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Doctoral Thesis

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Microbiota in Human Diseases

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Department of Biomedical Engineering

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Ulsan National Institute of Science and Technology

⁶

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CHURCH OF THE FLYING SPAGHETTI MONSTER

February 09, 2021

Letter of Good Standing

Dear Sir or Madam:

I am pleased to verify that _____

JAEWOONG LEE

is an ordained minister of the Church of the Flying Spaghetti Monster and recognized
within our organization as a member in good standing.

We hereby consent to this minister performing ceremonies and request that they are
granted all privileges and respect appropriate to a spiritual leader.

Any questions can be directed to the undersigned.

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

Representative,
Church of the Flying Spaghetti Monster
Bobby Henderson



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13

Abstract

14 (Microbiome)

15 (PTB) Section 2 introduces...

16 (Periodontitis) Section 3 describes...

17 (Colon) Setion 4...

18 (Conclusion)

19

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

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

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List of Abbreviations

103 **ACC** Accuracy

104 **ASV** Amplicon sequence variant

105 **AUC** Area-under-curve

106 **BA** Balanced accuracy

107 **C-section** Cesarean section

108 **DAT** Differentially abundant taxa

109 **F1** F1 score

110 **Faith PD** Faith's phylogenetic diversity

111 **FTB** Full-term birth

112 **GA** Gestational age

113 **MWU test** Mann-Whitney U-test

114 **PRE** Precision

115 **PROM** Prelabor rupture of membrane

116 **PTB** Preterm birth

117 **ROC curve** Receiver-operating characteristics curve

118 **rRNA** Ribosomal RNA

119 **SD** Standard deviation

120 **SEN** Sensitivity

121 **SPE** Specificity

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

123 **1 Introduction**

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

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

159 16S ribosomal RNA (rRNA) gene sequencing is one of the most extensively applied methods for
160 characterizing microbial communities by targeting the conserved 16S rRNA gene, which contains both

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

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

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

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

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

245 (Limitation & Novelty)

Table 1: Confusion matrix

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

246

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

247

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

248

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

249

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

250

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

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

251 **2 Predicting preterm birth using random forest classifier in salivary mi-**
252 **crobiome**

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

254 Hong, Y. M., **Lee, Jaewoong**, Cho, D. H., Jeon, J. H., Kang, J., Kim, M. G., ... & Kim, J. K. (2023).
255 Predicting preterm birth using machine learning techniques in oral microbiome. *Scientific Reports*, 13(1),
256 21105.

257 **2.1 Introduction**

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

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

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

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

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

290 Multiple unfavorable birth outcomes, including PROM and PTB, have been linked to periodontitis
291 as an independence risk factor, according to numerous epidemiological researches (Offenbacher et al.,
292 1996). It is expected that the oral microbiome will be able to explain additional hematogenous pathways
293 in light of these precedents; however, the oral microbiome composition of fetuses is limited understood.

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

297 **2.2 Materials and methods**

298 **2.2.1 Study design and study participants**

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

305 **2.2.2 Clinical data collection and grouping**

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

- 308 • maternal age at delivery
- 309 • diabetes mellitus
- 310 • hypertension
- 311 • overweight and obesity
- 312 • C-section
- 313 • history PROM or PTB
- 314 • gestational week on delivery
- 315 • birth weight
- 316 • sex

317 **2.2.3 Salivary microbiome sample collection**

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

326 **2.2.4 16s rRNA gene sequencing**

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

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

333 **2.2.5 Bioinformatics analysis**

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

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

345 Taxonomic assignment were implemented with HOMD (version 15.22) (T. Chen et al., 2010).
346 Afterward, DESeq2 was implemented to identify differentially abundant taxa (DAT) that could dis-
347 tinguish between salivary microbiome from PTB and FTB participants (Love et al., 2014). Taxa with
348 $|\log_2 \text{FoldChange}| > 1$ and $p < 0.05$ were considered as statistically significant.

349 The taxa for predicting PTB using salivary microbiome data were determined using a random forest
350 classifier (Breiman, 2001). Through stratified *k*-fold cross-validation (*k* = 5) that preserves the existence
351 rate of PTB and FTB participants, consistency and trustworthy classification were ensured (Wong & Yeh,
352 2019).

353 **2.2.6 Data and code availability**

354 All sequences from the 59 study participants have been added to the Sequence Read Archives (project ID
355 PRJNA985119): <https://dataview.ncbi.nlm.nih.gov/object/PRJNA985119>. Docker image that
356 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.

359 **2.3 Results**

360 **2.3.1 Overview of clinical information**

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

367 **2.3.2 Comparison of salivary microbiomes composition**

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

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

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

379 **2.3.3 Random forest classification to predict PTB risk**

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

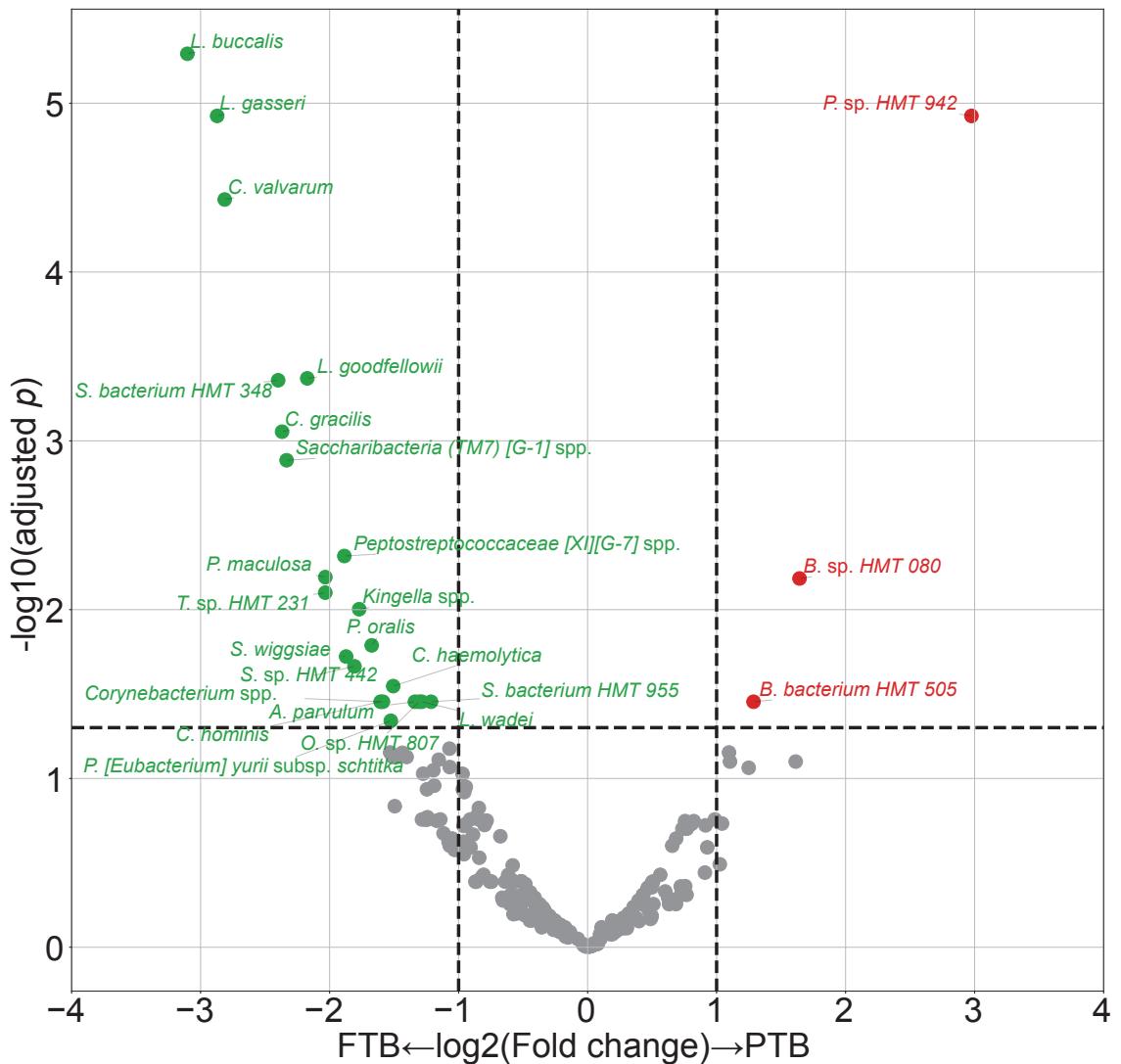


Figure 1: DAT volcano plot.

