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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). Therefore, the V3-V4 region offers sufficient variability to classify a wide range of bacteria taxa
157 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. Altogether, by providing a
178 thorough understanding of microbiome compositions, diversity indices, *e.g.* alpha-diversity and beta-
179 diversity, allow us to investigate factors that affecting community variability and structure.

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

191 adjustments are necessary to ensure reliable and stable detection of differentially abundant microbes.
192 Integrating DAT detection analysis with functional profiling further enhances our understanding of the
193 biological significance of microbial shifts or dysbiosis. As microbiome research advances, improving
194 methodologies for DAT selection remains essential for uncovering meaningful microbial association and
195 their potential roles in human diseases.

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

204 Random forest classification is one of the ensemble machine learning methods that constructs several
205 decision trees during training and aggregates their results to provide classification predictions (Breiman,
206 2001). A portion of the features and classes—known as bootstrapping (Jiang & Simon, 2007; Champagne,
207 McNairn, Daneshfar, & Shang, 2014; J.-H. Kim, 2009) and feature bagging (Bryll, Gutierrez-Osuna, &
208 Quek, 2003; Alelyani, 2021; Yaman & Subasi, 2019)—are utilized to construct each tree in the forest. The
209 majority vote from each tree determines the final classification, which lowers the possibility of overfitting
210 in comparison to a single decision tree. Furthermore, random forest classifier offers several advantages,
211 including its robustness to outliers and its ability to calculate the feature importance.

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

223 The receiver-operating characteristics (ROC) curve is a graphical representation used to evaluate
224 the performance of a classification model by plotting the sensitivity against (1-specificity) at multiple
225 threshold setting (Gonçalves, Subtil, Oliveira, & de Zea Bermudez, 2014; Obuchowski & Bullen, 2018;
226 Centor, 1991). The ROC curve illustrates the trade-off between detecting true positives while minimizing
227 false positives, suggesting determining the optimal decision threshold for classification. A key metric
228 derived from the ROC curve is the area-under-curve (AUC), which quantifies overall ability of the
229 classification model to discriminate between positive and negative predictions. An AUC value of 0.5

230 indicates a model performing no better than random chance, while value closer to 1.0 suggests high
231 predictive accuracy. Thus, by analyzing the AUC value of the ROC curve, researchers can compare
232 different models and select the better classification model that offers the best balance between sensitivity
233 and specificity for a given application.

234 (Limitation & Novelty)

Table 1: Confusion matrix

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

235

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

236

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

237

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

238

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

239

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

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

240 **2 Predicting preterm birth using random forest classifier in salivary mi-**
241 **crobiome**

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

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

246 **2.1 Introduction**

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

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

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

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

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

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

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

286 **2.2 Materials and methods**

287 **2.2.1 Study design and study participants**

288 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
289 Declaration of Helsinki (Goodyear, Krleza-Jeric, & Lemmens, 2007). The Institutional Review Board
290 authorized this study (IRB file No. 2019-01-024). Participants who were admitted for elective cesarean
291 sections (C-sections) or induction births, as well as those who had written informed consent obtained
292 with premature labor or PROM, were eligible.
293

294 **2.2.2 Clinical data collection and grouping**

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

297 • maternal age at delivery

298 • diabetes mellitus

299 • hypertension

300 • overweight and obesity

301 • C-section

302 • history PROM or PTB

303 • gestational week on delivery

304 • birth weight

305 • sex

306 **2.2.3 Salivary microbiome sample collection**

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

315 **2.2.4 16s rRNA gene sequencing**

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

322 **2.2.5 Bioinformatics analysis**

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

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

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

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

342 **2.2.6 Data and code availability**

343 All sequences from the 59 study participants have been added to the Sequence Read Archives (project
344 ID PRJNA985119): <https://dataview.ncbi.nlm.nih.gov/object/PRJNA985119?reviewer=6fdj2e9c8gp9vtf52n330e2h8j>. Docker image that employed throughout this study is available in the
345 DockerHub: https://hub.docker.com/r/fumire/helixco_premature. Every code used in this
346 study can be found on GitHub: https://github.com/CompbioLabUnist/Helixco_Premature.
347

348 **2.3 Results**

349 **2.3.1 Overview of clinical information**

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

356 **2.3.2 Comparison of salivary microbiomes composition**

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

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

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

368 **2.3.3 Random forest classification to predict PTB risk**

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

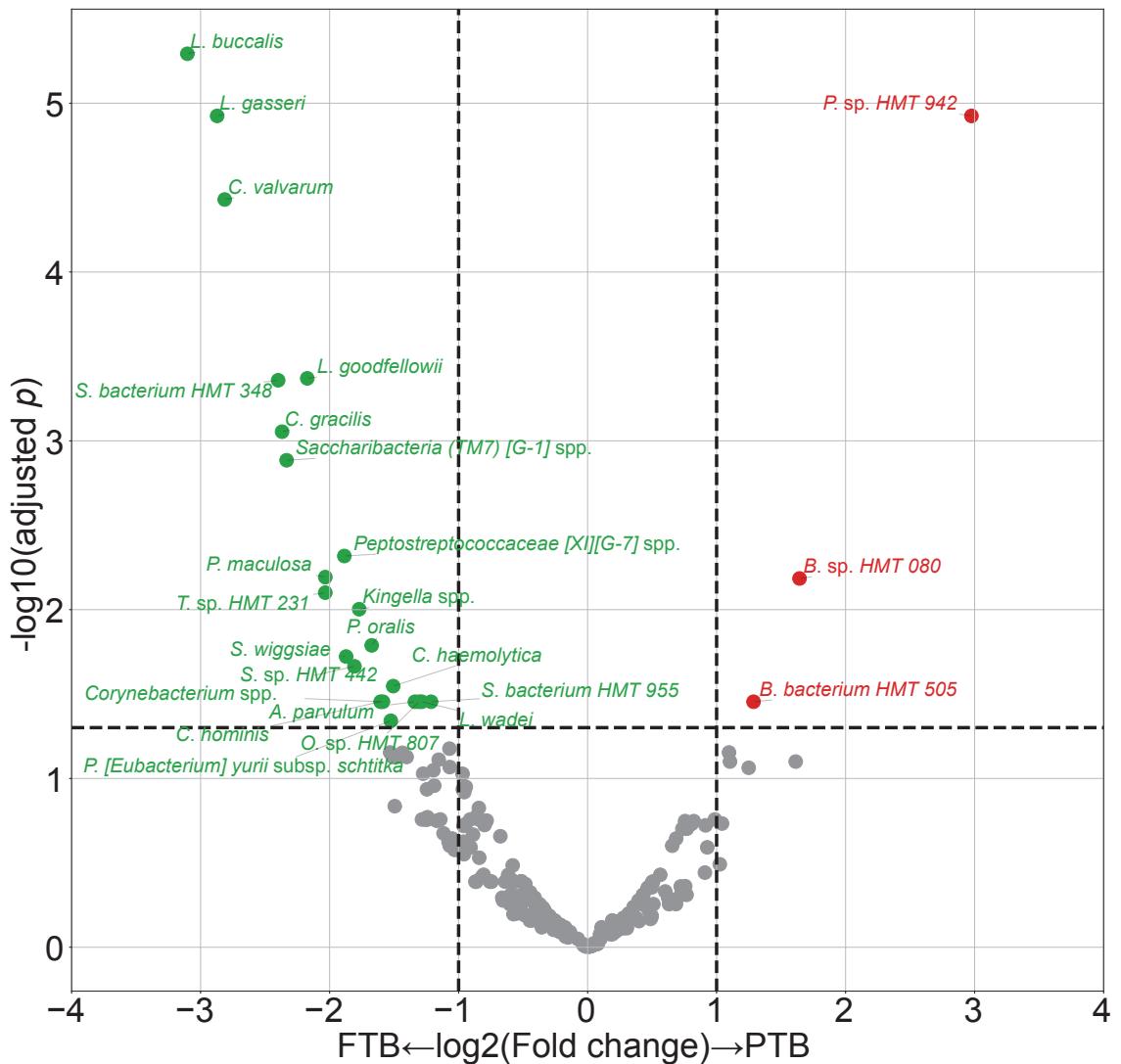


Figure 1: DAT volcano plot.

