probing, nor dental furcation involvement, even though we did gather information on
834 attachment level, probing depth, plaque index, and gingival index; this might have it challenging to present
835 thorough and in-depth data about periodontal health. Moreover, the broad age range may make it tougher
836 to evaluate the relationship between age and periodontitis statuses, providing the necessity for future
837 studies to consider into account more comprehensive clinical characteristics associated with periodontitis.
838 Additionally, potential confounders—*e.g.* body mass index and e-cigarette use—which might have affected
839 dental health and salivary microbiome composition were disregarding consideration in addition to smoking
840 status and systemic diseases. Thus, future research incorporating these components would offer a more
841 thorough knowledge of how lifestyle factors interact and affect the salivary microbiome composition and
842 periodontal health. Throughout, resolving these limitations will advance our understanding in pathogenesis
843 and development of periodontitis, offering significant novel insights on the causal connection between
844 systemic diseases and the salivary microbiome compositions.

845 **4 Colon microbiome**

846 **4.1 Introduction**

⁸⁴⁷ **4.2 Materials and methods**

848 **4.3 Results**

849 **4.4 Discussion**

850 **5 Conclusion**

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

852 In the section 2, I show that

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