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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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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*,
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List of Abbreviations

- 104 **ACC** Accuracy
105 **ASV** Amplicon sequence variant
106 **AUC** Area-under-curve
107 **BA** Balanced accuracy
108 **C-section** Cesarean section
109 **DAT** Differentially abundant taxa
110 **F1** F1 score
111 **Faith PD** Faith's phylogenetic diversity
112 **FTB** Full-term birth
113 **GA** Gestational age
114 **MSI** Microsatellite instability
115 **MSs** Microsatellite stable
116 **MWU test** Mann-Whitney U-test
117 **OS** Overall survival
118 **PRE** Precision
119 **PROM** Prelabor rupture of membrane
120 **PTB** Preterm birth
121 **ROC curve** Receiver-operating characteristics curve
122 **rRNA** Ribosomal RNA
123 **SD** Standard deviation
124 **SEN** Sensitivity
125 **SPE** Specificity
126 **t-SNE** t-distributed stochastic neighbor embedding

¹²⁷ **1 Introduction**

¹²⁸ The microbiome refers to the complex community of microorganisms, including bacteria, viruses, fungi,
¹²⁹ and other microbes, that inhabit various environment within living organisms (Ursell, Metcalf, Parfrey,
¹³⁰ & Knight, 2012; Gilbert et al., 2018). In humans, the microbiome plays a crucial role in maintaining
¹³¹ health (Lloyd-Price, Abu-Ali, & Huttenhower, 2016), influencing processes such as digestion (Lim, Park,
¹³² Tong, & Yu, 2020), immune response (Thaiss, Zmora, Levy, & Elinav, 2016; Kogut, Lee, & Santin, 2020;
¹³³ C. H. Kim, 2018), and even mental health (Mayer, Tillisch, Gupta, et al., 2015; X. Zhu et al., 2017;
¹³⁴ X. Chen, D'Souza, & Hong, 2013). These microbial communities are not static nor constant, but rather
¹³⁵ dynamic ecosystem that interacts with their host and respond to environmental changes. Recent studies
¹³⁶ have revealed that imbalances in the microbiome, known as dysbiosis, can contribute to a wide range of
¹³⁷ diseases, including obesity (John & Mullin, 2016; Tilg, Kaser, et al., 2011; Castaner et al., 2018), diabetes
¹³⁸ (Barlow, Yu, & Mathur, 2015; Hartstra, Bouter, Bäckhed, & Nieuwdorp, 2015; Sharma & Tripathi, 2019),
¹³⁹ infections (Whiteside, Razvi, Dave, Reid, & Burton, 2015; Alverdy, Hyoju, Weigerinck, & Gilbert, 2017),
¹⁴⁰ inflammatory conditions (Francescone, Hou, & Grivennikov, 2014; Peirce & Alviña, 2019; Honda &
¹⁴¹ Littman, 2012), and cancers (Helmink, Khan, Hermann, Gopalakrishnan, & Wargo, 2019; Cullin, Antunes,
¹⁴² Straussman, Stein-Thoeringer, & Elinav, 2021; Sepich-Poore et al., 2021; Schwabe & Jobin, 2013). Thus,
¹⁴³ understanding the composition of the human microbiomes is essential for developing new therapeutic
¹⁴⁴ approaches that target these microbial populations to promote health and prevent diseases.

¹⁴⁵ The microbiome participates a crucial role in overall health, influencing not only digestion and immune
¹⁴⁶ function but also systemic and neurological processes through the brain-gut axis (Martin, Osadchiy,
¹⁴⁷ Kalani, & Mayer, 2018; Aziz & Thompson, 1998; R. Li et al., 2024). The gut microbiota interact with
¹⁴⁸ the host through metabolic byproducts, immune signaling, and the production of neurotransmitters, *e.g.*
¹⁴⁹ serotonin and dopamine, which are essential for brain function and cognition. Disruptions in microbial
¹⁵⁰ composition, known as dysbiosis, have been linked to various diseases, including inflammatory bowel
¹⁵¹ disease (Sultan et al., 2021; Baldelli, Scaldaferrri, Putignani, & Del Chierico, 2021), obesity (Kang et al.,
¹⁵² 2022; Hamjane, Mechita, Nourouti, & Barakat, 2024; Pezzino et al., 2023), diabetes (Cai et al., 2024;
¹⁵³ X. Li et al., 2021; Y. Li et al., 2023), and cardiovascular diseases (Manolis, Manolis, Melita, & Manolis,
¹⁵⁴ 2022; Tian et al., 2021). Furthermore, the brain-gut axis, a bidirectional communication system between
¹⁵⁵ the gut microbiome composition and the central nervous system, has been implicated in mental disorders,
¹⁵⁶ *e.g.* anxiety disorder, depressive disorder, and neurodegenerative diseases. Emerging evidence suggested
¹⁵⁷ that alterations in the host microbiome can influence mood, cognitive function, and even behavior through
¹⁵⁸ immune modulation, vagus nerve signaling, and microbial metabolites. These findings highlight the
¹⁵⁹ microbiome as a critical factor in maintaining host health and suggest that targeted interventions, namely
¹⁶⁰ probiotics, antibiotics, dietary modification, and microbiome-based therapies, may hold promise for
¹⁶¹ improving both physical and mental comfort. Hence, understanding the microbial effects could lead to
¹⁶² novel therapeutic strategies for a wide range of health conditions.

¹⁶³ 16S ribosomal RNA (rRNA) gene sequencing is one of the most extensively applied methods for
¹⁶⁴ characterizing microbial communities by targeting the conserved 16S rRNA gene, which contains both

165 highly conserved and variable regions in bacteria (Tringe & Hugenholtz, 2008; Janda & Abbott, 2007).
166 The conserved regions enable universal primer binding, while the variable regions provide the specificity
167 needed to differentiate microbial taxa. Among these regions, the V3-V4 region is frequently selected for
168 sequencing due to its balance between phylogenetic resolution and sequencing efficiency (Johnson et al.,
169 2019; López-Aladid et al., 2023). Therefore, the V3-V4 region offers sufficient variability to classify a
170 wide range of bacteria taxa while maintaining compatibility with widely used sequencing platforms.

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

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

194 Differentially abundant taxa (DAT) detection is a key analytical approach in microbiome study to
195 identify microbial taxa that significantly differ in abundance between distinct study participant groups.
196 This DAT detection method is particularly valuable for understanding how microbial communities vary
197 across different conditions, such as disease states, environmental factors, and/or experimental treatments.
198 Various statistical and computational techniques, *e.g.* LEfSe (Segata et al., 2011), DESeq2 (Love, Huber,
199 & Anders, 2014), ANCOM (Lin & Peddada, 2020), and ANCOM-BC (Lin, Eggesbø, & Peddada,
200 2022; Lin & Peddada, 2024), are commonly used to assess differential abundance while accounting for
201 compositional and sparsity-related challenges in microbiome composition data (Swift, Cresswell, Johnson,
202 Stilianoudakis, & Wei, 2023; Cappellato, Baruzzo, & Di Camillo, 2022). Thus, identifying DAT can
203 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

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

249 (Limitation & Novelty)

Table 1: Confusion matrix

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

250

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

251

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

252

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

253

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

254

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

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

255 **2 Predicting preterm birth using random forest classifier in salivary mi-**
256 **crobiome**

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

258 Hong, Y. M., **Lee, Jaewoong**, Cho, D. H., Jeon, J. H., Kang, J., Kim, M. G., ... & Kim, J. K. (2023).
259 Predicting preterm birth using machine learning techniques in oral microbiome. *Scientific Reports*, 13(1),
260 21105.

261 **2.1 Introduction**

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

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

279 Preterm prelabor rupture of membranes (PROM) brought on by gestational inflammation and infection
280 contribute to about 70% of PTB cases (Romero, Dey, & Fisher, 2014). Nevertheless, as antibiotics and
281 anti-inflammatory therapeutic strategies were ineffective to decrease PTB occurrence rates, the pathology
282 of PTB has not been entirely elucidated by inflammatory and infectious pathways (Romero, Hassan, et al.,
283 2014). Recent researches on maternal microbiomes were beginning to examine unidentified connections
284 of PTB as a consequence of developmental processes in molecular biological technology (Fettweis et al.,
285 2019).

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

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

294 Multiple unfavorable birth outcomes, including PROM and PTB, have been linked to periodontitis
295 as an independence risk factor, according to numerous epidemiological researches (Offenbacher et al.,
296 1996). It is expected that the oral microbiome will be able to explain additional hematogenous pathways
297 in light of these precedents; however, the oral microbiome composition of fetuses is limited understood.

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

301 **2.2 Materials and methods**

302 **2.2.1 Study design and study participants**

303 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
304 Declaration of Helsinki (Goodyear, Krleza-Jeric, & Lemmens, 2007). The Institutional Review Board
305 authorized this study (IRB file No. 2019-01-024). Participants who were admitted for elective cesarean
306 sections (C-sections) or induction births, as well as those who had written informed consent obtained
307 with premature labor or PROM, were eligible.
308

309 **2.2.2 Clinical data collection and grouping**

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

- 312 • maternal age at delivery
- 313 • diabetes mellitus
- 314 • hypertension
- 315 • overweight and obesity
- 316 • C-section
- 317 • history PROM or PTB
- 318 • gestational week on delivery
- 319 • birth weight
- 320 • sex

321 **2.2.3 Salivary microbiome sample collection**

322 Salivary microbiome samples were collected 24 hours before to delivery using mouthwash. The standard
323 methods of sterilizing were performed. Medical experts oversaw each stage of the sample collecting
324 procedure. Participants received instruction not to eat, drink, or brush their teeth for 30 minutes before
325 sampling salivary microbiome. Saliva samples were gathered by washing the mouth for 30 seconds with
326 12 mL of a mouthwash solution (E-zen Gargle, JN Pharm, Pyeongtaek, Gyeonggi, Korea). The samples
327 were tagged with the anonymous ID for each participant and kept at 4 °C until they underwent further
328 processing. Genomic DNA was extracted using an ExgeneTM Clinic SV kit (GeneAll Biotechnology,
329 Seoul, Korea) following with the manufacturer instructions and store at -20 °C.

330 **2.2.4 16s rRNA gene sequencing**

331 Salivary microbiome samples were transported to the Department of Biomedical Engineering of the
332 Ulsan National Institute of Science and Technology . 16S rRNA sequencing was then carried out using a
333 commissioned Illumina MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA). Library methods were
334 utilized to amplify the V3-V4 areas. 300 base-pair paired-end reads were produced by sequencing the

335 pooled library using a v3 \times 600 cycle chemistry after the samples had been diluted to a final concentration
336 of 6 pM with a 20% PhiX control.

337 **2.2.5 Bioinformatics analysis**

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

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

349 Taxonomic assignment were implemented with HOMD (version 15.22) (T. Chen et al., 2010).
350 Afterward, DESeq2 was implemented to identify differentially abundant taxa (DAT) that could dis-
351 tinguish between salivary microbiome from PTB and FTB participants (Love et al., 2014). Taxa with
352 $|\log_2 \text{FoldChange}| > 1$ and $p < 0.05$ were considered as statistically significant.

353 The taxa for predicting PTB using salivary microbiome data were determined using a random forest
354 classifier (Breiman, 2001). Through stratified *k*-fold cross-validation (*k* = 5) that preserves the existence
355 rate of PTB and FTB participants, consistency and trustworthy classification were ensured (Wong & Yeh,
356 2019).

357 **2.2.6 Data and code availability**

358 All sequences from the 59 study participants have been published to the Sequence Read Archives
359 (project ID PRJNA985119): <https://dataview.ncbi.nlm.nih.gov/object/PRJNA985119>. Docker
360 image that 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.