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

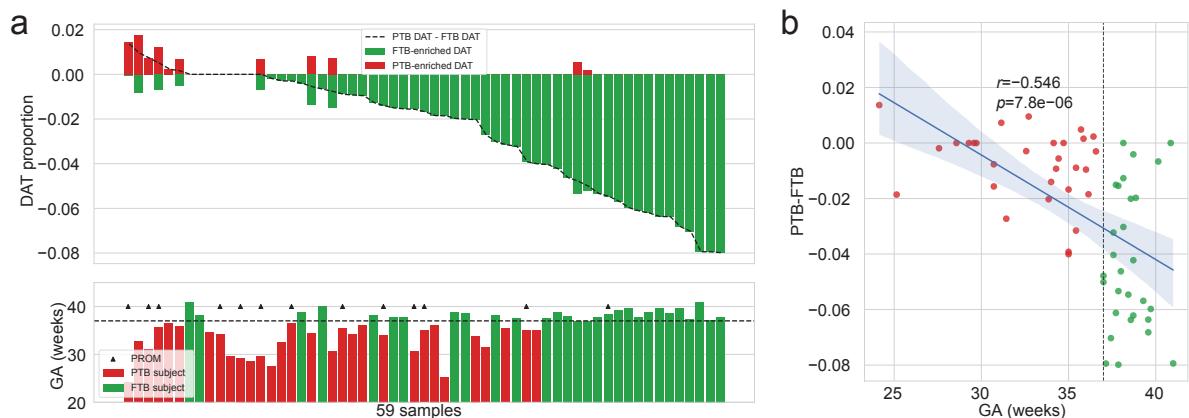


Figure 2: **Salivary microbiome compositions over DAT.**

(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.

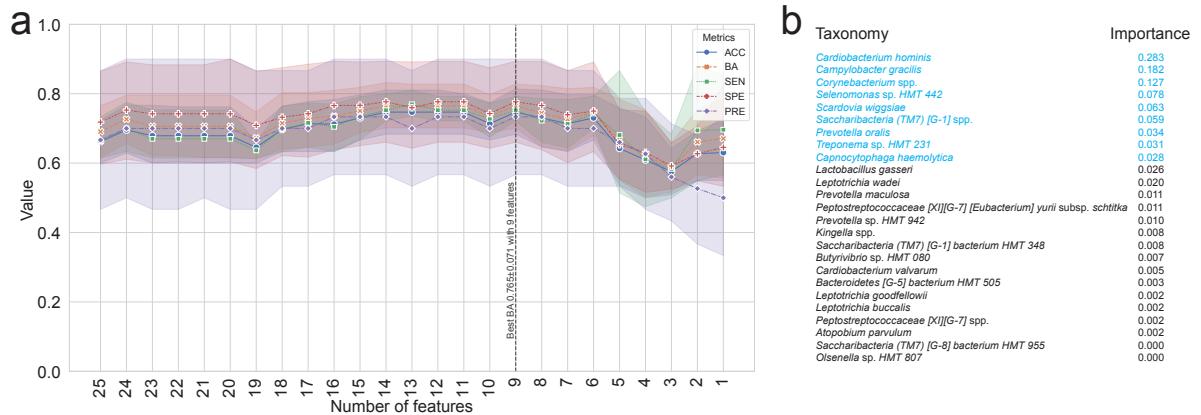


Figure 3: **Random forest-based PTB prediction model.**

(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.

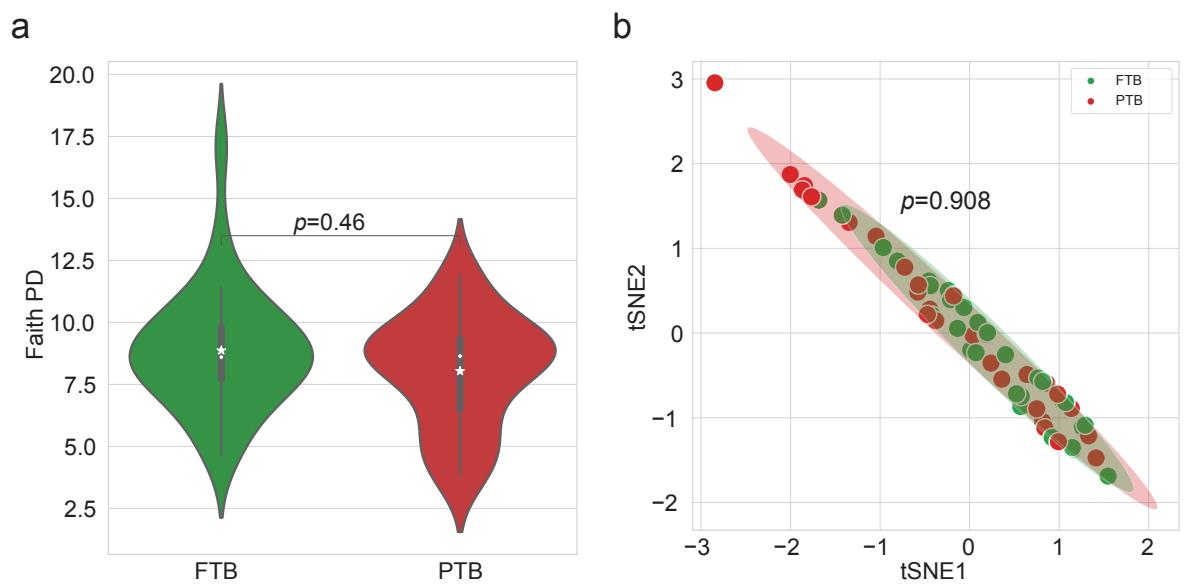


Figure 4: **Diversity indices.**

(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 $p = 0.908$)

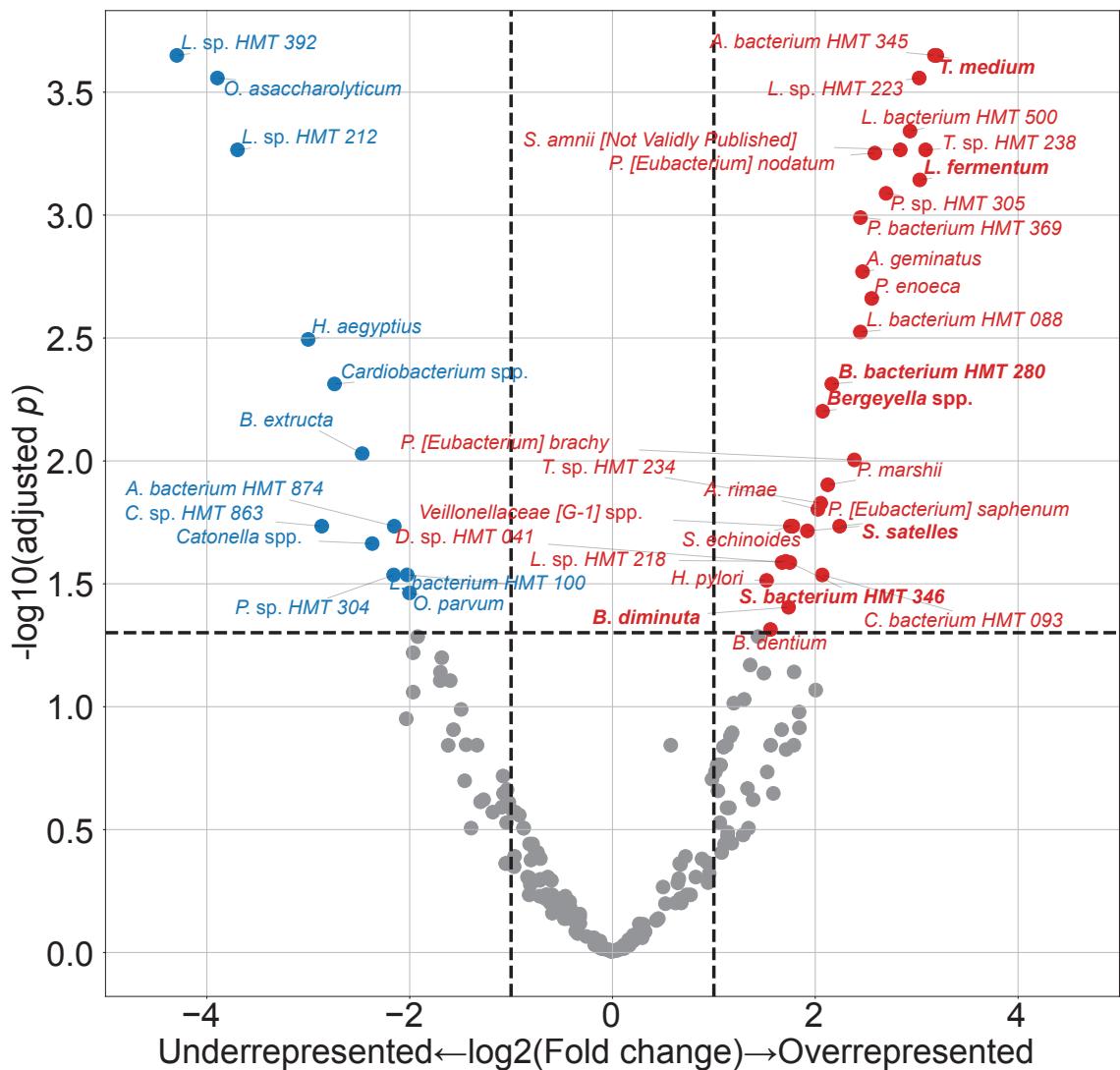


Figure 5: **PROM-related DAT**.