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

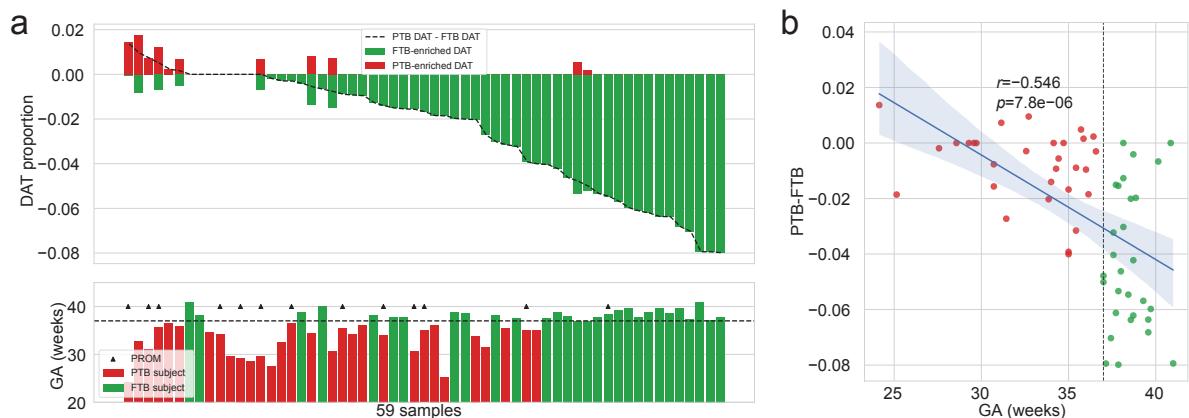


Figure 2: **Salivary microbiome compositions over DAT.**

(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.

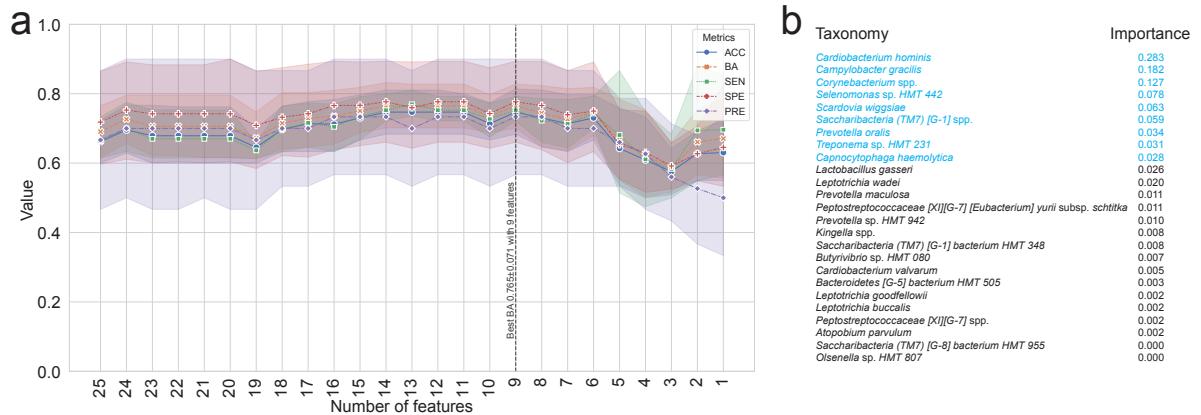


Figure 3: **Random forest-based PTB prediction model.**

(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.

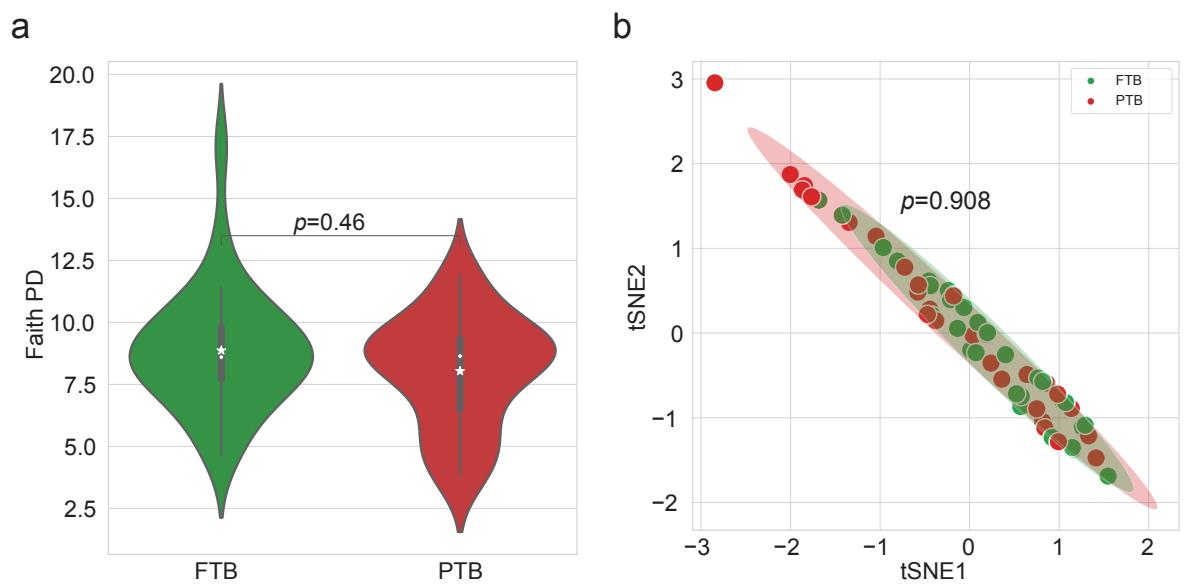


Figure 4: **Diversity indices.**

(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 $p = 0.908$)

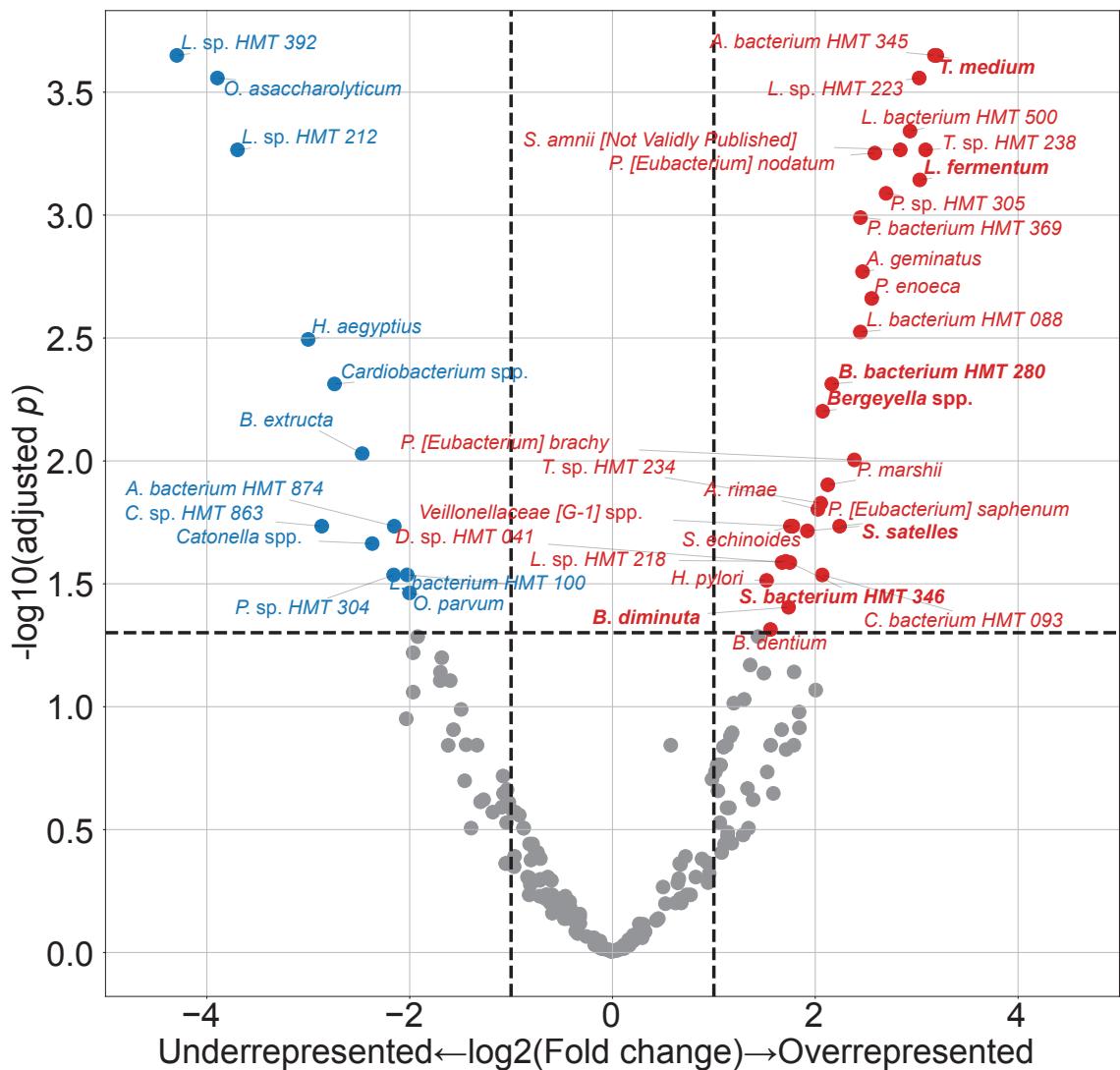


Figure 5: **PROM-related DAT**.

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.