363 **2.3 Results**

364 **2.3.1 Overview of clinical information**

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

371 **2.3.2 Comparison of salivary microbiomes composition**

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

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

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

383 **2.3.3 Random forest classification to predict PTB risk**

384 To classify PTB according to DAT, random forest classifiers were constructed. The nine most significant
385 DAT were used to obtain the best BA (0.765 ± 0.071 ; Figure 3a). Moreover, random forest classification
386 model determined each DAT's importance (Figure 3b). We conducted a validation procedure on nine
387 twin pregnancies that were excluded in the initial study design in order to confirm the reliability and
388 dependability of our random forest-based PTB prediction model (Figure 6). Comparable to the PTB
389 prediction model on the 59 initial singleton study participants, the validation classification on PTB risk of
390 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

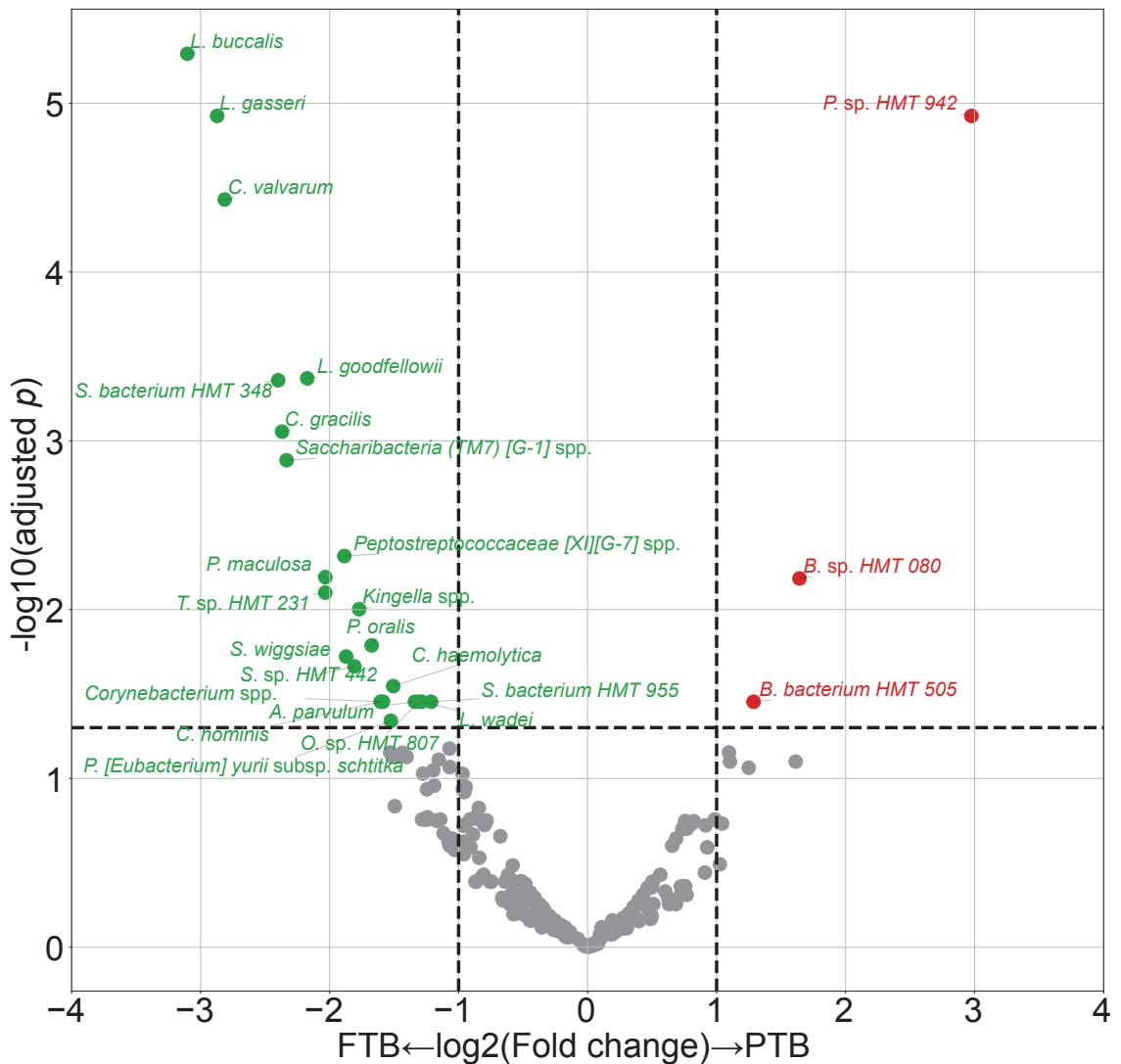


Figure 1: DAT volcano plot.

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

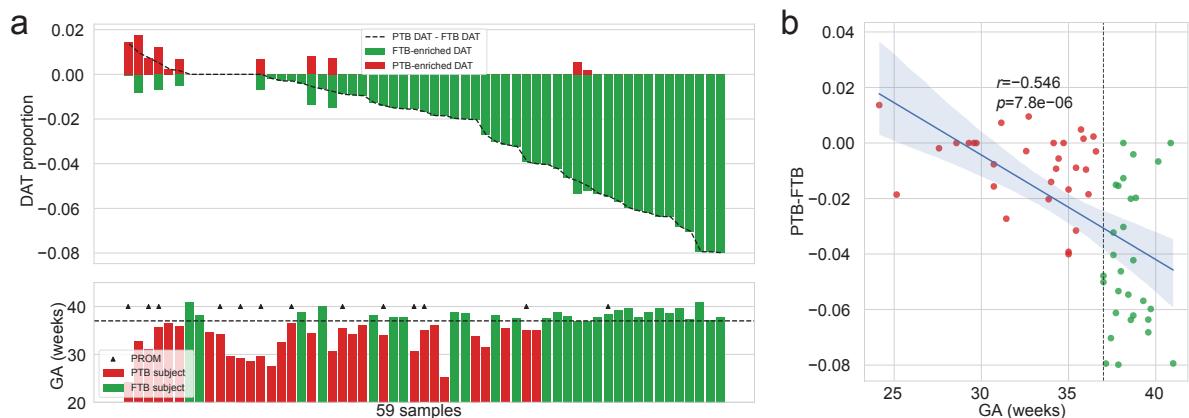


Figure 2: **Salivary microbiome compositions over DAT.**

(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.

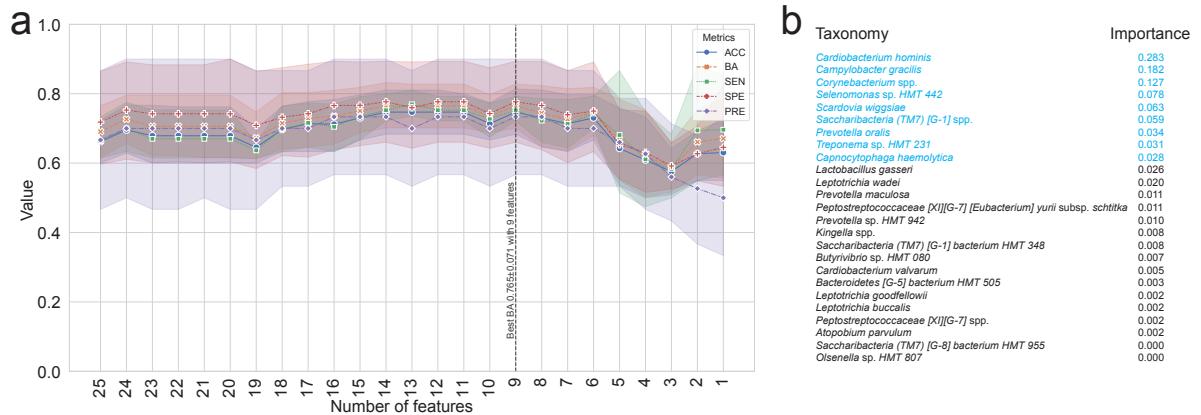


Figure 3: **Random forest-based PTB prediction model.**

(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.

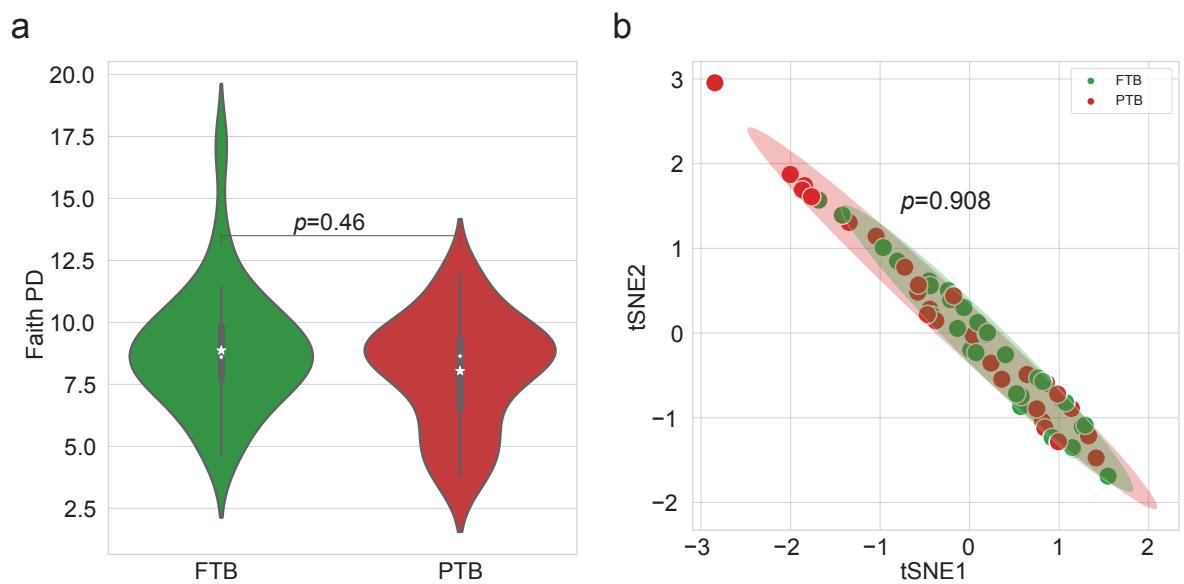


Figure 4: **Diversity indices.**

(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 $p = 0.908$)

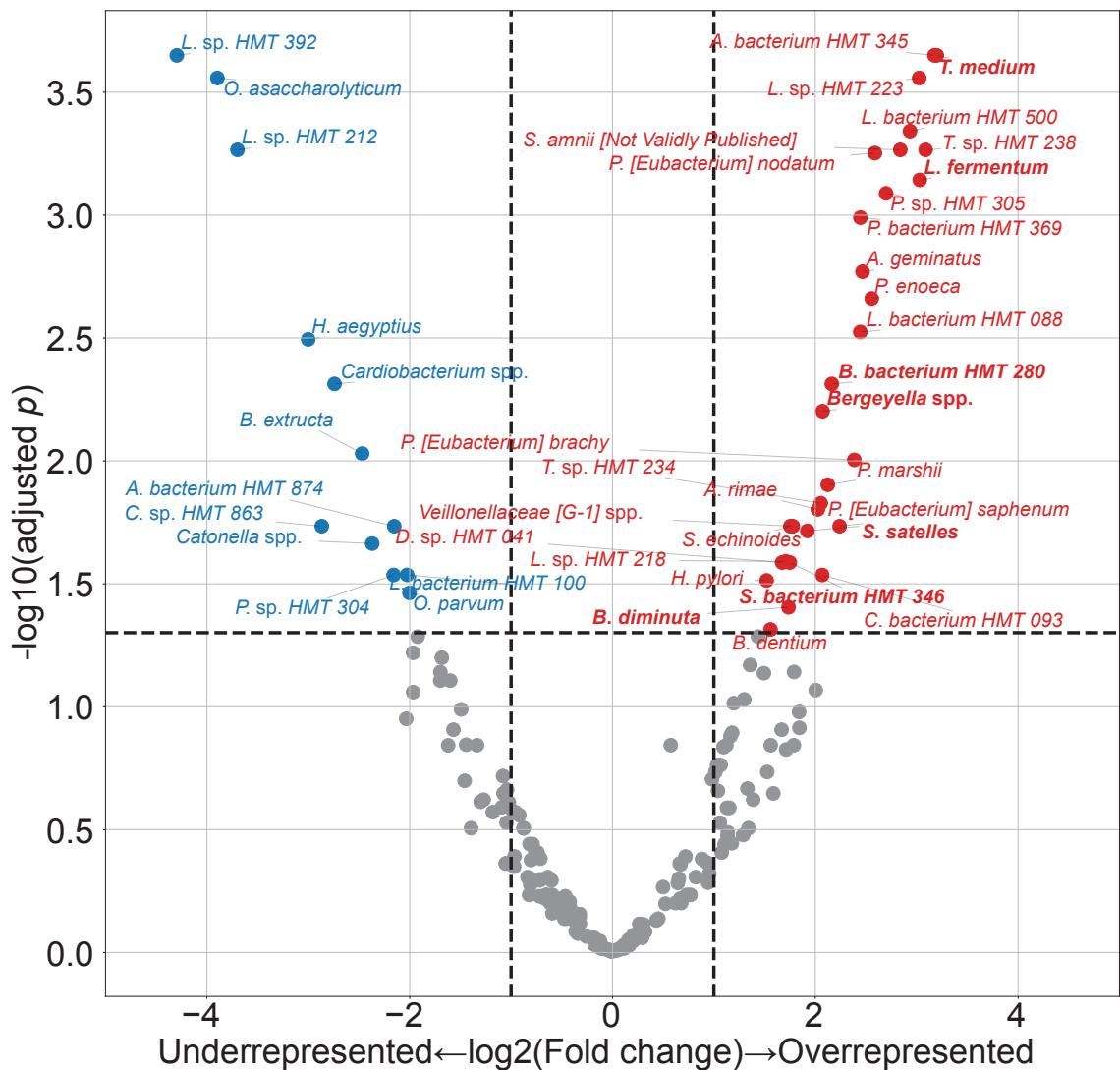


Figure 5: **PROM-related DAT**.