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.

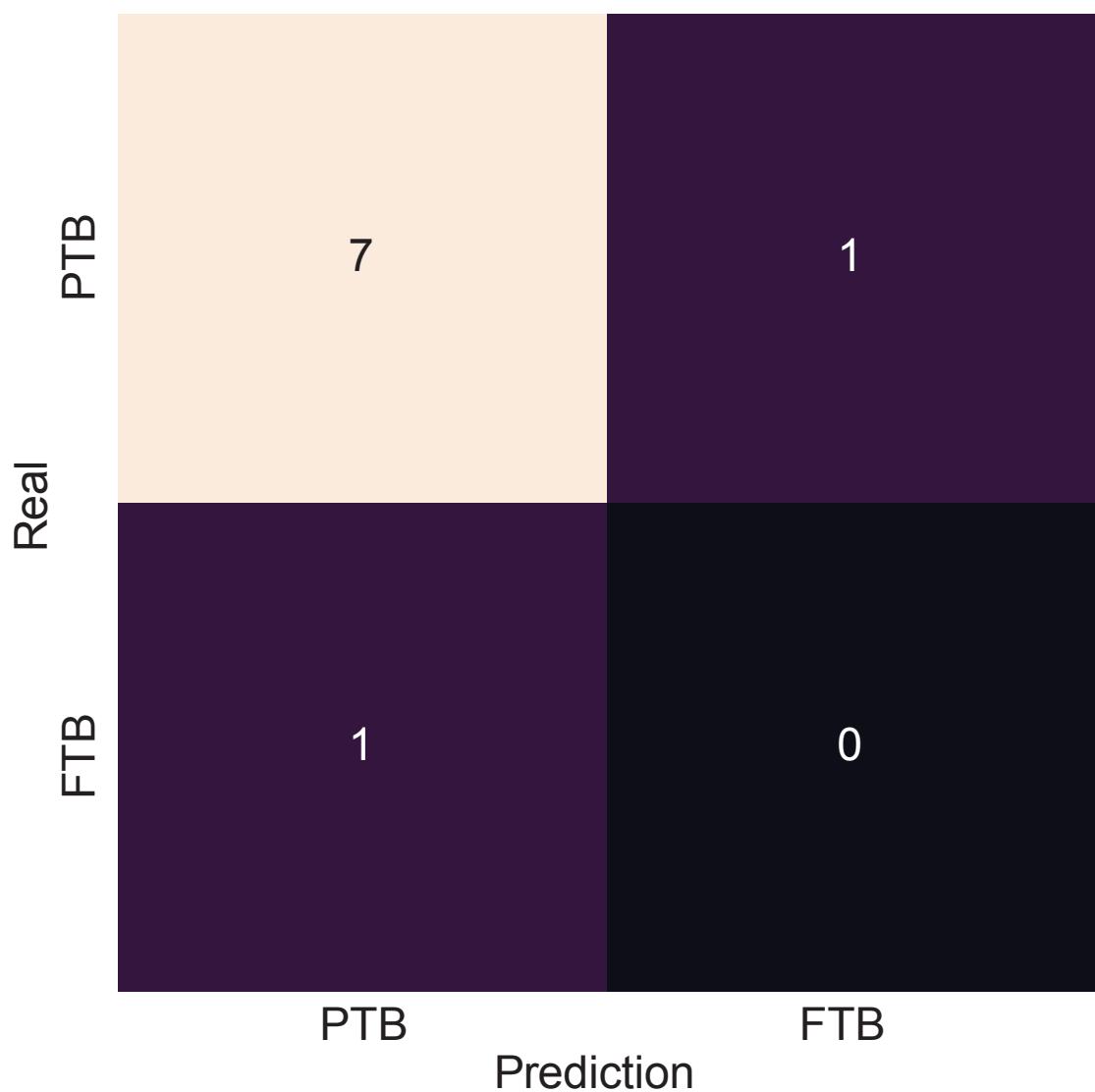


Figure 6: Validation of random forest-based PTB prediction model.

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)

376 **2.4 Discussion**

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

384 One of the most common salivary taxa that has been examined is *Fusobacterium nucleatum* (Han,
385 2015; Brennan & Garrett, 2019; Bolstad, Jensen, & Bakken, 1996), that is a Gram-negative, anaerobic, and
386 filamentous bacteria. *Fusobacterium nucleatum* can be separated from not only the salivary microbiome
387 but also the vaginal microbiome (Vander Haar, So, Gyamfi-Bannerman, & Han, 2018; Witkin, 2019). In
388 both animal and human investigation, *Fusobacterium nucleatum* infection has been linked to risk of PTB
389 (Doyle et al., 2014). According to recent researches, the placenta women who give birth prematurely may
390 include additional salivary microbiome dysbiosis, such as *Bergeyella* spp. and *Porphyromonas gingivalis*
391 (León et al., 2007; Katz, Chegini, Shiverick, & Lamont, 2009). Although *Bergeyella* spp. were one of the
392 PROM-overrepresented DAT (Figure 5), it was excluded in the final 25 PTB-related DAT. Furthermore,
393 *Porphyromonas gingivalis* and *Campylobacter gracilis* were pathogens of periodontitis in sub-gingival
394 microbiome (Yang et al., 2022). *Lactobacillus gasseri* was also one of the FTB-enriched DAT (Figure
395 1), and it is well established that early PTB risk can be reduced by *Lactobacillus gasseri* in the vaginal
396 microbiome (Basavaprabhu, Sonu, & Prabha, 2020; Payne et al., 2021).

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

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

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

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

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

428 **3 Random forest prediction model for periodontitis statuses based on the**
429 **salivary microbiomes**

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

431

432 **3.1 Introduction**

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

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

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

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

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

493 **3.2 Materials and methods**

494 **3.2.1 Study participants enrollment**

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

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

512 **3.2.2 Periodontal clinical parameter diagnosis**

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

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

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

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

530 0. Normal gingiva: without inflammation nor discoloration.

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

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

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

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

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

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

541 • Healthy:

542 1. Bleeding sites < 10%

543 2. Probing depth: \leq 3 mm

544 • Stage I:

545 1. No tooth loss because of periodontitis.

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

547 3. Radiographic bone loss: < 15%

548 • Stage II:

549 1. No tooth loss because of periodontitis.

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

551 3. Radiographic bone loss: 15-33%

552 • Stage III:

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

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

555 3. Radiographic bone loss: > 33%

556 **3.2.3 Saliva sampling and DNA extraction procedure**

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

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

565 • Forward: 5' -TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNNGCWGCAG-3'

566 • Reverse: 5' -GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'

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

569 1. Heat activation for 30 seconds at 95 °C.

570 2. 25 cycles for 30 seconds at 95 °C.

571 3. 30 seconds at 55 °C.

572 4. 30 seconds at 72 °C.

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

577 **3.2.4 Bioinformatics analysis**

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

582 • Abundance-based Coverage Estimator (ACE) (Chao & Lee, 1992)

583 • Chao1 (Chao, 1984)

584 • Fisher (Fisher, Corbet, & Williams, 1943)

585 • Margalef (Magurran, 2021)

586 • Observed ASVs (DeSantis et al., 2006)

587 • Berger-Parker d (Berger & Parker, 1970)

588 • 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.