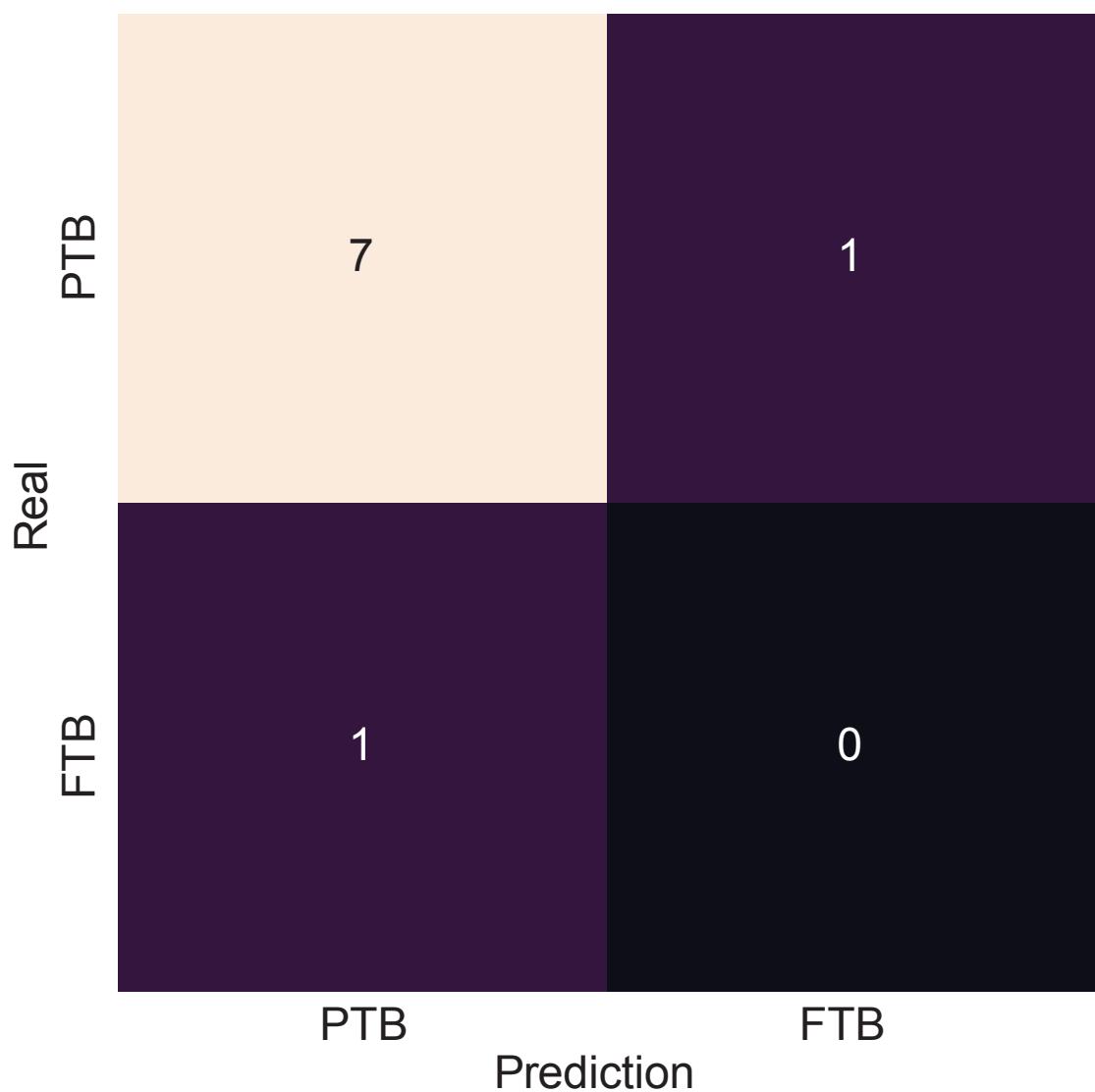


Figure 6: Validation of random forest-based PTB prediction model.

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)

387 **2.4 Discussion**

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

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

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

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

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

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

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

439 **3 Random forest prediction model for periodontitis statuses based on the**
440 **salivary microbiomes**

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

442

443 **3.1 Introduction**

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

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

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

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

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

504 **3.2 Materials and methods**

505 **3.2.1 Study participants enrollment**

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

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

523 **3.2.2 Periodontal clinical parameter diagnosis**

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

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

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

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

541 0. Normal gingiva: without inflammation nor discoloration.

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

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

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

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

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

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

552 • Healthy:

553 1. Bleeding sites < 10%

554 2. Probing depth: \leq 3 mm

555 • Stage I:

556 1. No tooth loss because of periodontitis.

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

558 3. Radiographic bone loss: < 15%

559 • Stage II:

560 1. No tooth loss because of periodontitis.

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

562 3. Radiographic bone loss: 15-33%

563 • Stage III:

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

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

566 3. Radiographic bone loss: > 33%

567 **3.2.3 Saliva sampling and DNA extraction procedure**

568 All study participants received instructions to avoid eating, drinking, brushing, and using mouthwash for
569 at least an hour prior to the saliva sample collection process. These collections were conducted between
570 09:00 and 11:00. Mouth rinse was collected by rinsing the mouth for 30 seconds with 12 mL of a solution
571 (E-zen Gargle, JN Pharm, Korea). All saliva samples were tagged with anonymous ID and stored at -4 °C.

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

- 576 • Forward: 5' -TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNNGCWGCAG-3'
577 • Reverse: 5' -GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'

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

- 580 1. Heat activation for 30 seconds at 95 °C.
581 2. 25 cycles for 30 seconds at 95 °C.
582 3. 30 seconds at 55 °C.
583 4. 30 seconds at 72 °C.

584 NexteraXT Indexed Primer was applied to amplification 10 µL of the purified initial PCR products for
585 the final library creation. The second PCR used the same conditions as the first PCR conditions but with
586 10 cycles. 16S rRNA gene sequencing was performed via 2×300 bp paired-end sequencing at Macrogen
587 Inc. (Macrogen, Seoul, Korea) using Illumina MiSeq platform (Illumina, San Diego, CA, USA).

588 **3.2.4 Bioinformatics analysis**

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

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

600 • Shannon (Weaver, 1963)
601 • Simpson (Simpson, 1949)
602 Aitchison index for a beta-diversity index was calculated using QIIME2 (version 2020.8) (Aitchison,
603 Barceló-Vidal, Martín-Fernández, & Pawlowsky-Glahn, 2000; Bolyen et al., 2019). We employed the
604 t-SNE algorithm to illustrate multi-dimensional data from the beta-diversity index computation (Van der
605 Maaten & Hinton, 2008). The beta-diversity index was compared using the PERMANOVA test (Anderson,
606 2014; Kelly et al., 2015) and MWU test.

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

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

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

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

634 **3.2.5 Data and code availability**

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

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

640 **3.3 Results**

641 **3.3.1 Summary of clinical information and sequencing data**

642 Among clinical information of the study participants, clinical attachment level, probing depth, plaque
643 index, and gingival index, were significantly increased with periodontitis severity (Kruskal-Wallis test
644 $p < 0.001$), while sex were observed no significant difference (Table 2). Notably, clinical attachment level
645 and probing depth have significant differences among the periodontitis severities (MWU test $p < 0.01$;
646 Figure 15). Additionally, 71461.00 ± 11792.30 and 45909.78 ± 11404.65 reads per sample were obtained
647 before and after filtering low-quality reads and trimming extra-long tails, respectively (Figure 16). In 250
648 study subjects, we have found a total of 425 bacterial taxa (Figure 13).

649 **3.3.2 Diversity indices reveal differences among the periodontitis severities**

650 Rarefaction curves showed that the sequencing depth was sufficient (Figure 12). Alpha-diversity in-
651 dices indicated significant differences between the healthy and the periodontitis stages (MWU test
652 $p < 0.01$; Figure 7a-e); however, there were no significant differences between the periodontitis stages.
653 This emphasizes how essential it is to classify the salivary microbiome compositions and distinguish
654 between the stages of periodontitis using machine learning approaches.

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

660 **3.3.3 DAT among multiple periodontitis severities and their correlation**

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

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

- 675 • Group 2
- 676 1. *Porphyromonas gingivalis*
- 677 2. *Campylobacter showae*
- 678 3. *Filifactor alocis*
- 679 4. *Treponema putidum*
- 680 5. *Tannerella forsythia*
- 681 6. *Prevotella intermedia*
- 682 7. *Porphyromonas* sp. HMT 285

- 683 • Group 3
- 684 1. *Actinomyces* spp.
- 685 2. *Corynebacterium durum*
- 686 3. *Actinomyces graevenitzii*

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

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

695 3.3.4 Classification of periodontitis severities by random forest models

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

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

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

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

711 perform a multi-label classification.