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.

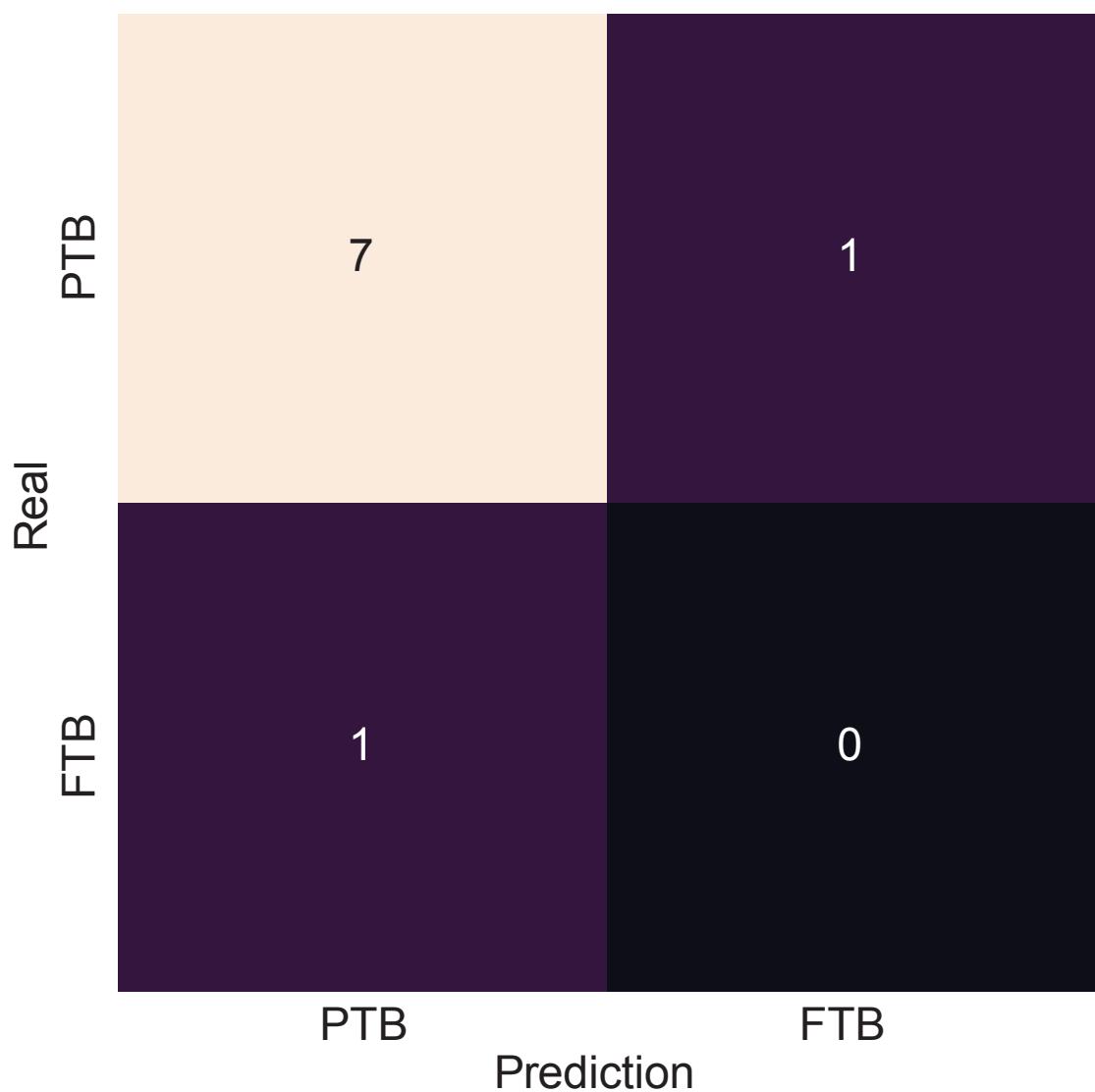


Figure 6: Validation of random forest-based PTB prediction model.

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)

391 **2.4 Discussion**

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

399 One of the most common salivary taxa that has been examined is *Fusobacterium nucleatum* (Han,
400 2015; Brennan & Garrett, 2019; Bolstad, Jensen, & Bakken, 1996), that is a Gram-negative, anaerobic, and
401 filamentous bacteria. *Fusobacterium nucleatum* can be separated from not only the salivary microbiome
402 but also the vaginal microbiome (Vander Haar, So, Gyamfi-Bannerman, & Han, 2018; Witkin, 2019). In
403 both animal and human investigation, *Fusobacterium nucleatum* infection has been linked to risk of PTB
404 (Doyle et al., 2014). According to recent researches, the placenta women who give birth prematurely may
405 include additional salivary microbiome dysbiosis, such as *Bergeyella* spp. and *Porphyromonas gingivalis*
406 (León et al., 2007; Katz, Chegini, Shiverick, & Lamont, 2009). Although *Bergeyella* spp. were one of the
407 PROM-overrepresented DAT (Figure 5), it was excluded in the final 25 PTB-related DAT. Furthermore,
408 *Porphyromonas gingivalis* and *Campylobacter gracilis* were pathogens of periodontitis in sub-gingival
409 microbiome (Yang et al., 2022). *Lactobacillus gasseri* was also one of the FTB-enriched DAT (Figure
410 1), and it is well established that early PTB risk can be reduced by *Lactobacillus gasseri* in the vaginal
411 microbiome (Basavaprabhu, Sonu, & Prabha, 2020; Payne et al., 2021).

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

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

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

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

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

443 **3 Random forest prediction model for periodontitis statuses based on the**
444 **salivary microbiomes**

445 This section includes the published contents:

446

447 **3.1 Introduction**

448 Saliva microbial dysbiosis brought on by the accumulation of plaque results in periodontitis, a chronic
449 inflammatory disease of the tissue that surrounds the tooth (Kinane, Stathopoulou, & Papapanou, 2017).
450 Loss of periodontal attachment is a consequence of periodontitis, which may lead to irreversible bone loss
451 and, eventually, permanent tooth loss if left untreated. A new classification criterion of periodontal diseases
452 was created in 2018, about 20 years after the 1999 statements of the previous one (Papapanou et al.,
453 2018). Even with this evolution, radiographic and clinical markers of periodontitis progression remain the
454 primary methods for diagnosing periodontitis (Papapanou et al., 2018). Such tools, nevertheless, frequently
455 demonstrate the prior damage from periodontitis rather than its present condition. Certain individuals have
456 a higher risk of periodontitis, a higher chance of developing severe generalized periodontitis, and a worse
457 response to common salivary bacteria control techniques utilized to prevent and treat periodontitis. As a
458 result, the 2017 framework for diagnosing periodontitis additionally allows for the potential development
459 of biomarkers to enhance diagnosis and treatment of periodontitis (Tonetti, Greenwell, & Kornman, 2018).
460 Instead of only depending on the progression of periodontitis, a new etiological indication based on the
461 current state must be introduced in order to enable appropriate intervention through early detection of
462 periodontitis. Thus, the current clinical diagnostic techniques that rely on periodontal probing can be
463 uncomfortable for patients with periodontitis (Canakci & Canakci, 2007).

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

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

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

496 Recently, we employed multiplex quantitative-PCR and machine learning-based classification model
497 to predict the severity of periodontitis based on the amount of nine pathogens of periodontitis from
498 saliva collections (E.-H. Kim et al., 2020). On the other hand, the fact that we focused merely at nine
499 pathogens for periodontitis and neglected the variety bacterial species associated to the various severities
500 of periodontitis constrained the breadth of our investigation. By developing a machine learning model
501 that could classify multiple severities of periodontitis based on the salivary microbiome composition,
502 this study aims to fill these knowledge gaps and produce more accurate and therapeutically useful
503 guidance to evaluate progression of periodontitis. Hence, in order to examine the salivary microbiome
504 composition of both healthy controls and patients with periodontitis in multiple stages, we applied
505 16S rRNA gene sequencing. Furthermore, employing the 2018 classification criteria, we sought to find
506 biomarkers (species) for the precise prediction of periodontitis severities (Papapanou et al., 2018; Chapple
507 et al., 2018).

508 **3.2 Materials and methods**

509 **3.2.1 Study participants enrollment**

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

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

527 **3.2.2 Periodontal clinical parameter diagnosis**

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

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

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

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

545 0. Normal gingiva: without inflammation nor discoloration.

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

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

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

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

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

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

556 • Healthy:

557 1. Bleeding sites < 10%

558 2. Probing depth: \leq 3 mm

559 • Stage I:

560 1. No tooth loss because of periodontitis.

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

562 3. Radiographic bone loss: < 15%

563 • Stage II:

564 1. No tooth loss because of periodontitis.

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

566 3. Radiographic bone loss: 15-33%

567 • Stage III:

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

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

570 3. Radiographic bone loss: > 33%

571 **3.2.3 Saliva sampling and DNA extraction procedure**

572 All study participants received instructions to avoid eating, drinking, brushing, and using mouthwash for
573 at least an hour prior to the saliva sample collection process. These collections were conducted between
574 09:00 and 11:00. Mouth rinse was collected by rinsing the mouth for 30 seconds with 12 mL of a solution
575 (E-zen Gargle, JN Pharm, Korea). All saliva samples were tagged with anonymous ID and stored at -4 °C.

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

- 580 • Forward: 5' -TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNNGCWGCAG-3'
581 • Reverse: 5' -GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'

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

- 584 1. Heat activation for 30 seconds at 95 °C.
585 2. 25 cycles for 30 seconds at 95 °C.
586 3. 30 seconds at 55 °C.
587 4. 30 seconds at 72 °C.

588 NexteraXT Indexed Primer was applied to amplification 10 µL of the purified initial PCR products for
589 the final library creation. The second PCR used the same conditions as the first PCR conditions but with
590 10 cycles. 16S rRNA gene sequencing was performed via 2×300 bp paired-end sequencing at Macrogen
591 Inc. (Macrogen, Seoul, Korea) using Illumina MiSeq platform (Illumina, San Diego, CA, USA).

592 **3.2.4 Bioinformatics analysis**

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

- 597 • Abundance-based Coverage Estimator (ACE) (Chao & Lee, 1992)
598 • Chao1 (Chao, 1984)
599 • Fisher (Fisher, Corbet, & Williams, 1943)
600 • Margalef (Magurran, 2021)
601 • Observed ASVs (DeSantis et al., 2006)
602 • Berger-Parker d (Berger & Parker, 1970)
603 • Gini index (Gini, 1912)

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

604 Aitchison index for a beta-diversity index was calculated using QIIME2 (version 2020.8) (Aitchison,
605 Barceló-Vidal, Martín-Fernández, & Pawlowsky-Glahn, 2000; Bolyen et al., 2019). We employed the
606 t-SNE algorithm to illustrate multi-dimensional data from the beta-diversity index computation (Van der
607 Maaten & Hinton, 2008). The beta-diversity index was compared using the PERMANOVA test (Anderson,
608 2014; Kelly et al., 2015) and MWU test.

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

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

621 As previously stated (E.-H. Kim et al., 2020), we used stratified k -fold cross-validation ($k = 10$)
622 by severity of periodontitis to achieve consistent and trustworthy classification results (Wong & Yeh,
623 2019). Additionally, we utilized various features with confusion matrices and their derivations to evaluate
624 the classification outcomes in order to identify which features optimize classification evaluations and
625 decrease sequencing efforts. Using the DAT discovered by ANCOM, we iteratively removed the least
626 significant taxa from the input features (taxa) of the random forest (Breiman, 2001) and gradient boosting
627 (Friedman, 2002) classification models using the backward elimination method. Random forest classifier
628 builds multiple decision trees independently using bootstrapped samples and aggregates their predictions,
629 enhancing stability and reducing overfitting problems. In contrast, Gradient boosting constructs trees
630 sequentially, where each new tree improves the errors of the previous ones using gradient descent, leading
631 to higher classification evaluations.

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

636 3.2.5 Data and code availability

637 All sequences from the 250 study participants have been published to the Sequence Read Archives (project
638 ID PRJNA976179): <https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA976179>. Docker

641 image that employed throughout this study is available in the DockerHub: <https://hub.docker.com/>
642 repository/docker/fumire/periodontitis_16s. Every code used in this study can be found on
643 GitHub: https://github.com/CompbioLabUnist/Periodontitis_16S.