625 **3.3 Results**

626 **3.3.1 Summary of clinical information and sequencing data**

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

633 **3.3.2 Diversity indices reveal differences among the periodontitis severities**

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

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

644 **3.3.3 DAT among multiple periodontitis severities and their correlation**

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

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

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

- 667 • Group 3
- 668 1. *Actinomyces* spp.
- 669 2. *Corynebacterium durum*
- 670 3. *Actinomyces graevenitzii*

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

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

679 3.3.4 Classification of periodontitis severities by random forest models

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

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

689 Third, based on the findings that the salivary microbiome composition in stage II is more comparable
 690 to those in stage III than to other severities (Figure 7f and Figure 7j), we combined stage II and stage III
 691 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>	0.095	<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>	0.062	<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>	0.052	<i>Filifactor alocis</i>	0.036	<i>Filifactor alocis</i>	0.032		
6	<i>Campylobacter showae</i>	0.042	<i>Porphyromonas sp. HMT 285</i>	0.040	<i>Corynebacterium durum</i>	0.040	<i>Prevotella intermedia</i>	0.050	<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>	0.038	<i>Tannerella forsythia</i>	0.025	<i>Porphyromonas sp. HMT 285</i>	0.022		
8	<i>Corynebacterium durum</i>	0.032	<i>Tannerella forsythia</i>	0.026	<i>Tannerella forsythia</i>	0.037	<i>Treponema spp.</i>	0.023	<i>Prevotella intermedia</i>	0.022		
9	<i>Treponema spp.</i>	0.032	<i>Prevotella intermedia</i>	0.025	<i>Prevotella intermedia</i>	0.029	<i>Treponema spp.</i>	0.021	<i>Treponema spp.</i>	0.022		
10	<i>Tannerella forsythia</i>	0.026	<i>Prevotella intermedia</i>	0.025	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.026	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.018	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.015		
11	<i>Treponema putidum</i>	0.025	<i>Freibacterium spp.</i>	0.023	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.018	<i>Lachnospiraceae (G-8) bacterium HMT 500</i>	0.014	<i>Lachnospiraceae (G-8) bacterium HMT 500</i>	0.010		
12	<i>Freibacterium spp.</i>	0.023	<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		
13	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.021	<i>Treponema putidum</i>	0.019	<i>Treponema putidum</i>	0.014	<i>Treponema putidum</i>	0.010	<i>Freibacterium spp.</i>	0.009		
14	<i>Treponema sp. HMT 260</i>	0.019	<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>Prevotella putidum</i>	0.006		
15	<i>Prevotella sp. HMT 526</i>	0.018	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.018	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.008	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.008	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.004		
16	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.018	<i>Prevotella sp. HMT 304</i>	0.017	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.008	<i>Freibacterium spp.</i>	0.008	<i>Treponema sp. HMT 260</i>	0.004		
17	<i>Prevotella sp. HMT 304</i>	0.017	<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		
18	<i>Mycoplasma faecium</i>	0.014	<i>Prevotella sp. HMT 304</i>	0.014	<i>Prevotella sp. HMT 304</i>	0.003	<i>Mycoplasma faecium</i>	0.005	<i>Prevotella sp. HMT 304</i>	0.003		
19	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.013	<i>Lachnospiraceae (G-8) bacterium HMT 500</i>	0.013	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.003	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.004	<i>Prevotella sp. HMT 304</i>	0.001		

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

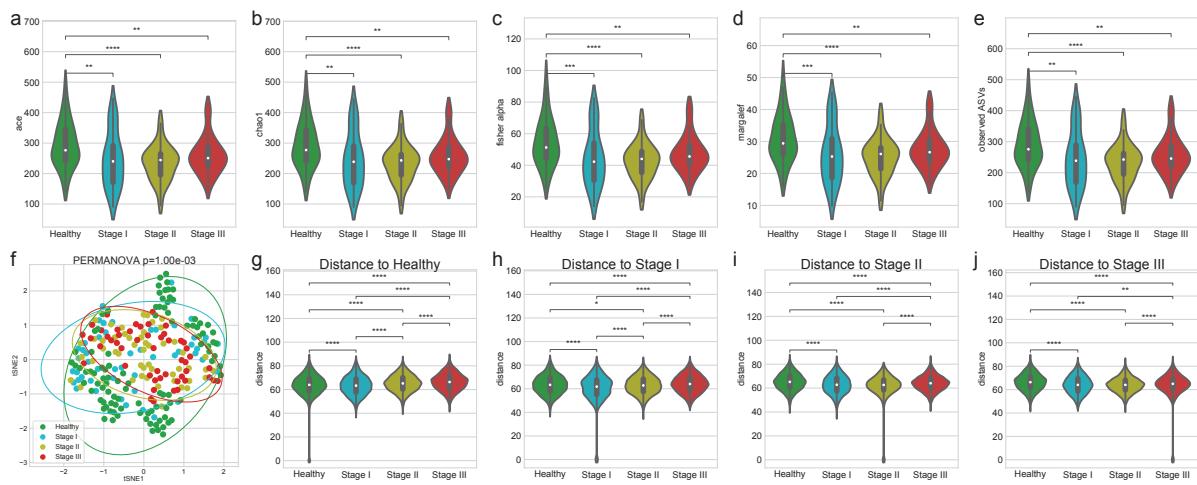


Figure 7: Diversity indices.

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

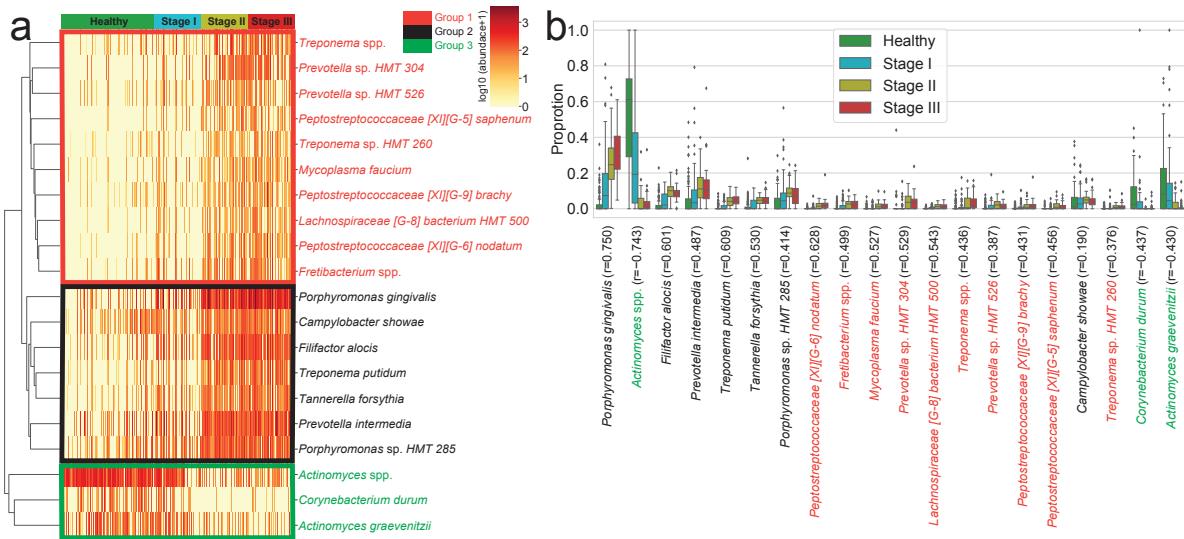


Figure 8: **Differentially abundant taxa (DAT).**

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.