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

718 Finally, to confirm the reliability and consistency of our random forest classifier, we validated our
719 classification model using openly accessible 16S rRNA gene sequencing from Spanish participants
720 (Iniesta et al., 2023) and Portuguese participants (Relvas et al., 2021) (Figure 11). Although some
721 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

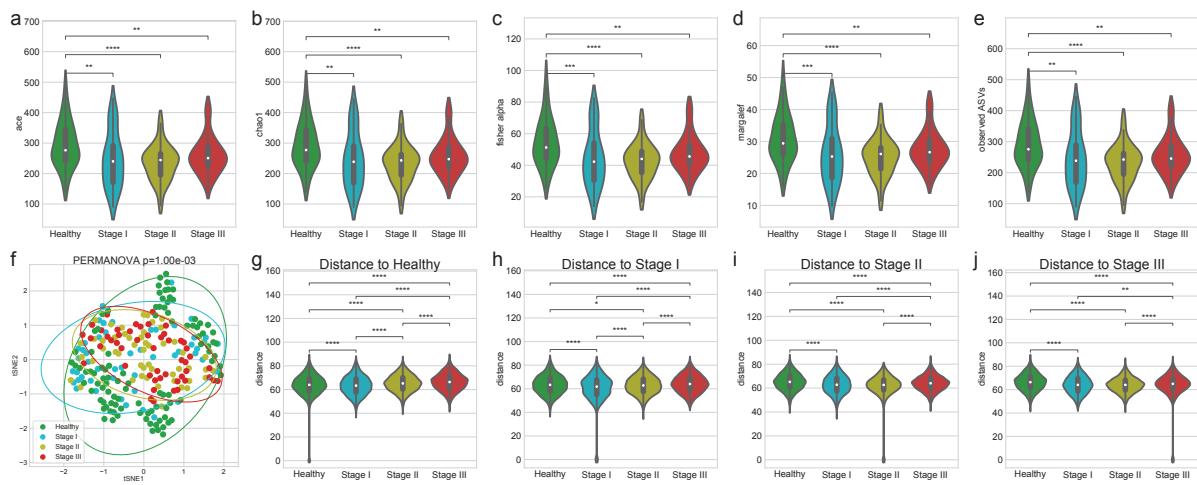


Figure 7: Diversity indices.

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

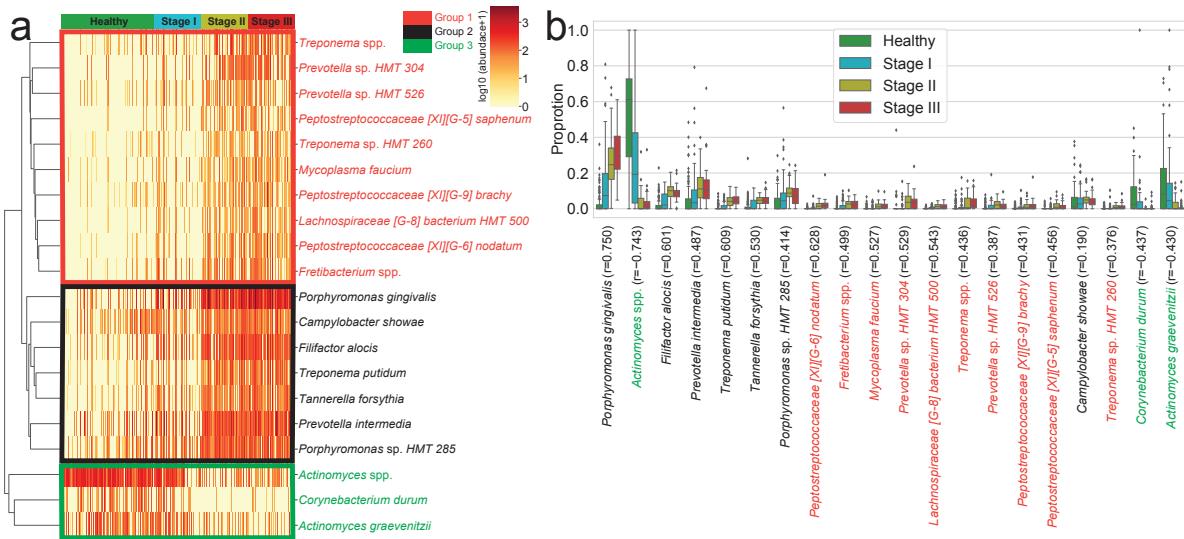


Figure 8: **Differentially abundant taxa (DAT).**

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.

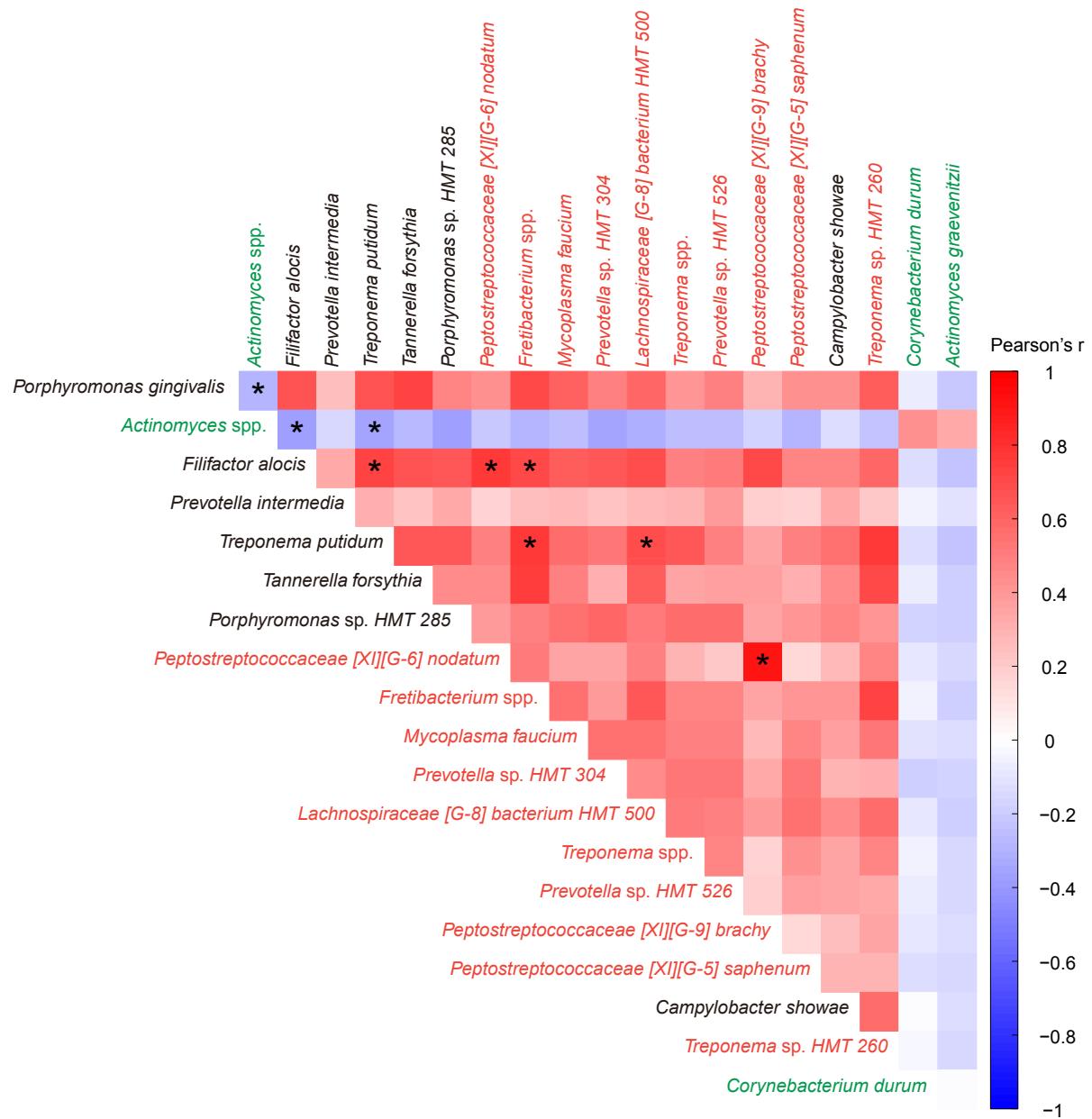


Figure 9: Correlation heatmap.

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

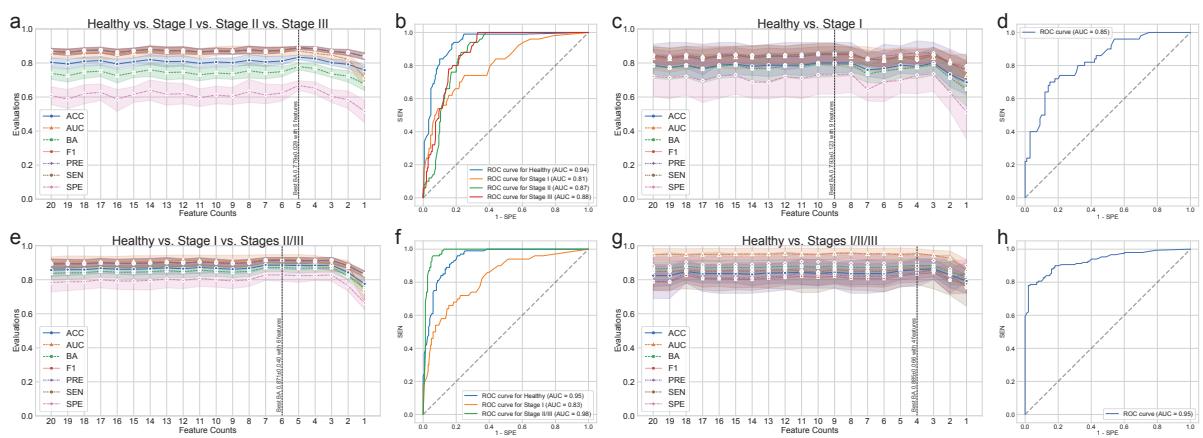


Figure 10: Random forest classification metrics.