644 **3.3 Results**

645 **3.3.1 Summary of clinical information and sequencing data**

646 Among clinical information of the study participants, clinical attachment level, probing depth, plaque
647 index, and gingival index, were significantly increased with periodontitis severity (Kruskal-Wallis test
648 $p < 0.001$), while sex were observed no significant difference (Table 2). Notably, clinical attachment level
649 and probing depth have significant differences among the periodontitis severities (MWU test $p < 0.01$;
650 Figure 15). Additionally, 71461.00 ± 11792.30 and 45909.78 ± 11404.65 reads per sample were obtained
651 before and after filtering low-quality reads and trimming extra-long tails, respectively (Figure 16). In 250
652 study subjects, we have found a total of 425 bacterial taxa (Figure 13).

653 **3.3.2 Diversity indices reveal differences among the periodontitis severities**

654 Rarefaction curves showed that the sequencing depth was sufficient (Figure 12). Alpha-diversity in-
655 dices indicated significant differences between the healthy and the periodontitis stages (MWU test
656 $p < 0.01$; Figure 7a-e); however, there were no significant differences between the periodontitis stages.
657 This emphasizes how essential it is to classify the salivary microbiome compositions and distinguish
658 between the stages of periodontitis using machine learning approaches.

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

664 **3.3.3 DAT among multiple periodontitis severities and their correlation**

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

- 668 • Group 1
 - 669 1. *Treponema* spp.
 - 670 2. *Prevotella* sp. HMT 304
 - 671 3. *Prevotella* sp. HMT 526
 - 672 4. *Peptostreptococcaceae [XI][G-5]* saphenum
 - 673 5. *Treponema* sp. HMT 260
 - 674 6. *Mycoplasma faecium*
 - 675 7. *Peptostreptococcaceae [XI][G-9]* brachy
 - 676 8. *Lachnospiraceae [G-8]* bacterium HMT 500
 - 677 9. *Peptostreptococcaceae [XI][G-6]* nodatum
 - 678 10. *Fretibacterium* spp.

- 679 • Group 2
- 680 1. *Porphyromonas gingivalis*
- 681 2. *Campylobacter showae*
- 682 3. *Filifactor alocis*
- 683 4. *Treponema putidum*
- 684 5. *Tannerella forsythia*
- 685 6. *Prevotella intermedia*
- 686 7. *Porphyromonas* sp. HMT 285

- 687 • Group 3
- 688 1. *Actinomyces* spp.
- 689 2. *Corynebacterium durum*
- 690 3. *Actinomyces graevenitzii*

691 Ten DAT that were significant enriched in stage II and stage III, but deficient in healthy formed Group
692 1 (Figure 8). Furthermore, in comparison to the healthy, the seven DAT of Group 2 were significantly
693 enriched in each of the stages of periodontitis. On the other hand, three DAT in Group 3 were deficient in
694 stage II and stage III, but significantly enriched in healthy. The relative proportions of the DAT further
695 supported these findings (Figure 8b), suggesting that the DAT is primarily linked to periodontitis rather
696 than other salivary bacteria.

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

699 3.3.4 Classification of periodontitis severities by random forest models

700 To confirm that using selected DAT bacterial profiles could have enhanced sequencing expenses without
701 losing the classification evaluations, we built the random forest classification models based on DAT and
702 full microbiome compositions (Figure 18). DAT based classifier showed non-significant different or better
703 evaluations, by removing confounding taxa.

704 Based on the proportion of DAT, random forest classifier were trained to classify the periodontitis
705 severities (Table 6). We conducted multi-label classification for the multiple periodontitis severities,
706 namely healthy, stage I, stage II, and stage III. In this setting, we classified multiple periodontitis
707 severities with the highest BA of 0.779 ± 0.029 (Table 4). AUC ranged between 0.81 and 0.94 (Figure
708 10b).

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

713 Based on the findings that the salivary microbiome composition in stage II is more comparable to
714 those in stage III than to other severities (Figure 7f and Figure 7j), we combined stage II and stage III to

715 perform a multi-label classification.

716 To examine alternative classification algorithms in comparison to random forest classification, we
717 selected gradient boost algorithm because it is another algorithm of the few classification algorithms
718 that can provide feature importances, which is essential for identifying key taxa contributing to the
719 classification of periodontitis severities. Thus, we assessed gradient boosting algorithms (Figure 20).
720 However, the classification evaluations obtained from gradient boosting have non-significant differences
721 compared to random forest classification.

722 Finally, to confirm the reliability and consistency of our random forest classifier, we validated our
723 classification model using openly accessible 16S rRNA gene sequencing from Spanish participants
724 (Iniesta et al., 2023) and Portuguese participants (Relvas et al., 2021) (Figure 11). Although some
725 evaluations, *e.g.* SPE, were low, the other were comparable.

Table 3: Clinical characteristics of the study participants.

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 I/II/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 graevenitzii</i>	0.059		
4	<i>Actinomyces graevenitzii</i>	0.052	<i>Lachnospiraceae (G-8) bacterium HMT 500</i>	0.050	<i>Porphyromonas sp. HMT 285</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>	0.052	<i>Prevotella intermedia</i>	0.033	<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>Prevotella intermedia</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 sp. HMT 526</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>Freibacterium spp.</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>Treponema sp. HMT 260</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>	0.003		
18	<i>Mycoplasma faecium</i>	0.014	<i>Prevotella sp. HMT 304</i>	0.014	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.003	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.005	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.002		
19	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.014	<i>Lachnospiraceae (G-8) bacterium HMT 500</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		
20	<i>Lachnospiraceae (G-8) bacterium HMT 500</i>	0.013										

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

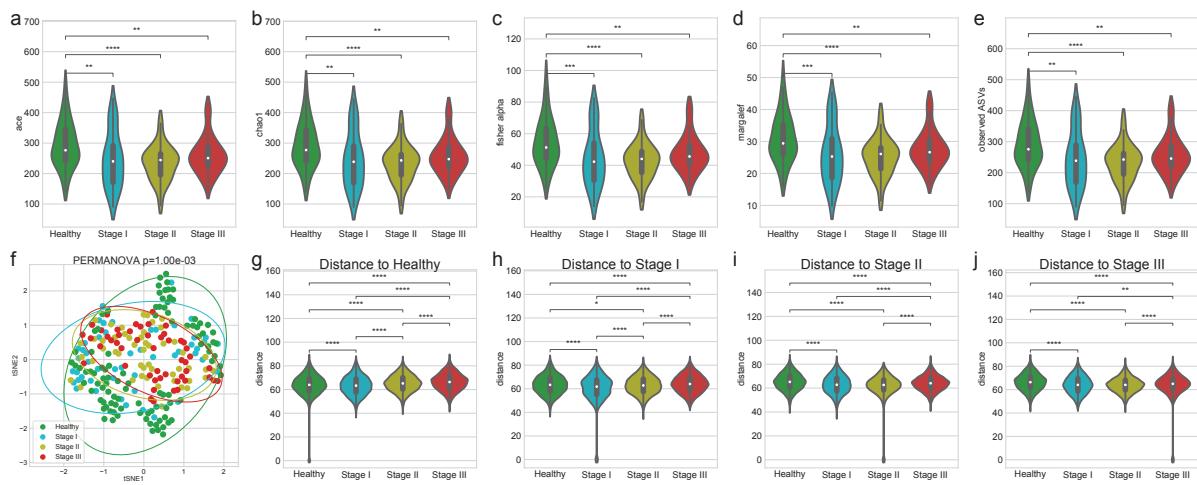


Figure 7: Diversity indices.

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

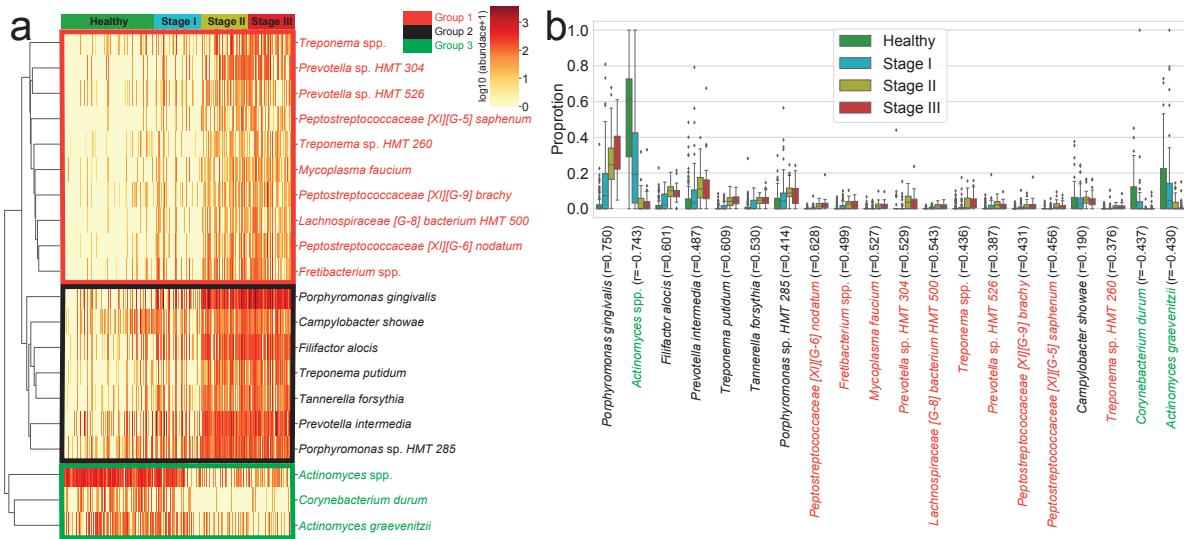


Figure 8: **Differentially abundant taxa (DAT).**

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.

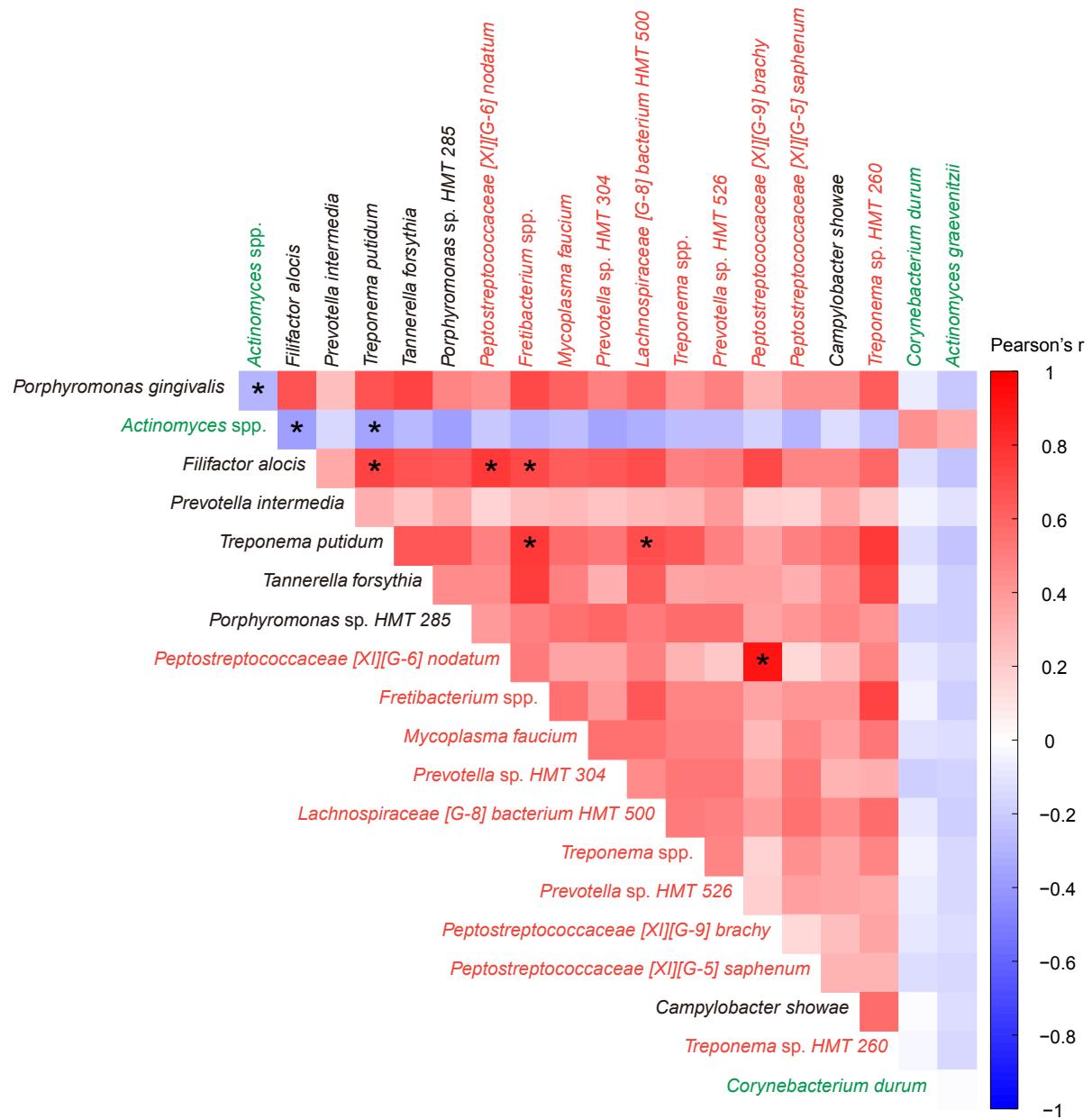


Figure 9: Correlation heatmap.