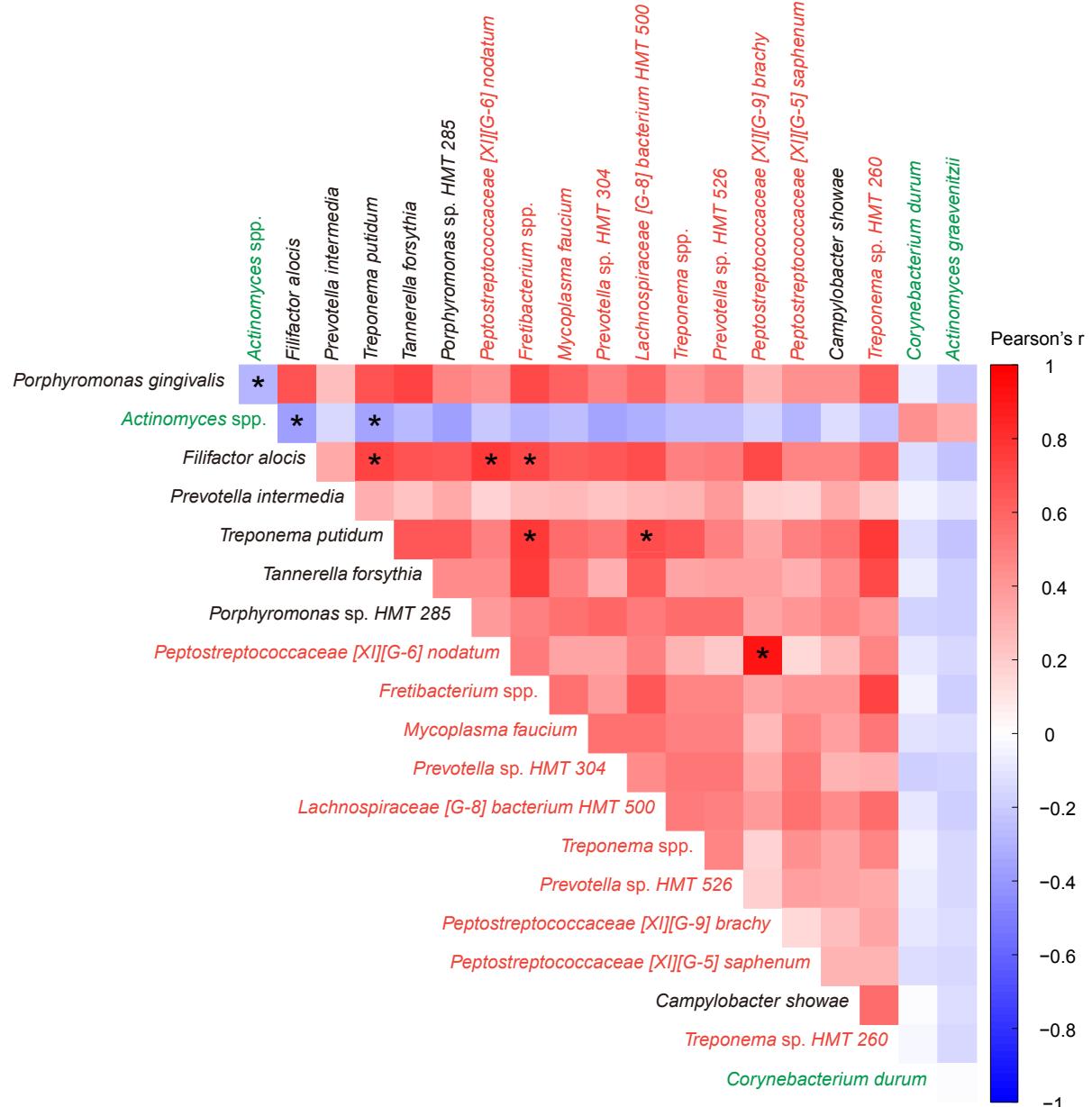


Figure 9: Correlation heatmap.

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

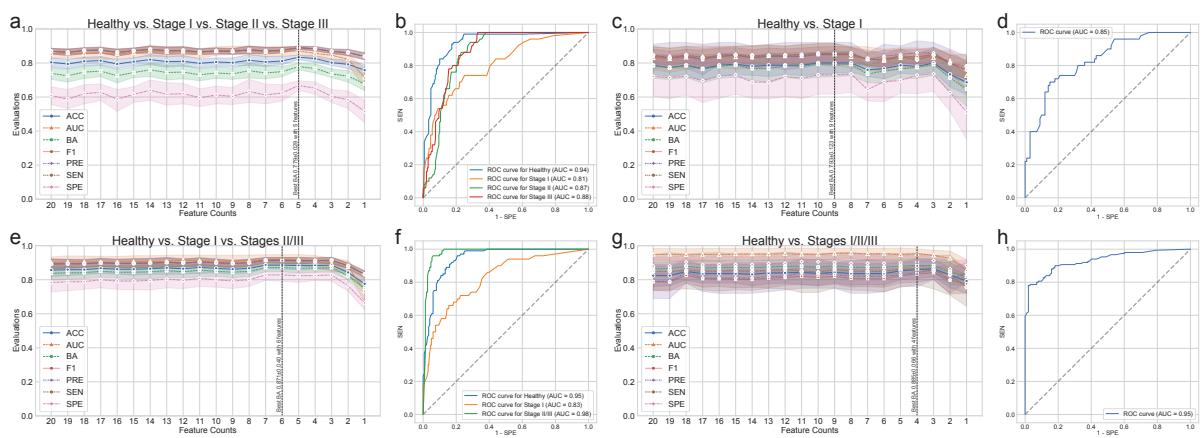


Figure 10: Random forest classification metrics.

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).

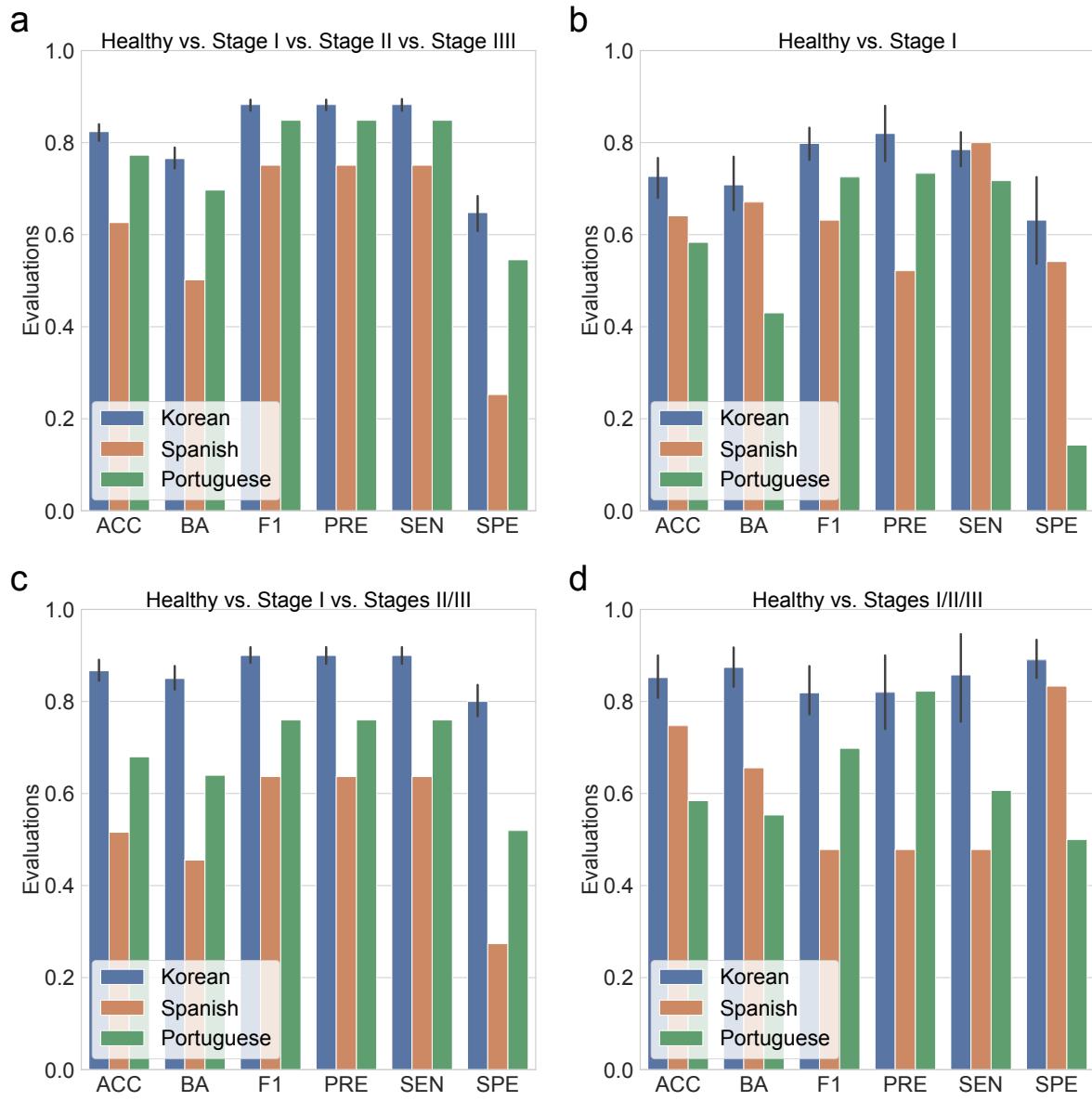


Figure 11: **Random forest classification metrics from external datasets.**

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.