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).

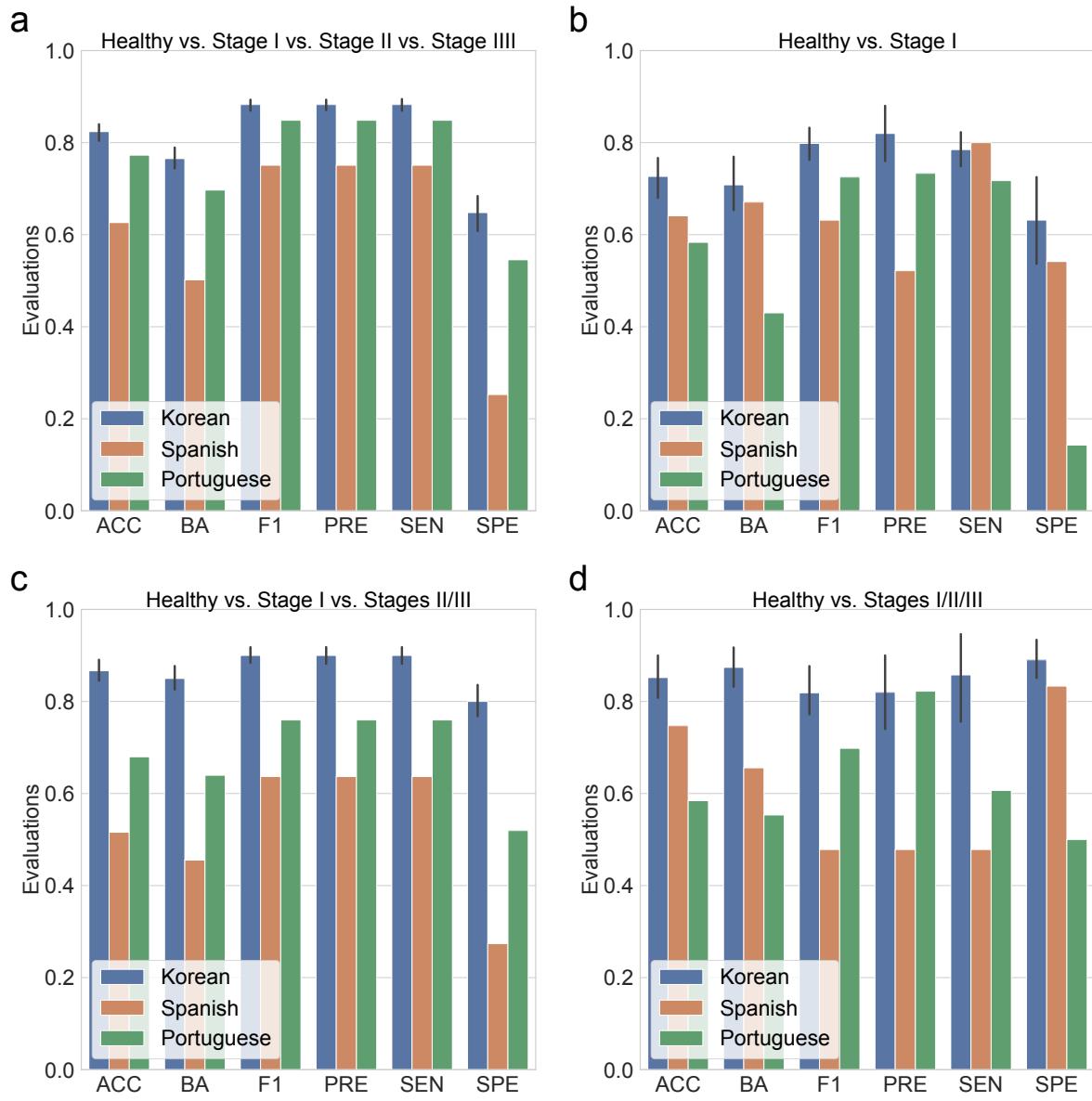


Figure 11: **Random forest classification metrics from external datasets.**

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.

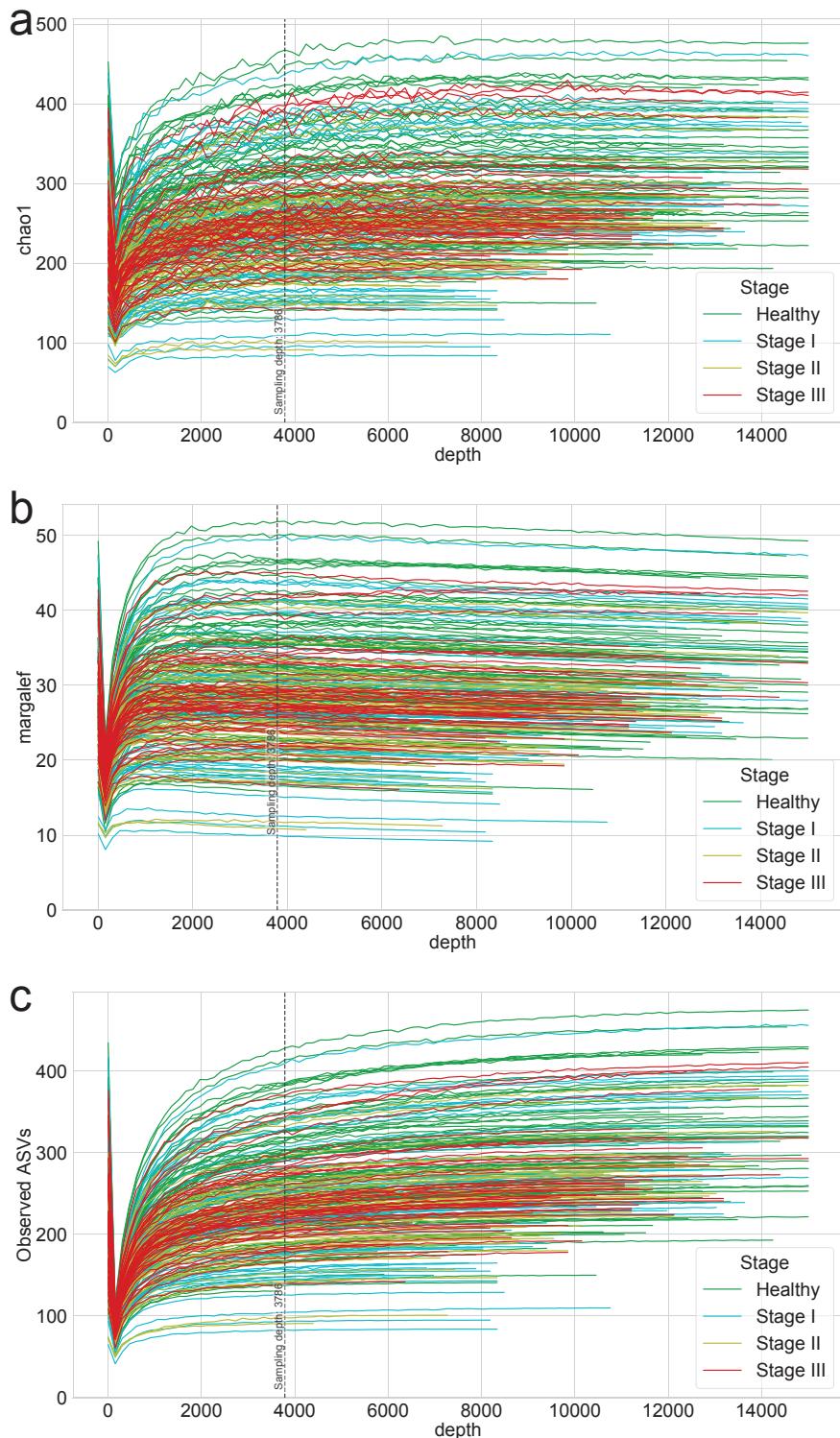


Figure 12: Rarefaction curves for alpha-diversity indices.