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

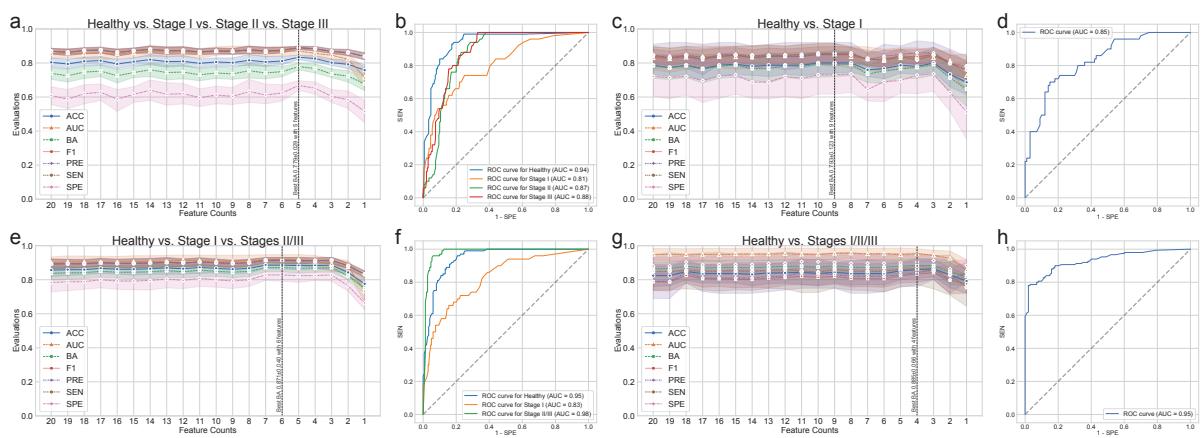


Figure 10: Random forest classification metrics.

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).

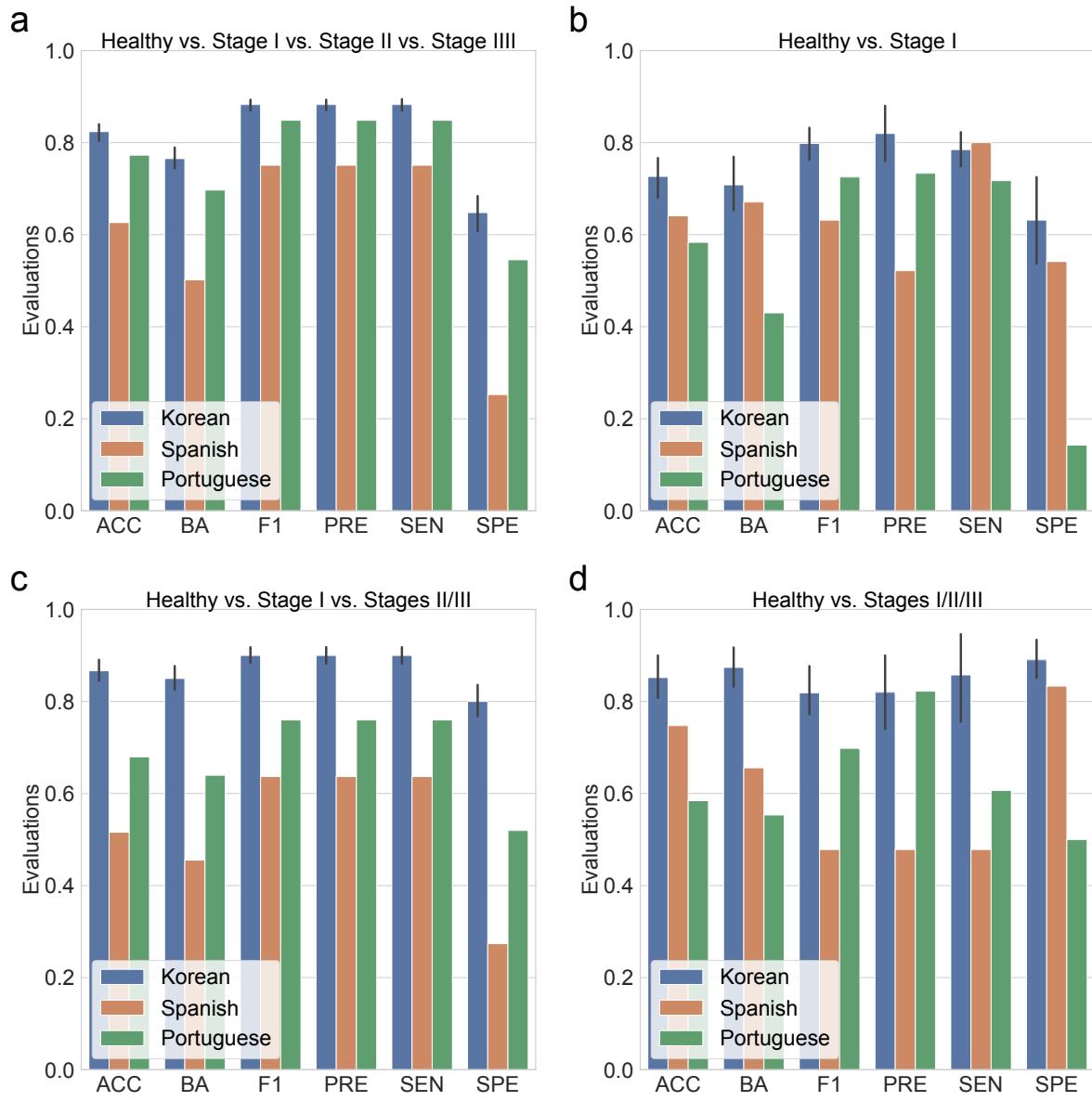


Figure 11: **Random forest classification metrics from external datasets.**

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.

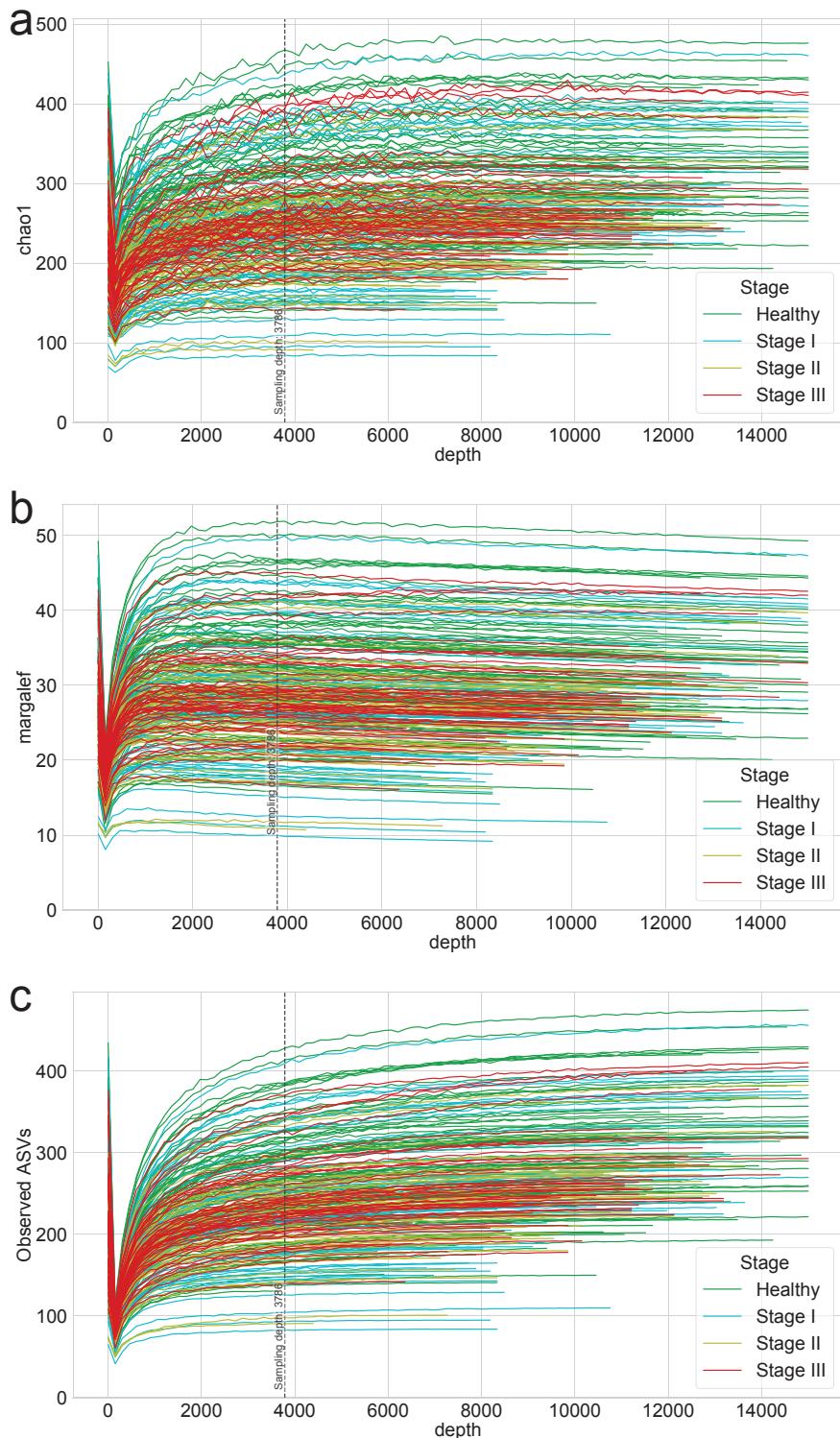


Figure 12: Rarefaction curves for alpha-diversity indices.

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

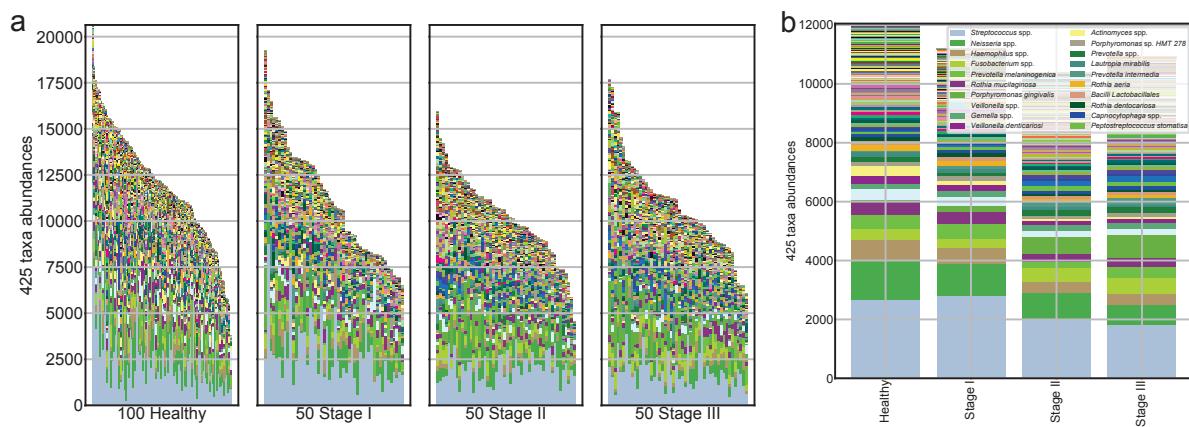


Figure 13: Salivary microbiome compositions in the different periodontal statuses.

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**).