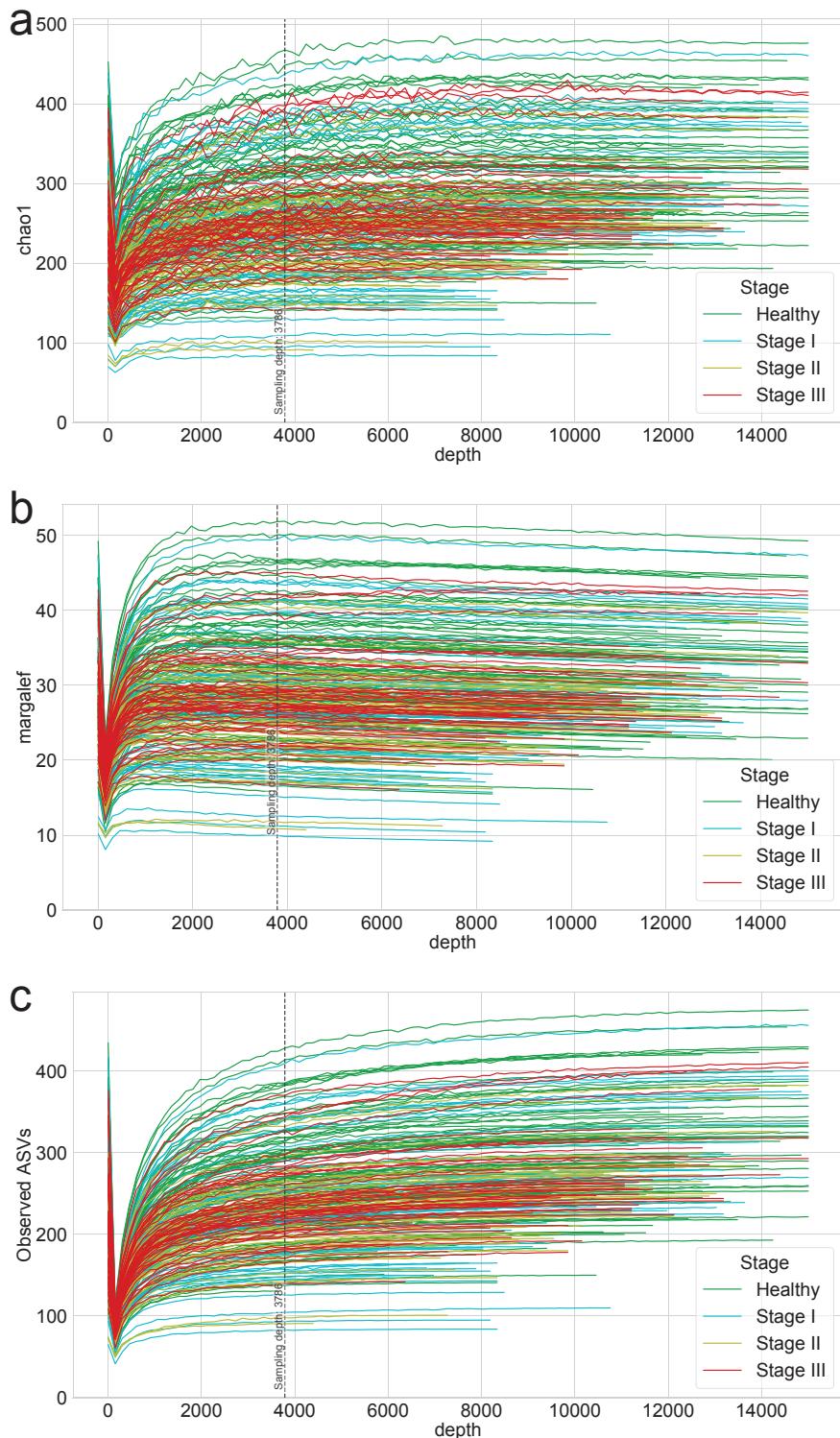


Figure 12: Rarefaction curves for alpha-diversity indices.

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

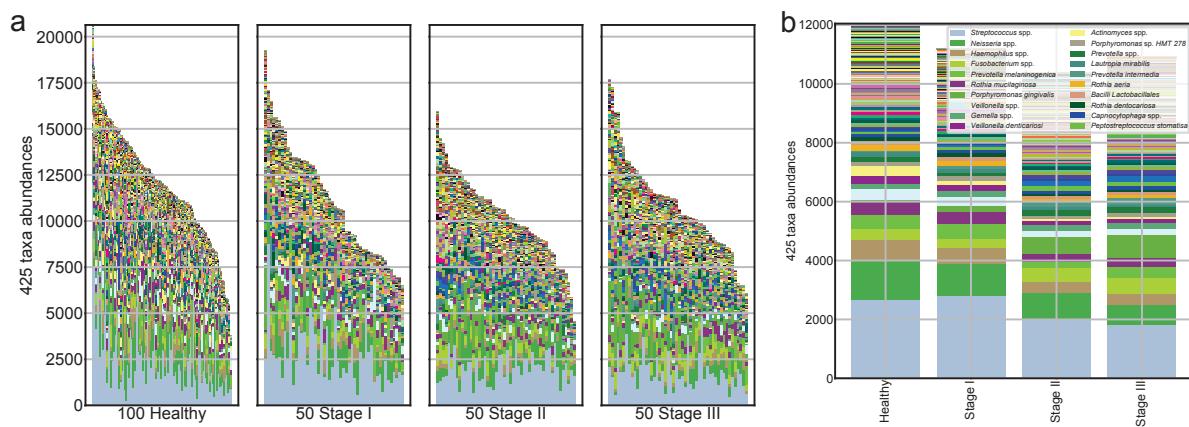


Figure 13: Salivary microbiome compositions in the different periodontal statuses.

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).

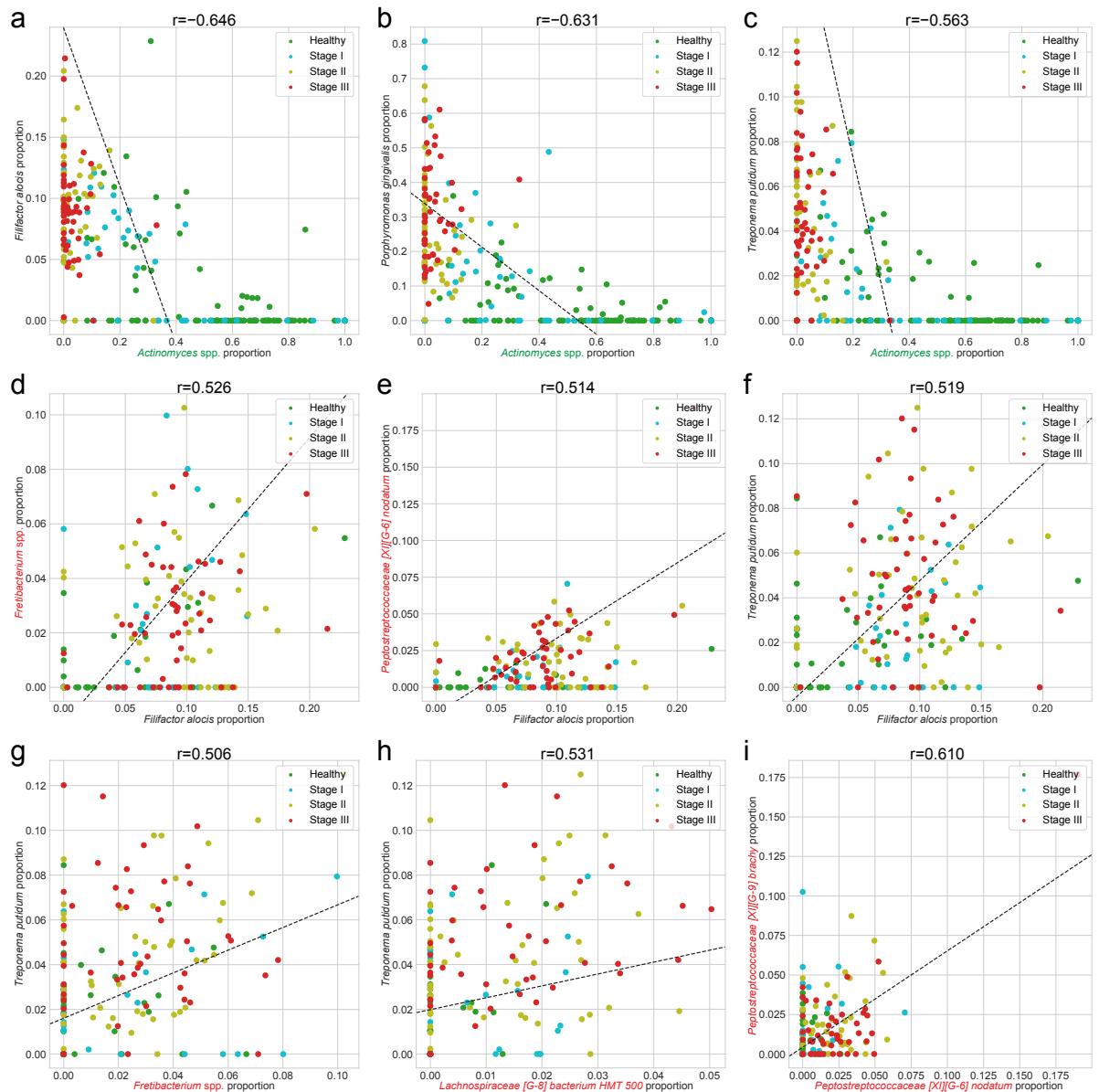


Figure 14: Correlation plots for differentially abundant taxa.