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

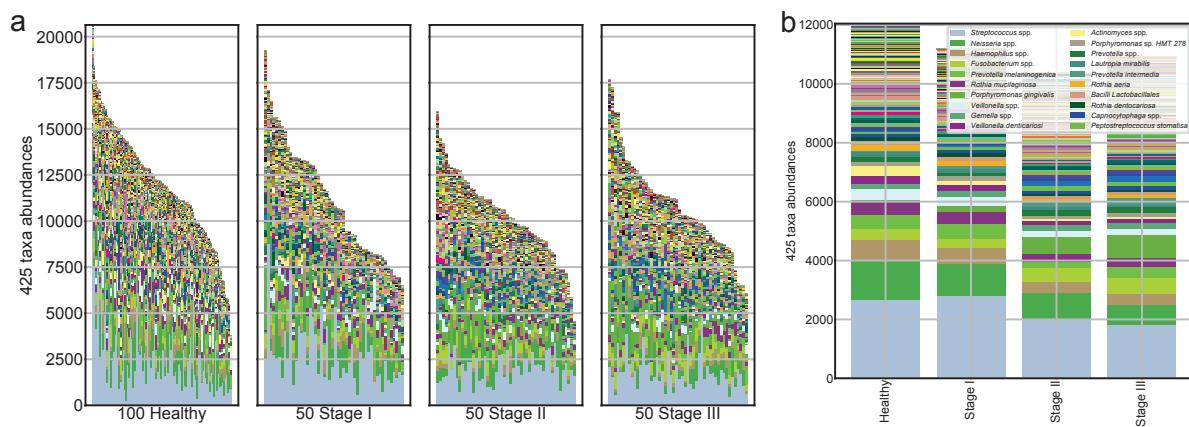


Figure 13: Salivary microbiome compositions in the different periodontal statuses.

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**).

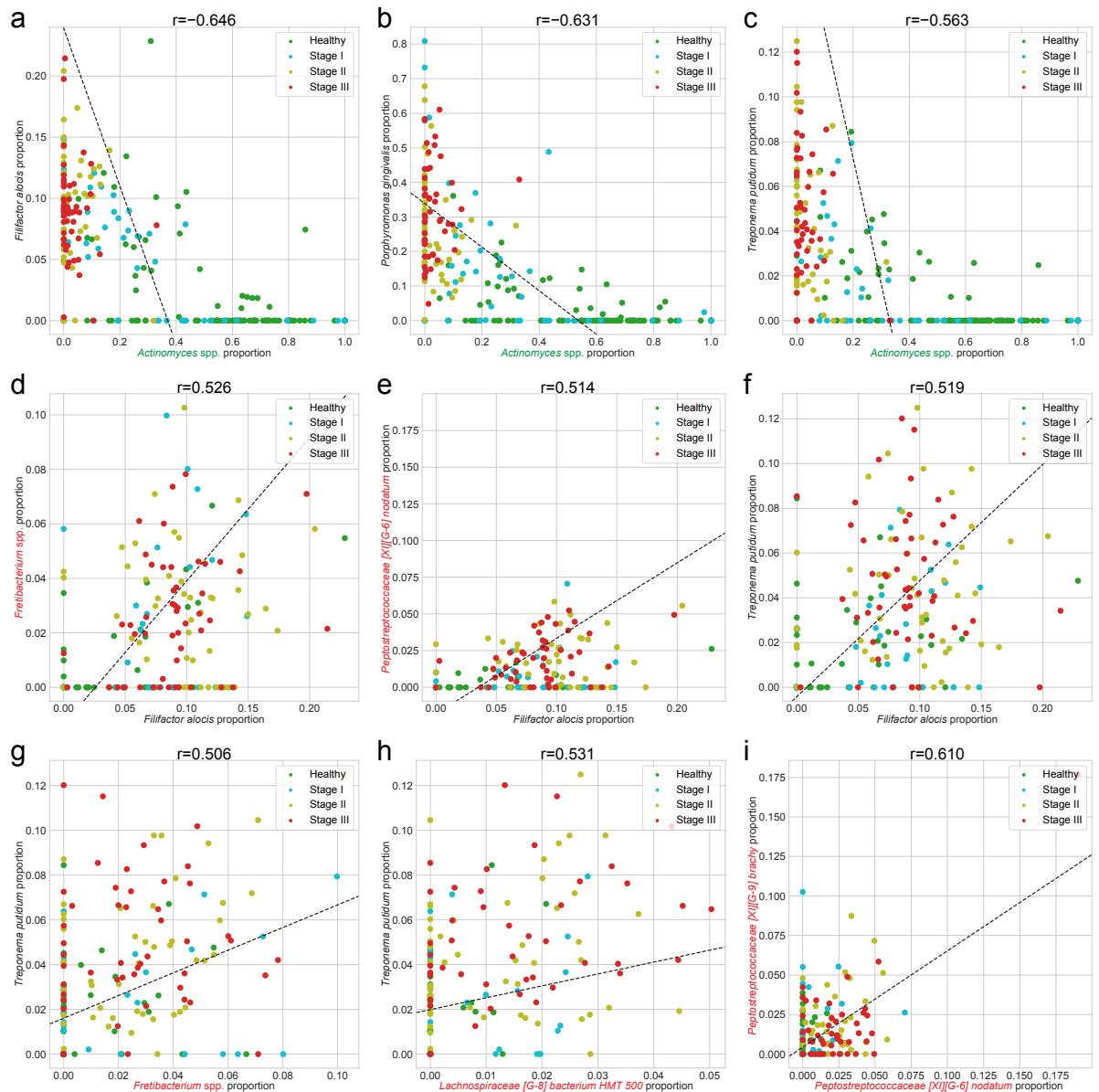


Figure 14: Correlation plots for differentially abundant taxa.

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).

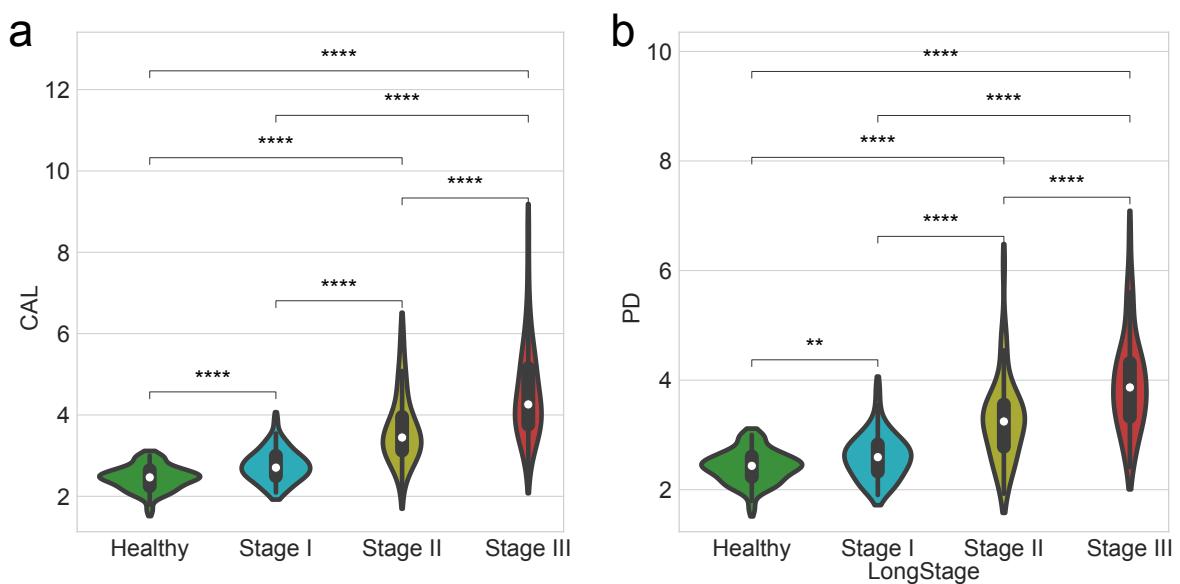


Figure 15: Clinical measurements by the periodontitis statuses.