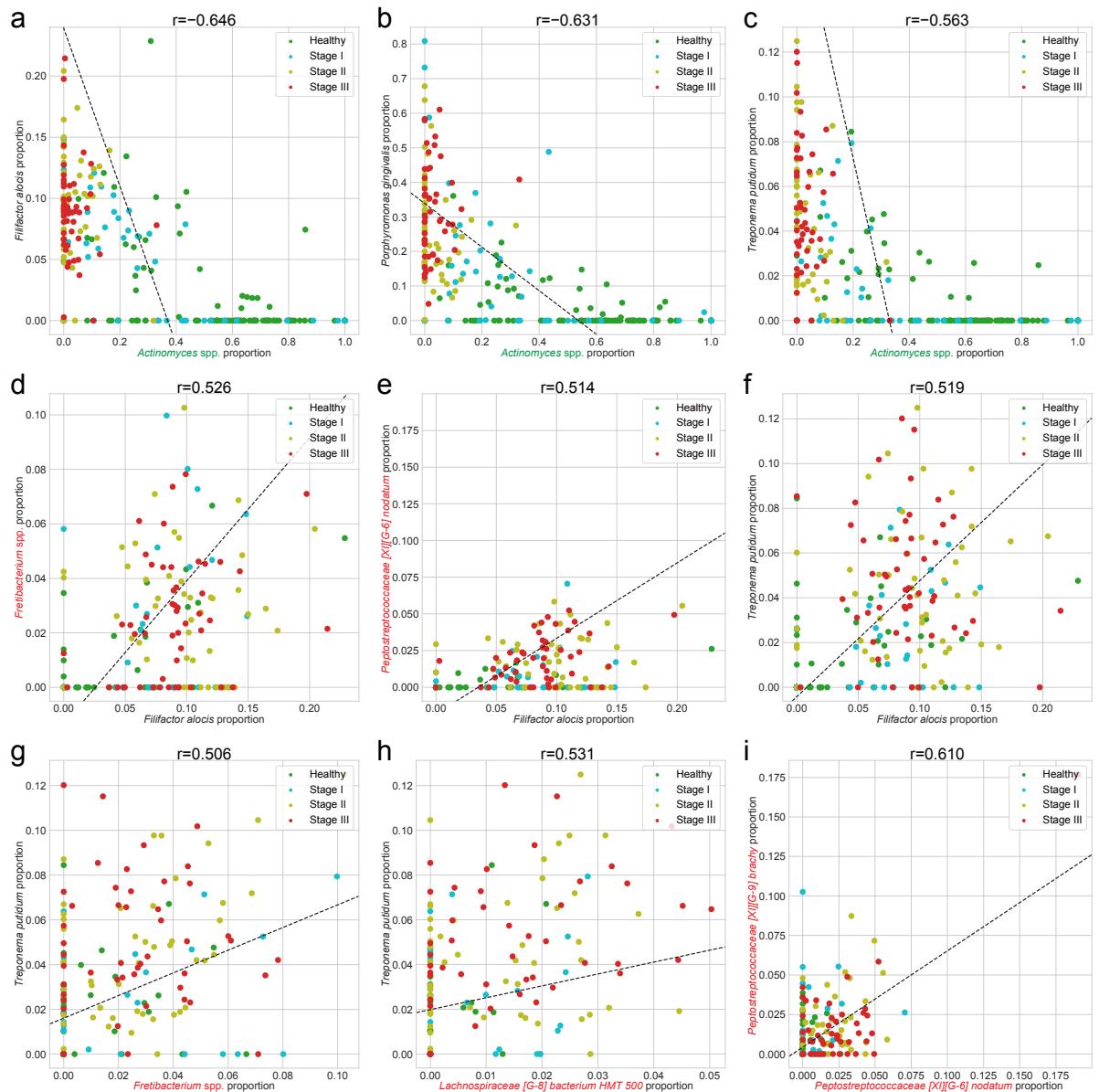


Figure 14: Correlation plots for differentially abundant taxa.

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).

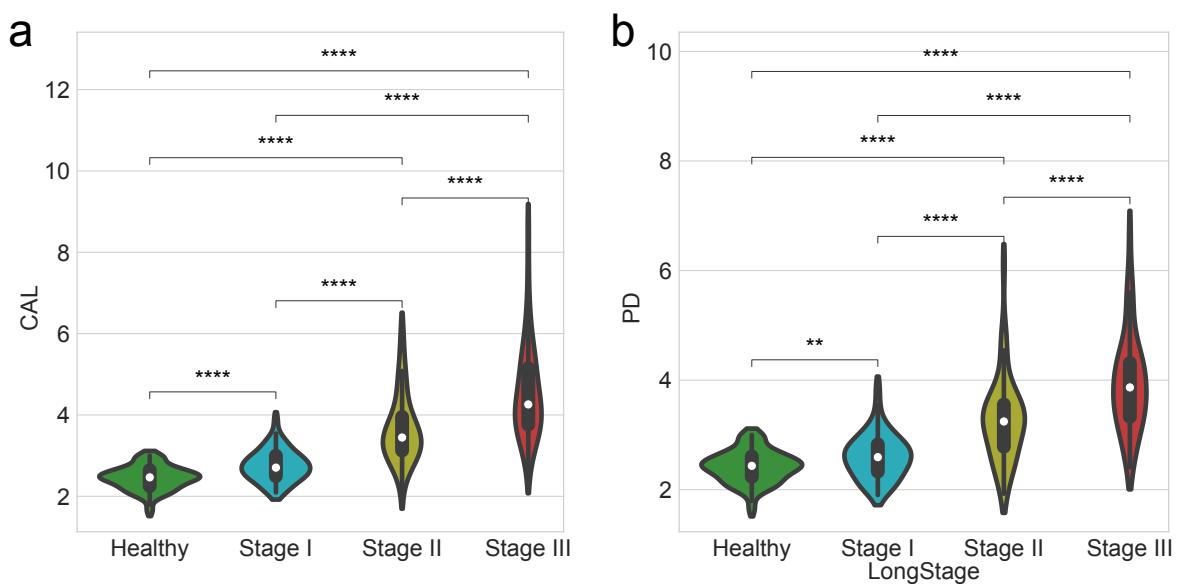


Figure 15: Clinical measurements by the periodontitis statuses.

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

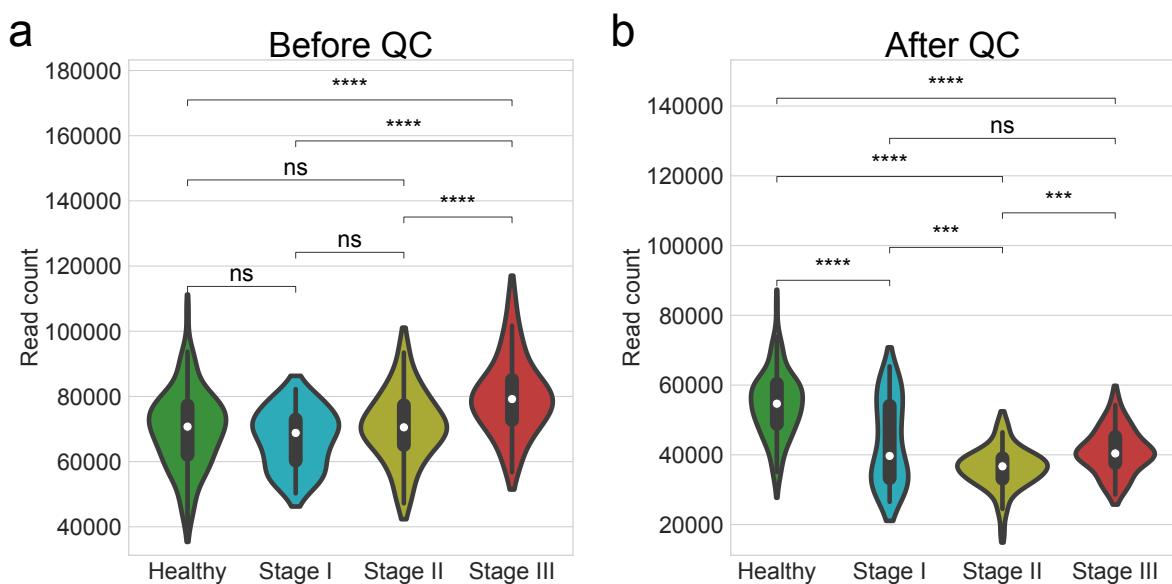


Figure 16: Number of read counts by the periodontitis statuses.

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

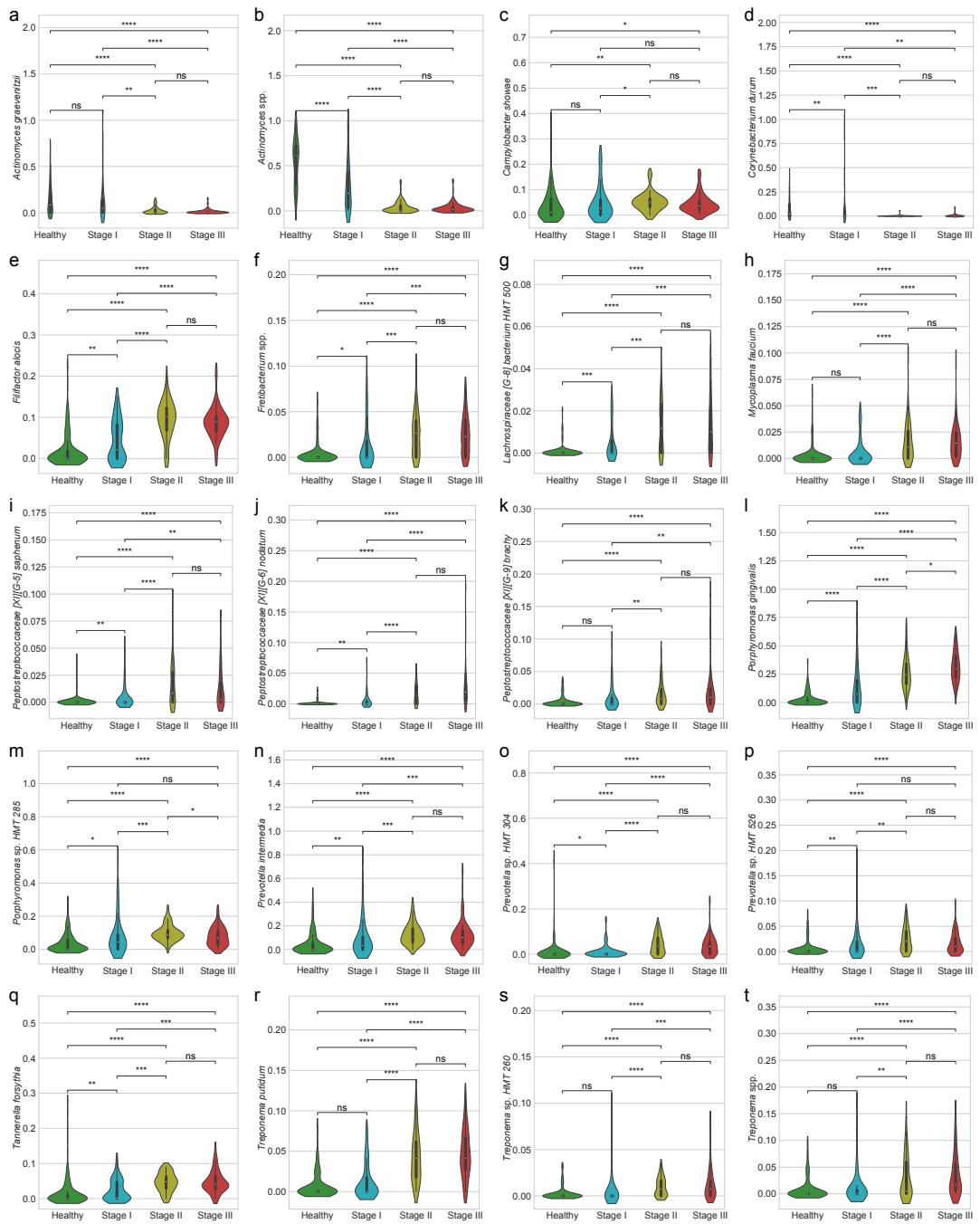


Figure 17: Proportion of DAT.

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

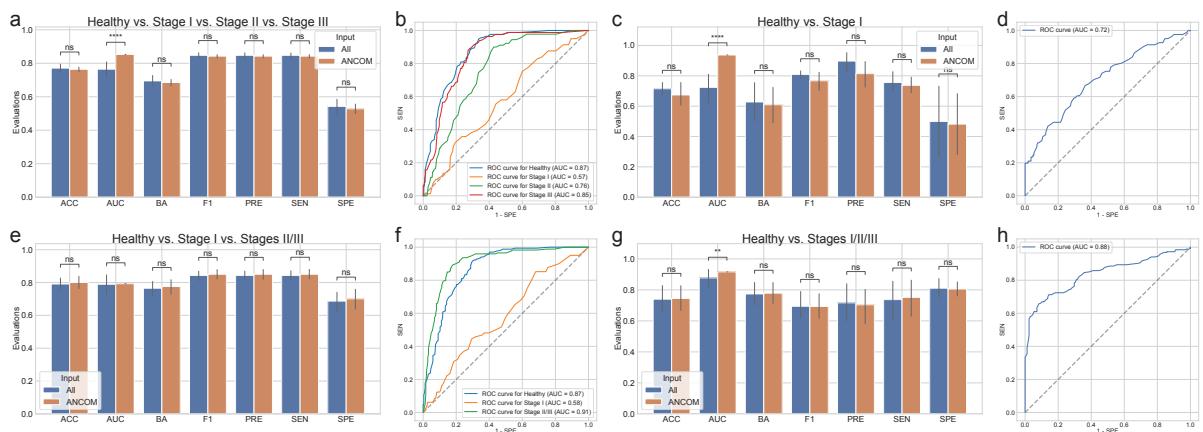


Figure 18: Random forest classification metrics with the full microbiome compositions and ANCOM-selected DAT compositions.