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).

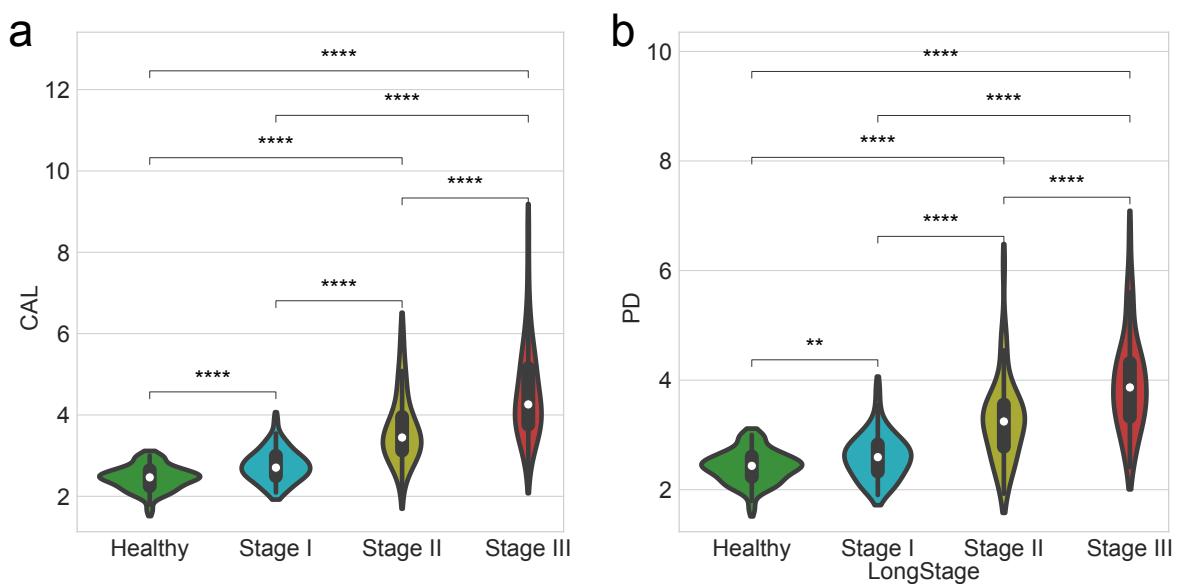


Figure 15: Clinical measurements by the periodontitis statuses.

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

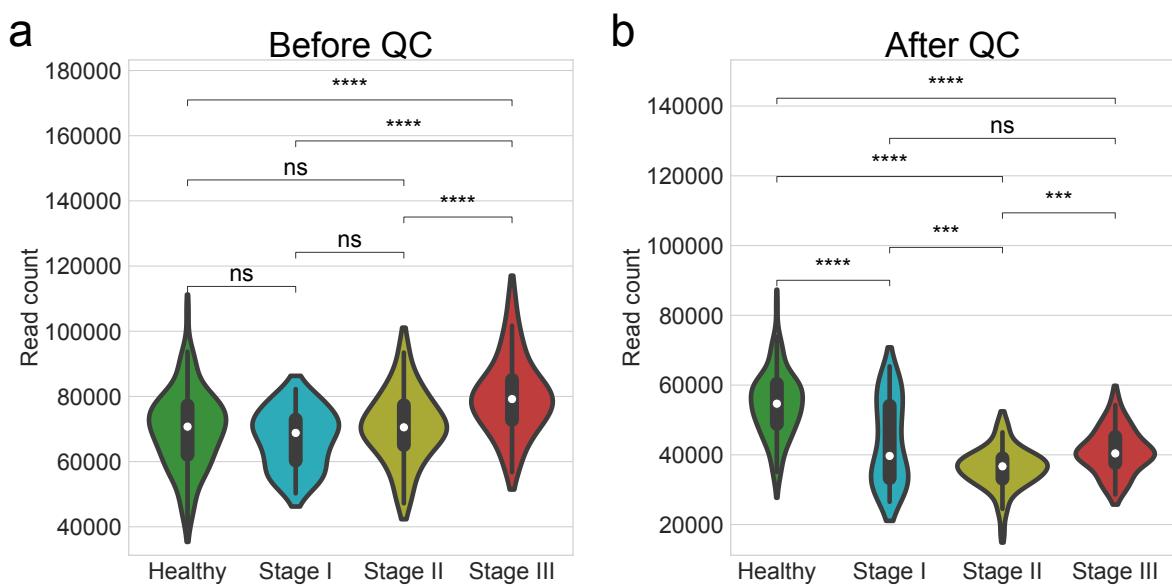


Figure 16: **Number of read counts by the periodontitis statuses.**

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

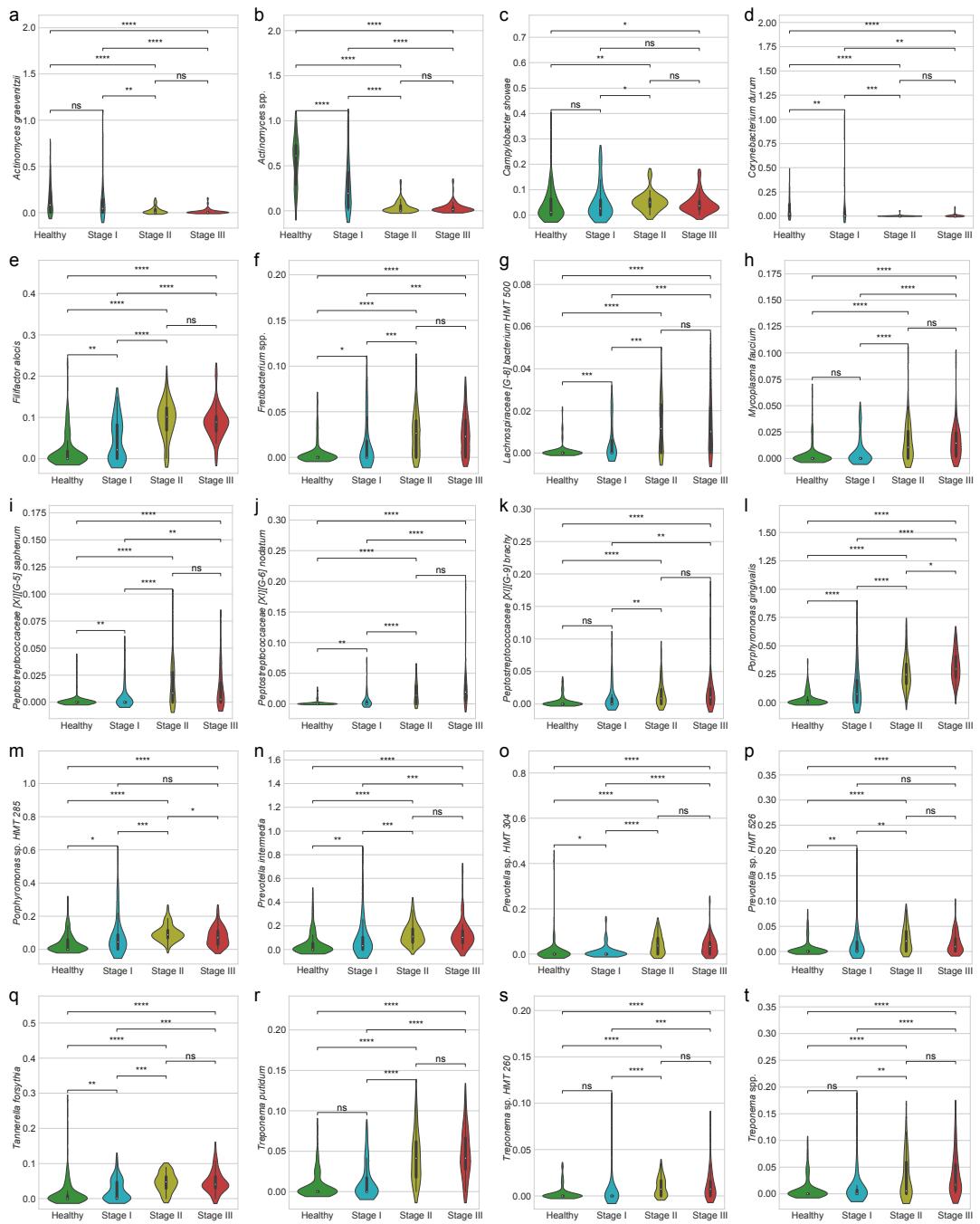


Figure 17: Proportion of DAT.