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

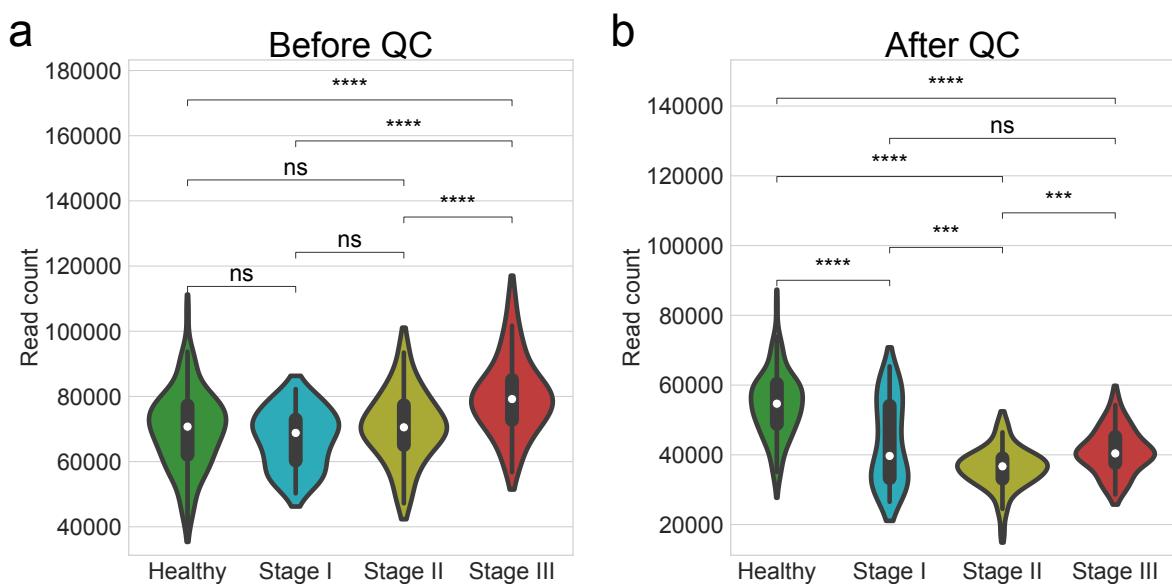


Figure 16: **Number of read counts by the periodontitis statuses.**

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

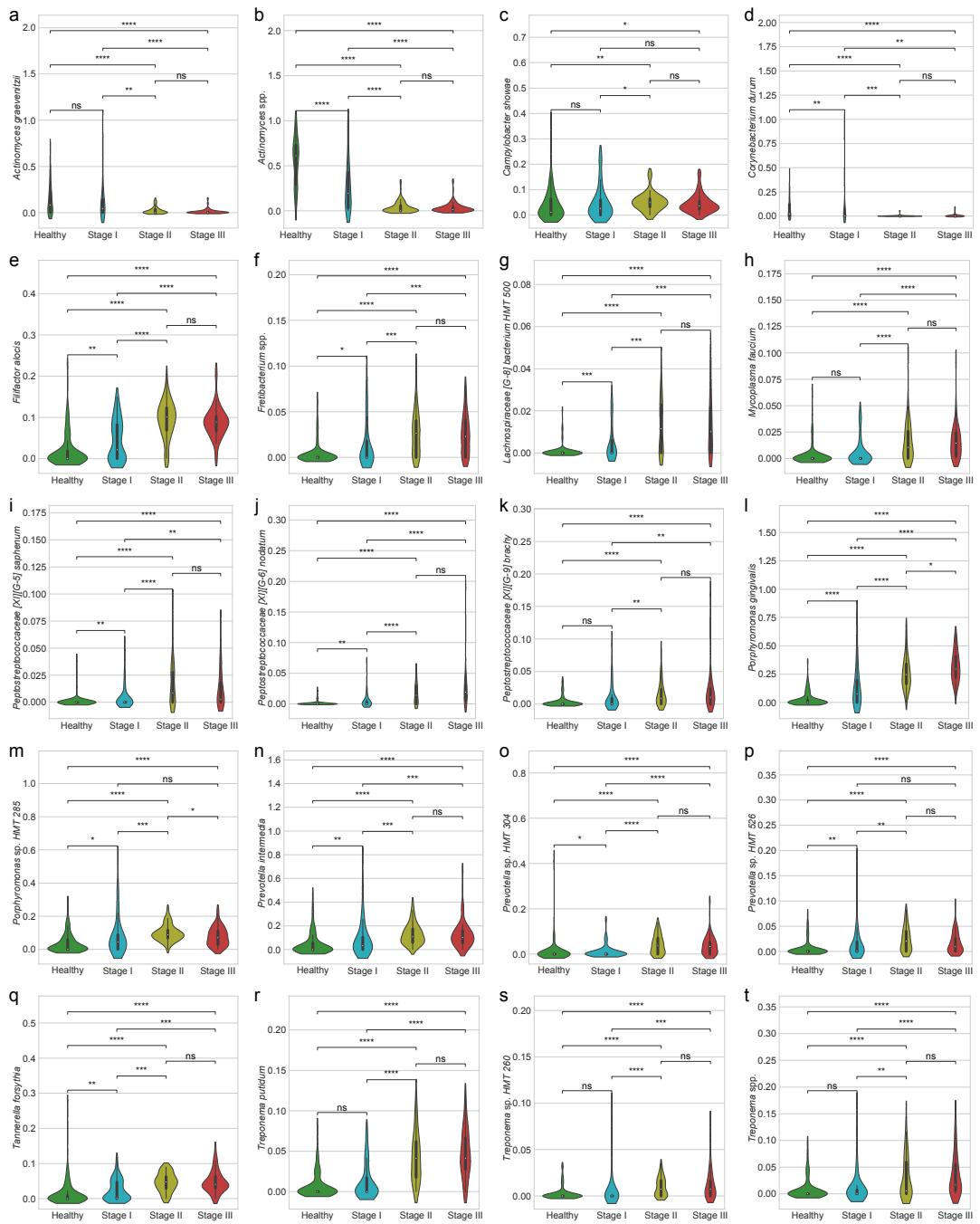


Figure 17: Proportion of DAT.

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

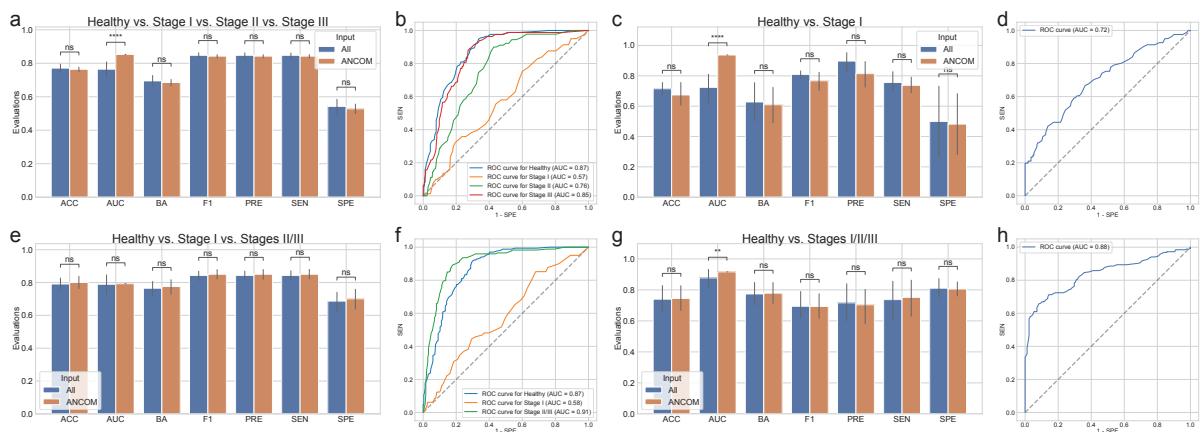


Figure 18: Random forest classification metrics with the full microbiome compositions and ANCOM-selected DAT compositions.

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

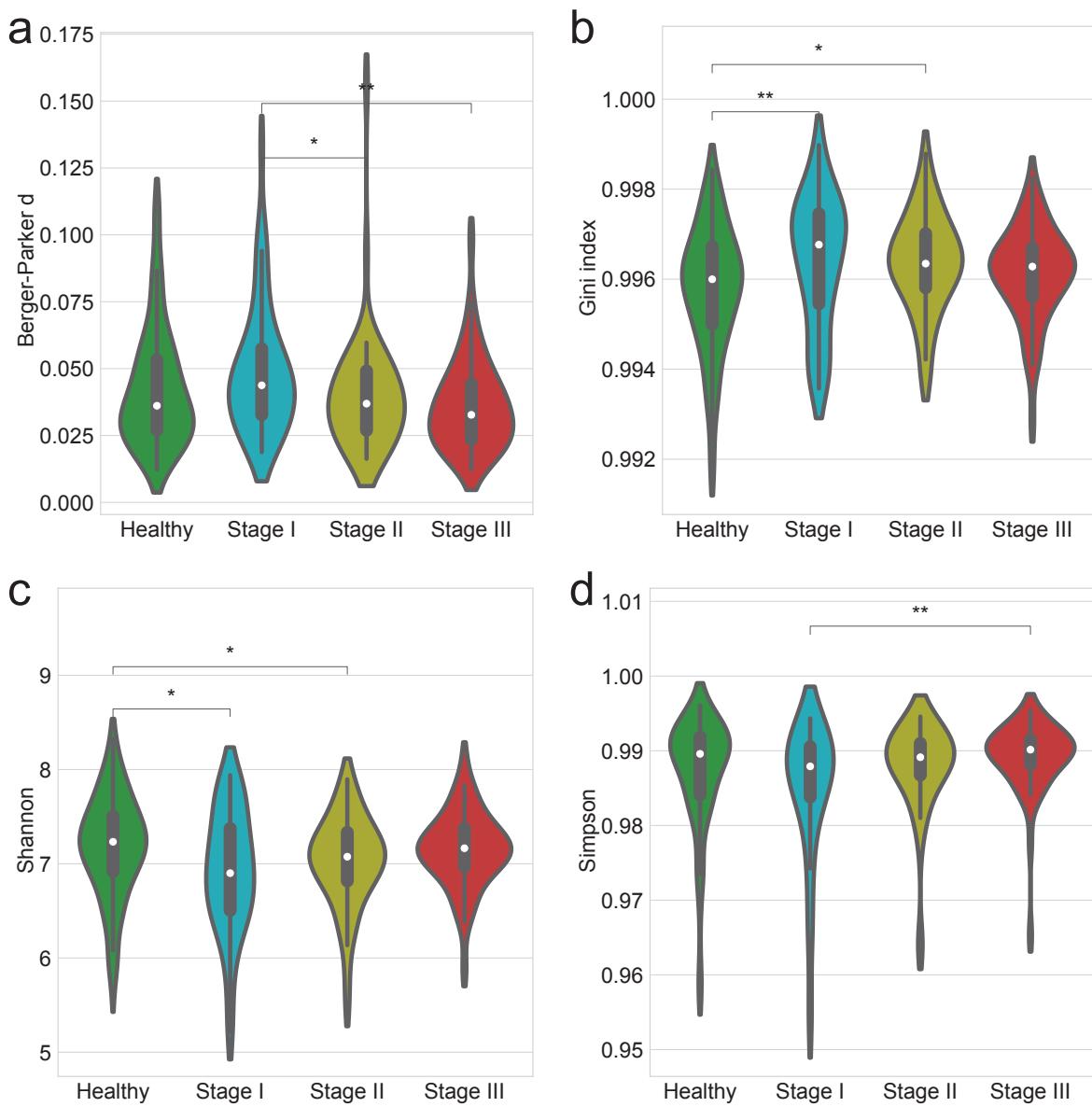


Figure 19: **Alpha-diversity indices account for evenness.**

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 $p \leq 0.01$ (**)