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

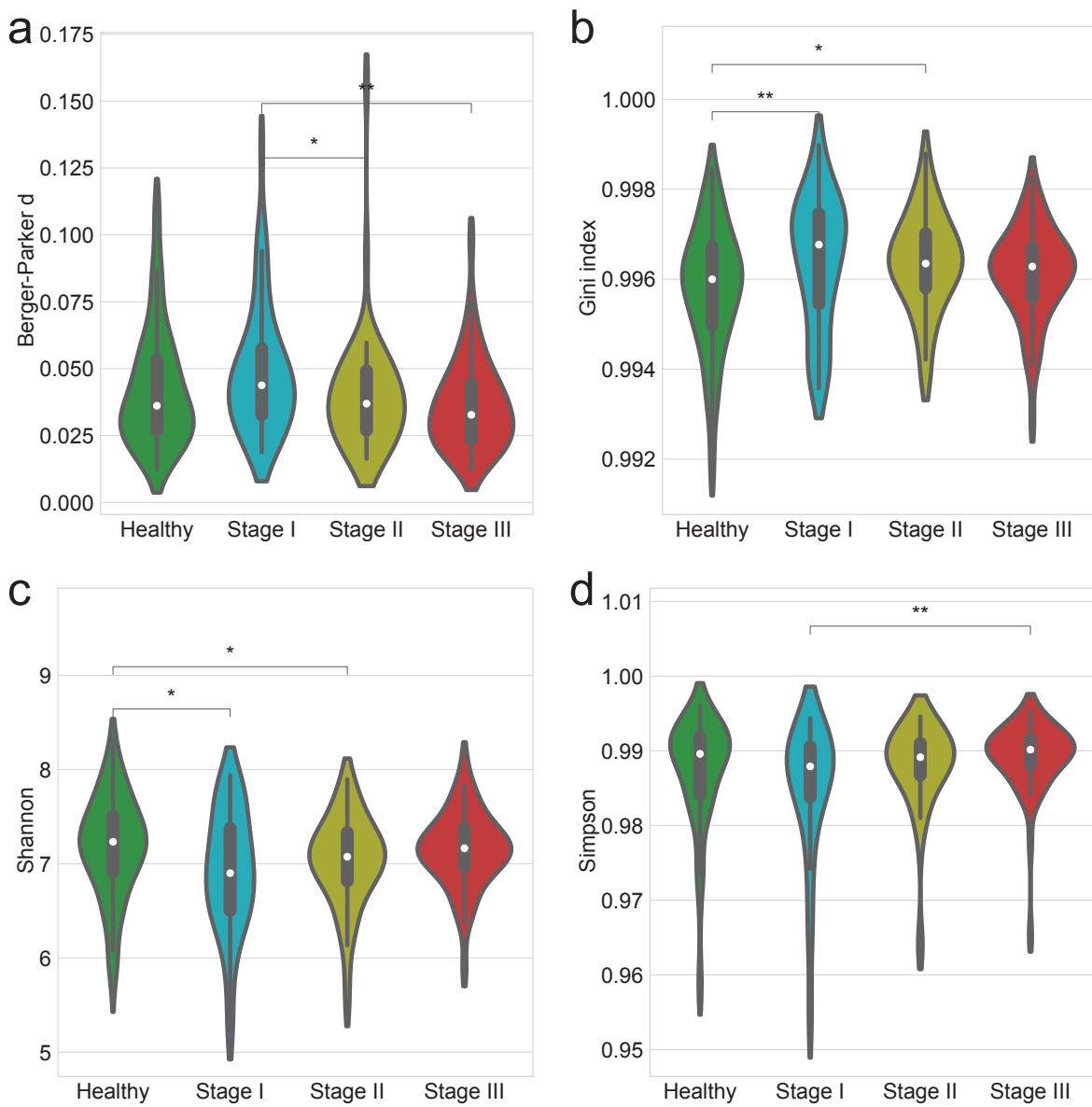


Figure 19: **Alpha-diversity indices account for evenness.**

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 $p \leq 0.01$ (**)

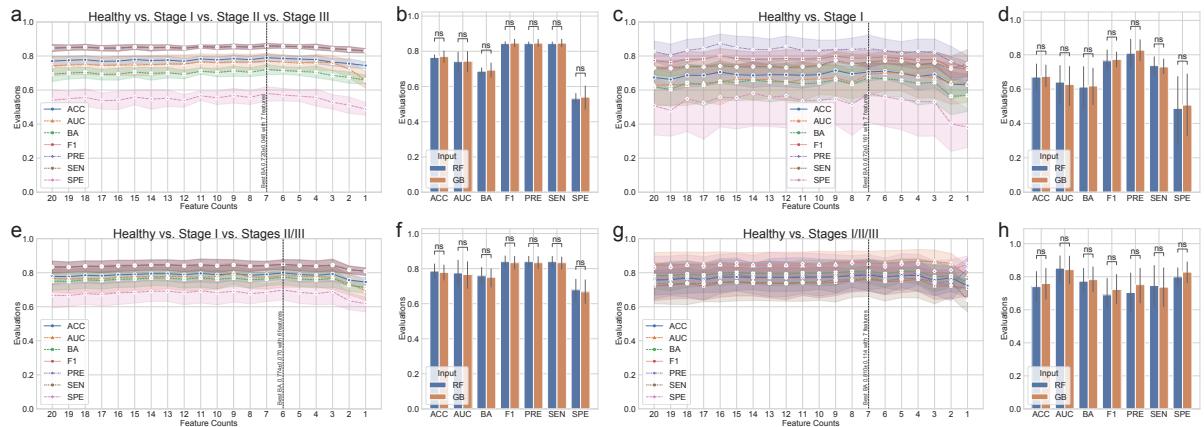


Figure 20: Gradient Boosting classification metrics.

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.

726 **3.4 Discussion**

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

735 Previous research identified the red complex as the primary pathogens of periodontitis (Listgarten,
736 1986): *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. Other studies, however,
737 have shown that periodontal pathogens communicate with other bacteria in the salivary microbiome
738 networks to generate dental plaque prior to the pathogenesis and development of periodontitis (Lamont &
739 Jenkinson, 2000; Rosan & Lamont, 2000; Yoshimura, Murakami, Nishikawa, Hasegawa, & Kawaminami,
740 2009).

741 Using subgingival plaque collections, recent researches have suggested a connection between the
742 periodontitis severity and the salivary microbiome compositions (Altabtbaei et al., 2021; Iniesta et al.,
743 2023; Nemoto et al., 2021). Therefore, we have examined the salivary microbiome compositions of
744 patients with multiple severities of periodontitis and periodontally healthy controls, extending on earlier
745 studies.

746 According to our findings, the salivary microbiome compositions have 425 taxa (Figure 13). We
747 computed the alpha-diversity indices to determine the variability within each salivary microbiome
748 composition, including ace (Chao & Lee, 1992), chao1 (Chao, 1984), fisher alpha (Fisher et al., 1943),
749 margalef (Magurran, 2021), observed ASVs (DeSantis et al., 2006), Berger-Parker *d* (Berger & Parker,
750 1970), Gini index (Gini, 1912), Shannon (Weaver, 1963), and Simpson (Simpson, 1949) (Figure 7 and
751 Figure 19). Alpha-diversity indices suggested that the microbial richness of periodontally healthy controls
752 was higher than that of patients with periodontitis (Figure 7a-e and Figure 19). These results are in line with
753 findings with that patients with advanced periodontitis, namely stage II and stage III, have less diversified
754 communities than periodontally healthy controls (Jorth et al., 2014). Recognizing that the periodontitis
755 severity increases the amount of *Porphyromonas gingivalis*, the salivary microbiome compositions from
756 periodontally healthy controls conserved microbial networks dominated by *Streptococcus* spp. (Figure
757 13). *Porphyromonas gingivalis* is one of the known periodontal pathogen that could cause dysbiosys
758 in the salivary microbiomes, suggesting in the pathophysiology of periodontitis. Despite this finding,
759 earlier research found that subgingival microbiome of patients with periodontitis had a greater alpha-
760 diversity index (observed ASVs) than that of healthy controls (Iniesta et al., 2023), might due to the
761 different sampling sites between saliva and subgingival plaque. On the other hand, another research
762 has addressed significant discrepancies in alpha-diversity indices from subgingival plaque, saliva, and
763 tongue biofilms from healthy controls and periodontitis patients, resulting the highest alpha-diversity

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

773 Therefore, we calculated beta-diversity indices to analyze the differences between the study partici-
774 pants. The distances for the multiple stages of periodontitis, including stage I, stage II, and stage III, as
775 well as healthy controls (Figure 4g-j and Table 7), suggesting notable differences among the multiple
776 periodontitis severities. In other words, the composition of the salivary microbiome compositions varies
777 depending on the periodontitis stages, so that supporting the findings from a previous study (Iniesta et al.,
778 2023). Taken together that it is nearly impossible to fully restore the attachment level after it has been lost
779 due to the progression and development of periodontitis, the ability to rapidly screen for periodontitis in
780 its early phases using saliva collections would be highly beneficial for effective disease management and
781 treatment.

782 Of the total of 425 taxa in the salivary microbiome composition that have been identified (Figure 13),
783 ANCOM was applied to select 20 taxa as the DAT that indicated notable abundance variation among
784 the periodontitis severities (Figure 8 and Table 5). Three sub-groups were formed from the DAT using
785 hierarchical clustering (Figure 8a). Surprisingly, two of the red complex pathogens (Rôças, Siqueira Jr,
786 Santos, Coelho, & de Janeiro, 2001), *Porphyromonas gingivalis* and *Tannerella forsythia*, were classified
787 in Group 2 and were more prevalent in stage II and stage II periodontitis compared to healthy controls.
788 *Campylobacter showae* was additionally placed in Group 2 of the orange complex pathogens (Gambin et
789 al., 2021). Furthermoe, some of the DAT in Group 2 have reported their crucial roles in pathogenesis
790 and development of periodontitis: *Filifactor alocis* (Aruni et al., 2015), *Treponema putidum* (Wyss et
791 al., 2004), *Tannerella forsythia* (Stafford, Roy, Honma, & Sharma, 2012; W. Zhu & Lee, 2016), and
792 *Prevotella intermedia* (Karched, Bhardwaj, Qudeimat, Al-Khabbaz, & Ellepol, 2022). Taken together,
793 this indicates that DAT in Group 2 is essential to periodontitis. The portion of some Group 1 DAT,
794 including *Peptostreptococcaceae[XI][G-5] saphenum*, *Peptostreptococcaceae[XI][G-6] nodatum*, and
795 *Peptostreptococcaceae[XI][G-9] brachy*, in healthy controls and patients with periodontitis significantly
796 differed, according to earlier research (Lafaurie et al., 2022). These outcomes support our research,
797 implying that Group 1 DAT are also essential to the etiology and progression of periodontitis. However,
798 in contrast to patients with periodontitis, Group 3 DAT, namely *Corynebacterium durum* and *Actinomyces*
799 *graevenitzii*, were enriched in healthy controls, which is consistent with earlier research (Redanz et al.,
800 2021; Nibali et al., 2020).