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

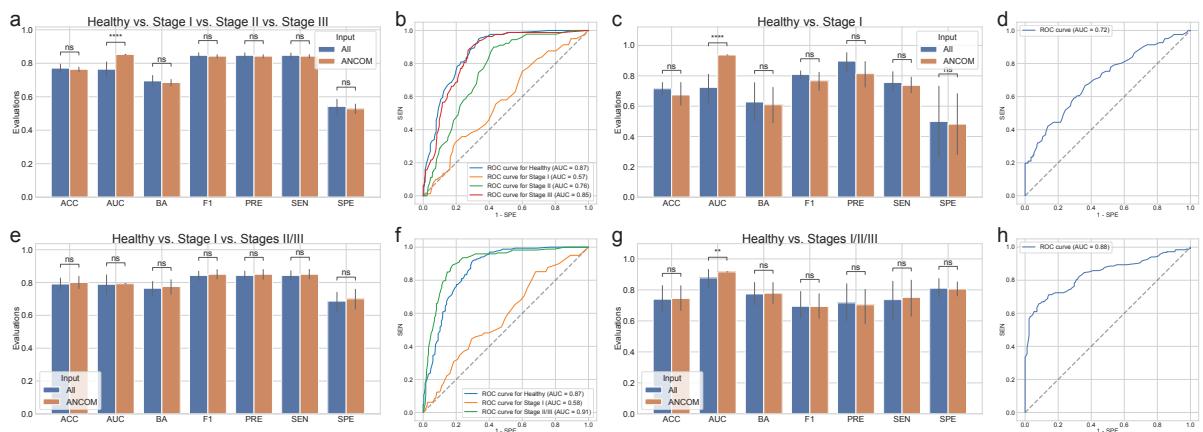


Figure 18: Random forest classification metrics with the full microbiome compositions and ANCOM-selected DAT compositions.

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

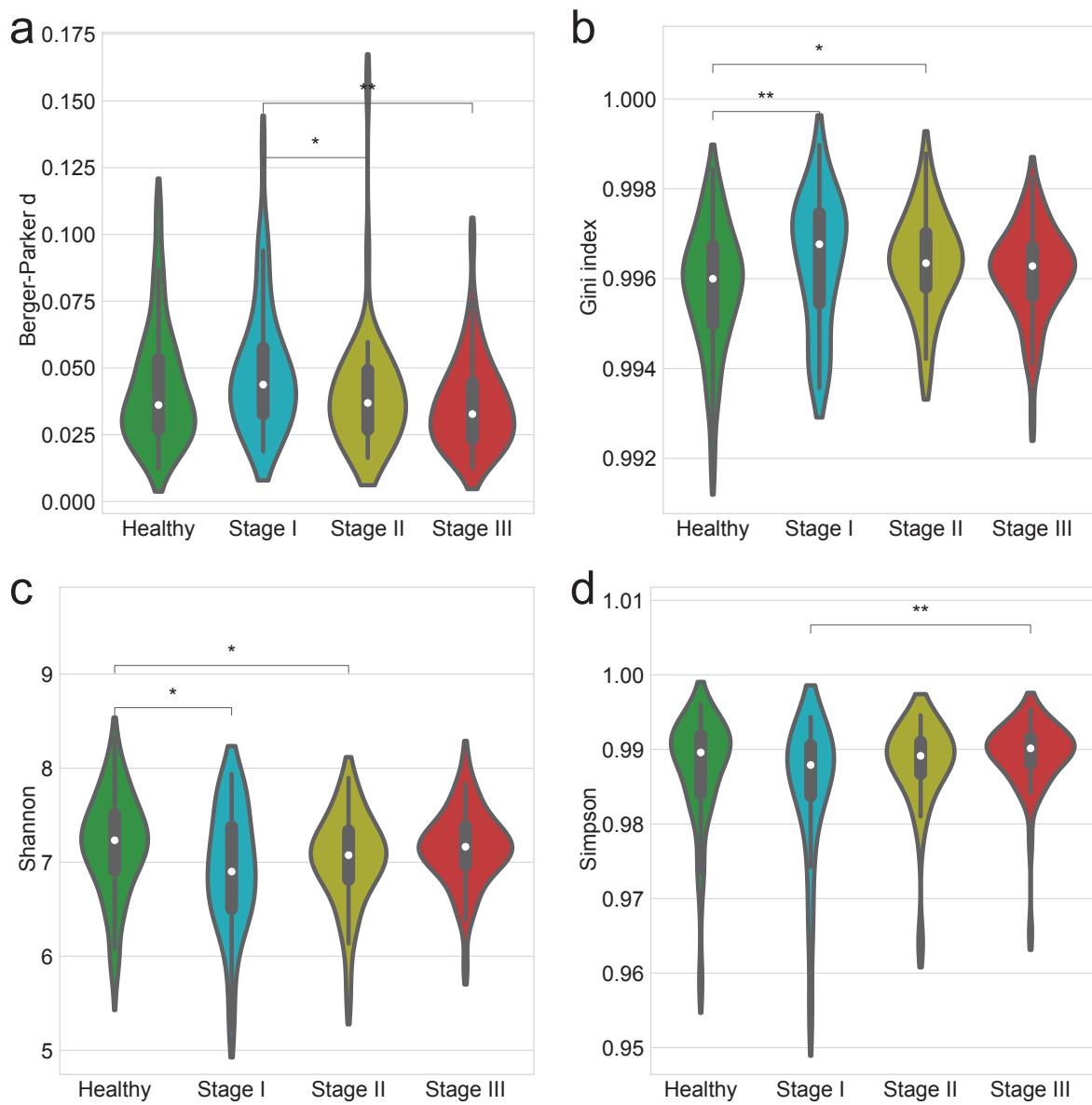


Figure 19: **Alpha-diversity indices account for evenness.**

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 $p \leq 0.01$ (**)

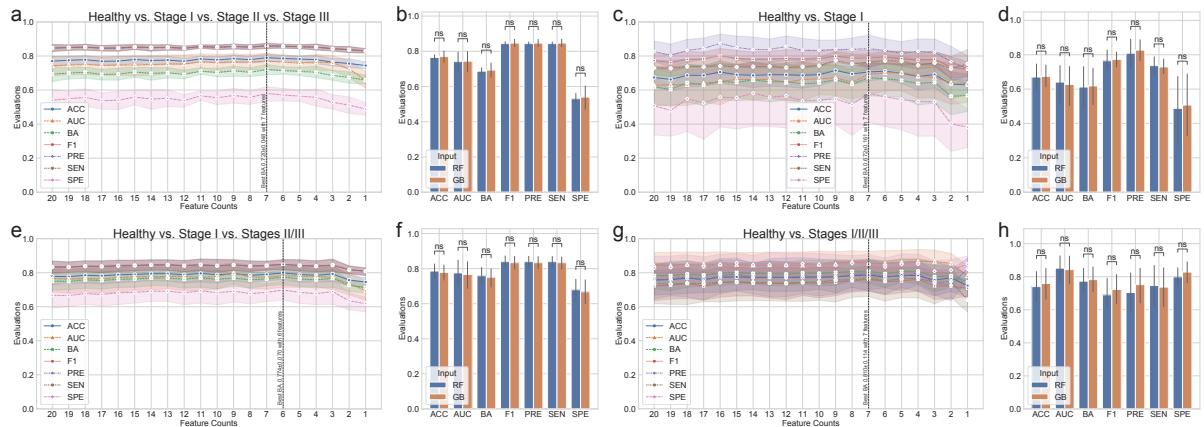


Figure 20: Gradient Boosting classification metrics.

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.

692 **3.4 Discussion**

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

701 Previous research identified the red complex as the primary pathogens of periodontitis (Listgarten,
702 1986): *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. Other studies, however,
703 have shown that periodontal pathogens communicate with other bacteria in the salivary microbiome
704 networks to generate dental plaque prior to the pathogenesis and development of periodontitis (Lamont &
705 Jenkinson, 2000; Rosan & Lamont, 2000; Yoshimura, Murakami, Nishikawa, Hasegawa, & Kawaminami,
706 2009).

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

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

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

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

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

767 In our correlation analysis (Figure 9), we have discovered strongly negative correlations (coefficient \leq
768 -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%

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

843 **4 Colon microbiome**

844 **4.1 Introduction**

845 **4.2 Materials and methods**

846 **4.3 Results**

847 **4.4 Discussion**

848 **5 Conclusion**

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

850 In the section 2, I show that

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