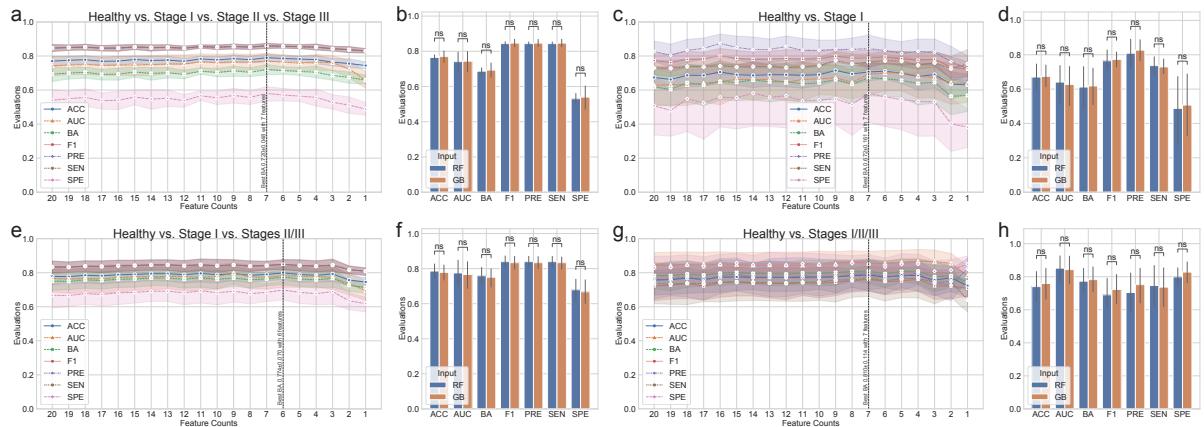


Figure 20: Gradient Boosting classification metrics.

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.

722 **3.4 Discussion**

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

731 Previous research identified the red complex as the primary pathogens of periodontitis (Listgarten,
732 1986): *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. Other studies, however,
733 have shown that periodontal pathogens communicate with other bacteria in the salivary microbiome
734 networks to generate dental plaque prior to the pathogenesis and development of periodontitis (Lamont &
735 Jenkinson, 2000; Rosan & Lamont, 2000; Yoshimura, Murakami, Nishikawa, Hasegawa, & Kawaminami,
736 2009).

737 Using subgingival plaque collections, recent researches have suggested a connection between the
738 periodontitis severity and the salivary microbiome compositions (Altabtbaei et al., 2021; Iniesta et al.,
739 2023; Nemoto et al., 2021). Therefore, we have examined the salivary microbiome compositions of
740 patients with multiple severities of periodontitis and periodontally healthy controls, extending on earlier
741 studies.

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

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

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

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

797 In our correlation analysis (Figure 9), we have discovered strongly negative correlations (coefficient \leq
798 -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%

- 838 • BA: 84%
839 • SEN: 94%
840 • SPE: 74%

841 By separating patients with periodontitis from healthy controls using only four DAT, *e.g.* *Actinomyces*
842 *graevenitzii*, *Actinomyces* spp., *Corynebacterium durum*, and *Porphyromonas gingivalis*, our machine
843 learning model performed better than previously published models (Figure 10, Table 4, and Table 6):

- 844 • AUC: $95.3\% \pm 4.9\%$
845 • BA: $88.5\% \pm 6.6\%$
846 • SEN: $86.4\% \pm 15.7\%$
847 • SPE: $90.5\% \pm 7.0\%$

848 This result showed that by detecting Group 3 bacteria that were substantially abundant in health
849 controls than patients with periodontitis, our study increased BA by at least 5% and SPE by at least 17%.

850 Furthermore, we have validated our machine-learning prediction model using openly accessible 16S
851 gene rRNA sequencing data from Portuguese (Iniesta et al., 2023) and Spanish participants (Relvas et
852 al., 2021) in order to ensure the consistency of our random forest classification model (Figure 11). Our
853 classification models employed in this study were primarily developed and assessed on Korean study par-
854 ticipants, which may limit their generalizability to other ethnic groups with different salivary microbiome
855 compositions (Premaraj et al., 2020; Renson et al., 2019). Therefore, the evaluations of this periodonti-
856 tis classification models can be affected by ethnic-specific variances and differences, highlighting the
857 necessity for additional validation and adjustment across a spectrum of ethnic backgrounds.

858 Regarding the clinical characteristics and potential confounders influencing the analysis of salivary
859 microbiome compositions connected with periodontitis severity, this study had a number of limitations
860 that were pointed out. We did not offer clinical information, such as the percentage of teeth, the percentage
861 of bleeding on probing, nor dental furcation involvement, even though we did gather information on
862 attachment level, probing depth, plaque index, and gingival index; this might have it challenging to present
863 thorough and in-depth data about periodontal health. Moreover, the broad age range may make it tougher
864 to evaluate the relationship between age and periodontitis statuses, providing the necessity for future
865 studies to consider into account more comprehensive clinical characteristics associated with periodontitis.
866 Additionally, potential confounders—*e.g.* body mass index and e-cigarette use—which might have affected
867 dental health and salivary microbiome composition were disregarding consideration in addition to smoking
868 status and systemic diseases. Thus, future research incorporating these components would offer a more
869 thorough knowledge of how lifestyle factors interact and affect the salivary microbiome composition and
870 periodontal health. Throughout, resolving these limitations will advance our understanding in pathogenesis
871 and development of periodontitis, offering significant novel insights on the causal connection between
872 systemic diseases and the salivary microbiome compositions.

873 **4 Colon microbiome**

874 **4.1 Introduction**

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

888 The gut microbiome plays a fundamental role in maintaining host health by helping digestion
889 (Joscelyn & Kasper, 2014; Cerqueira, Photenhauer, Pollet, Brown, & Koropatkin, 2020), regulating
890 metabolism (Dabke, Hendrick, Devkota, et al., 2019; Utzschneider, Kratz, Damman, & Hullarg, 2016;
891 Magnúsdóttir & Thiele, 2018), adjusting immune function (Kau, Ahern, Griffin, Goodman, & Gordon,
892 2011; Shi, Li, Duan, & Niu, 2017; Broom & Kogut, 2018), and even coordinating neurological processes
893 by the brain-gut axis (Martin et al., 2018; Aziz & Thompson, 1998; R. Li et al., 2024). Comprising
894 these gut microbiota, including, archaea, bacteria, fungi, and viruses, the gut microbiome contributes
895 to the synthesis of essential vitamins, and production of fatty acids, which influence intestinal integrity
896 and immune responses. Thus, well-balanced gut microbiome composition modulates systemic immune
897 function by interacting with gut-associated lymphoid tissue, shaping immune tolerance and response
898 to infections. Hence, emerging evidence suggests that dysbiosis in the gut microbiome composition are
899 associated not only a narrow range of diseases, e.g. diarrhea and enteritis (Paganini & Zimmermann,
900 2017; Gao, Yin, Xu, Li, & Yin, 2019) but also a wide range of diseases, e.g. obesity, diabetes, and cancers
901 (Barlow et al., 2015; Hartstra et al., 2015; Helmink et al., 2019; Cullin et al., 2021).

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

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

915 (Novelty)

916 **4.2 Materials and methods**

917 **4.2.1 Study participants enrollment**

918 **4.2.2 DNA extraction procedure**

919 **4.2.3 Bioinformatics analysis**

920 **4.2.4 Data and code availability**

921 **4.3 Results**

922 **4.3.1 Summary of clinical characteristics**

923 **4.3.2 Gut microbiome compositions**

924 **4.3.3 Diversity indices**

925 **4.3.4 DAT selection**

Table 8: Clinical characteristics of the study participants

926 **4.4 Discussion**

927 **5 Conclusion**

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

929 In the section 2, I show that

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