801 In our correlation analysis (Figure 9), we have discovered strongly negative correlations (coefficient \leq
802 -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%

- 842 • BA: 84%
 843 • SEN: 94%
 844 • SPE: 74%
- 845 By separating patients with periodontitis from healthy controls using only four DAT, *e.g.* *Actinomyces*
 846 *graevenitzii*, *Actinomyces* spp., *Corynebacterium durum*, and *Porphyromonas gingivalis*, our machine
 847 learning model performed better than previously published models (Figure 10, Table 4, and Table 6):
- 848 • AUC: $95.3\% \pm 4.9\%$
 849 • BA: $88.5\% \pm 6.6\%$
 850 • SEN: $86.4\% \pm 15.7\%$
 851 • SPE: $90.5\% \pm 7.0\%$
- 852 This result showed that by detecting Group 3 bacteria that were substantially abundant in health
 853 controls than patients with periodontitis, our study increased BA by at least 5% and SPE by at least 17%.
- 854 Furthermore, we have validated our machine-learning prediction model using openly accessible 16S
 855 gene rRNA sequencing data from Portuguese (Iniesta et al., 2023) and Spanish participants (Relvas et
 856 al., 2021) in order to ensure the consistency of our random forest classification model (Figure 11). Our
 857 classification models employed in this study were primarily developed and assessed on Korean study par-
 858 ticipants, which may limit their generalizability to other ethnic groups with different salivary microbiome
 859 compositions (Premaraj et al., 2020; Renson et al., 2019). Therefore, the evaluations of this periodonti-
 860 tis classification models can be affected by ethnic-specific variances and differences, highlighting the
 861 necessity for additional validation and adjustment across a spectrum of ethnic backgrounds.
- 862 Regarding the clinical characteristics and potential confounders influencing the analysis of salivary
 863 microbiome compositions connected with periodontitis severity, this study had a number of limitations
 864 that were pointed out. We did not offer clinical information, such as the percentage of teeth, the percentage
 865 of bleeding on probing, nor dental furcation involvement, even though we did gather information on
 866 attachment level, probing depth, plaque index, and gingival index; this might have it challenging to present
 867 thorough and in-depth data about periodontal health. Moreover, the broad age range may make it tougher
 868 to evaluate the relationship between age and periodontitis statuses, providing the necessity for future
 869 studies to consider into account more comprehensive clinical characteristics associated with periodontitis.
 870 Additionally, potential confounders—*e.g.* body mass index (Bombin, Yan, Bombin, Mosley, & Ferguson,
 871 2022) and e-cigarette use (Suzuki, Nakano, Yoneda, Hirofushi, & Hanioka, 2022)—which might have
 872 affected dental health and salivary microbiome composition were disregarding consideration in addition to
 873 smoking status and systemic diseases. Thus, future research incorporating these components would offer a
 874 more thorough knowledge of how lifestyle factors interact and affect the salivary microbiome composition
 875 and periodontal health. Throughout, resolving these limitations will advance our understanding in
 876 pathogenesis and development of periodontitis, offering significant novel insights on the causal connection
 877 between systemic diseases and the salivary microbiome compositions.

878 **4 Metagenomic signature analysis of Korean colorectal cancer**

879 **4.1 Introduction**

880 Colorectal cancer (CRC) is one of the most prevalent and life-threatening malignancies worldwide
881 (Kuipers et al., 2015; Center, Jemal, Smith, & Ward, 2009; N. Li et al., 2021), with its incidence
882 influenced by a combination of genetic (Zhuang et al., 2021; Peltomaki, 2003), environmental (O'Sullivan
883 et al., 2022; Raut et al., 2021), and lifestyle factors (X. Chen et al., 2021; Bai et al., 2022; Zhou et
884 al., 2022; X. Chen, Li, Guo, Hoffmeister, & Brenner, 2022). Established risk factors include a often
885 diet in red and processed meats (Kennedy, Alexander, Taillie, & Jaacks, 2024; Abu-Ghazaleh, Chua,
886 & Gopalan, 2021), obesity (Mandic, Safizadeh, Niedermaier, Hoffmeister, & Brenner, 2023; Bardou
887 et al., 2022), cigarette smoking (X. Chen et al., 2021; Bai et al., 2022), alcohol consumption (Zhou et
888 al., 2022; X. Chen et al., 2022), and a sedentary lifestyle (An & Park, 2022), all of which contribute to
889 chronic inflammation, mutagenesis, and metabolic regulation. Additionally, underlying conditions, e.g.
890 Lynch syndrome (Vasen, Mecklin, Khan, & Lynch, 1991; Hampel et al., 2008) and familial adenomatous
891 polyposis (Inra et al., 2015; Burt et al., 2004), significantly increase risk of CRC due to persistent mucosal
892 inflammation and somatic mutations that promote tumorigenesis.

893 The gut microbiome plays a fundamental role in maintaining host health by helping digestion
894 (Joscelyn & Kasper, 2014; Cerqueira, Photenhauer, Pollet, Brown, & Koropatkin, 2020), regulating
895 metabolism (Dabke, Hendrick, Devkota, et al., 2019; Utzschneider, Kratz, Damman, & Hullarg, 2016;
896 Magnúsdóttir & Thiele, 2018), adjusting immune function (Kau, Ahern, Griffin, Goodman, & Gordon,
897 2011; Shi, Li, Duan, & Niu, 2017; Broom & Kogut, 2018), and even coordinating neurological processes
898 by the brain-gut axis (Martin et al., 2018; Aziz & Thompson, 1998; R. Li et al., 2024). Comprising
899 these gut microbiota, including, archaea, bacteria, fungi, and viruses, the gut microbiome contributes
900 to the synthesis of essential vitamins, and production of fatty acids, which influence intestinal integrity
901 and immune responses. Thus, well-balanced gut microbiome composition modulates systemic immune
902 function by interacting with gut-associated lymphoid tissue, shaping immune tolerance and response
903 to infections. Hence, emerging evidence suggests that dysbiosis in the gut microbiome composition are
904 associated not only a narrow range of diseases, e.g. diarrhea and enteritis (Paganini & Zimmermann,
905 2017; Gao, Yin, Xu, Li, & Yin, 2019) but also a wide range of diseases, e.g. obesity, diabetes, and cancers
906 (Barlow et al., 2015; Hartstra et al., 2015; Helmink et al., 2019; Cullin et al., 2021).

907 Recent studies have highlighted the crucial role of the gut microbiome in tumorigenesis and progres-
908 sion of CRC (Song, Chan, & Sun, 2020; Rebersek, 2021), with dysbiosis emerging as a potential risk
909 factor. Dysbiosis in gut microbiome compositions can promote tumorigenesis of many cancers, including
910 CRC, through several signaling cascades, including inflammation, mutagenesis, and altered metabolism
911 in host. Certain bacteria species, such as *Fusobacterium* genus (Hashemi Goradel et al., 2019; Bullman et
912 al., 2017; Flanagan et al., 2014), *Bacteroides* genus (Ulger Toprak et al., 2006; Boleij et al., 2015), and
913 *Escherichia coli* (Swidsinski et al., 1998; Bonnet et al., 2014), have been associated with development
914 and progression of CRC by producing pro-inflammatory signals, generating toxins including mutagens,

915 and disrupting the intestinal barriers including mucous surface. In contrast, beneficial bacteria, such as
916 *Lactobacillus* genus (Ghorbani et al., 2022; Ghanavati et al., 2020) and *Bifidobacterium* genus (Le Leu,
917 Hu, Brown, Woodman, & Young, 2010; Fahmy et al., 2019), are regarded to apply protective roles by
918 maintaining homeostasis of gut microbiome compositions and regulating immune responses including
919 inflammation.

920 Furthermore, identifying metagenome biomarkers in Korean CRC patients is essential, as the gut
921 microbiome compositions significantly vary by ethnicity due to genetic, dietary, and environmental
922 factor (Fortenberry, 2013; Merrill & Mangano, 2023; Parizadeh & Arrieta, 2023). Additionally, ethnicity-
923 specific microbiome composition signatures may affect the reliability of previously established biomarkers
924 derived from predominantly Western CRC cohorts (Network et al., 2012), necessitating population-
925 specific investigations. By identifying metagenomic biomarkers tailored to Korean CRC patients, we
926 can improve early detection rate of early-stage CRC, develop more accurate risk of CRC, and explore
927 microbiome-targeted therapies that consider host-microbiome interactions within the Korean population.

928 Accordingly, this study aims to identify microbiome-based biomarkers specific to CRC within
929 the Korean population, addressing the critical demand for ethnicity-specific microbiome research. By
930 leveraging metagenomic sequencing and advanced computational biology analysis, this study seeks to
931 uncover novel microbial signatures associated with Korean CRC patients. As part of the larger "Multi-
932 genomic analysis for biomarker development in colon cancer" project (NTIS No. 1711055951), this study
933 investigates microbial signatures within next-generation sequencing data to enhance precision medicine
934 approaches for CRC and to develop robust microbiome-based biomarkers for early detection, prognosis,
935 and therapeutic stratification, complementing genomic and epigenomic markers. Hence, this research
936 represents a crucial step toward personalized cancer diagnostic and therapeutic strategies tailored to the
937 Korean population.

938 **4.2 Materials and methods**

939 **4.2.1 Study participants enrollment**

940 To achieve metagenomic observations of CRC, a total of 211 Korean CRC patients were enrolled (Table
941 8). The tissue samples were collected from both the tumor lesion and its corresponding adjacent normal
942 lesion to enable comparative metagenomic analyses. Tumor tissue samples were obtained from confirmed
943 CRC lesions, ensuring adequate representation of CRC-associated microbial alterations. Adjacent normal
944 tissues were collected from non-cancerous regions away from the tumor margin to serve as a control
945 for baseline molecular and microbial composition. Moreover, clinical information was collected for all
946 study participants included in this study to investigate potential associations between gut microbiome
947 compositions and clinical outcomes. Key clinical characteristics recorded included overall survival (OS),
948 recurrence, age at diagnosis and sex. Additionally, microsatellite instability (MSI) status, a critical
949 molecular feature of CRC (Boland & Goel, 2010; Söreide, Janssen, Söiland, Körner, & Baak, 2006; Vilar
950 & Gruber, 2010), was evaluated using next-generation sequencing methods to classify CRC as MSI-high,
951 MSI-low, or microsatellite stable (MSS). These clinical parameters were integrated with metagenomic
952 data to explore potential microbiome-based biomarkers for CRC prognosis and progression. Ethical
953 approval was obtained for clinical data collection, and all patient information was anonymized to ensure
954 confidentiality in accordance with institutional guidelines.

955 **4.2.2 DNA extraction procedure**

956 Tissue samples were immediately processed under sterile conditions to prevent contamination and
957 preserved in low temperature (-80 °C) storage for downstream DNA extraction and whole-genome
958 sequencing. Furthermore, produced sequencing data were provided by the "Multi-genomic analysis
959 for biomarker development in colon cancer" project (NTIS No. 1711055951) in mapped BAM format,
960 aligned to the hg38 human reference genome. The preprocessing pipeline utilized by the main project
961 included high-throughput whole-genome sequencing using standardized alignment algorithm, BWA
962 (H. Li & Durbin, 2009). In addition to the mapped human sequences, our whole-genome sequencing
963 data retained unmapped sequences, which contain potential microbial reads that were not aligned to the
964 human reference genome.

965 **4.2.3 Bioinformatics analysis**

966 To identify microbial signatures associated with CRC, we employed PathSeq (Kostic et al., 2011; Walker
967 et al., 2018), a computational pipeline designed for metagenomic analysis of high-throughput sequencing
968 data including the whole-genome sequences. After processing these sequencing data through the PathSeq
969 pipeline, a comprehensive bioinformatics analyses were conducted to characterize microbial signatures
970 associated with CRC. Prevalent taxa identification was performed by determining microbial taxa present
971 in the majority of the study participants, filtering out low-abundance and rare taxa to ensure robust down-
972 stream analyses. To assess microbial community structure, diversity indices were calculated, including

973 alpha-diversity to evaluate single-sample diversity and beta-diversity to compare microbial composition
974 between the tumor tissues and their corresponding adjacent normal tissues. Differentially abundant taxa
975 (DAT) were identified using statistical method, (DESeq2, ANCOM), adjusting for sequencing depth and
976 potential confounders to highlight taxa significantly associated with CRC. To explore functional implica-
977 tions, microbial pathway prediction was performed using (PICRUSt3, HUMAnN3), linking microbial
978 composition to metabolic and functional pathways relevant to carcinogenesis and progression of CRC.
979 This multi-layered bioinformatics approach enabled a comprehensive investigation of gut microbiome
980 alteration in CRC, facilitating the identification of potential microbial biomarkers for diagnosis and
981 prognosis of CRC.

982 **4.2.4 Data and code availability**

983 All sequences from the 211 study participants have been published to the Korea Bioinformation Center
984 (data ID KGD10008857): <https://kbds.re.kr/KGD10008857>. Docker image that employed through-
985 out this study is available in the DockerHub: <https://hub.docker.com/repository/docker/fumire/unist-crc-copm/general>. Every code used in this study can be found on GitHub: <https://github.com/CompbioLabUnist/CoPM-ColonCancer>.

988 **4.3 Results**

989 **4.3.1 Summary of clinical characteristics**

990 **4.3.2 Gut microbiome compositions**

991 **4.3.3 Diversity indices**

992 **4.3.4 DAT selection**

993 **4.3.5 Pathway prediction**

Table 8: Clinical characteristics of CRC study participants

994 **4.4 Discussion**

⁹⁹⁵ **5 Conclusion**

⁹⁹⁶ In conclusion, the research described in this doctoral dissertation was conducted to identify significant ...

⁹⁹⁷ In the section 2, I show that

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