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Doctoral Thesis

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Microbiota in Human Diseases

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Department of Biomedical Engineering

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CHURCH OF THE FLYING SPAGHETTI MONSTER

February 09, 2021

Letter of Good Standing

Dear Sir or Madam:

I am pleased to verify that _____

JAEWOONG LEE

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

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

Any questions can be directed to the undersigned.

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

Representative,
Church of the Flying Spaghetti Monster
Bobby Henderson



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13

Abstract

14 (Microbiome)

15 (PTB) Section 2 introduces...

16 (Periodontitis) Section 3 describes...

17 (Colon) Setion ??...

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.

Contents

26	1	Introduction	2
27	2	Predicting preterm birth using random forest classifier in salivary microbiome	8
28	2.1	Introduction	8
29	2.2	Materials and methods	10
30	2.2.1	Study design and study participants	10
31	2.2.2	Clinical data collection and grouping	10
32	2.2.3	Salivary microbiome sample collection	10
33	2.2.4	16s rRNA gene sequencing	10
34	2.2.5	Bioinformatics analysis	11
35	2.2.6	Data and code availability	11
36	2.3	Results	12
37	2.3.1	Overview of clinical information	12
38	2.3.2	Comparison of salivary microbiomes composition	12
39	2.3.3	Random forest classification to predict PTB risk	12
40	2.4	Discussion	20
41	3	Random forest prediction model for periodontitis statuses based on the salivary microbiomes	22
42	3.1	Introduction	22
43	3.2	Materials and methods	24
44	3.2.1	Study participants enrollment	24
45	3.2.2	Periodontal clinical parameter diagnosis	24
46	3.2.3	Saliva sampling and DNA extraction procedure	26
47	3.2.4	Bioinformatics analysis	26
48	3.2.5	Data and code availability	27
49	3.3	Results	29

50	3.3.1	Summary of clinical information and sequencing data	29
51	3.3.2	Diversity indices reveal differences among the periodontitis severities .	29
52	3.3.3	DAT among multiple periodontitis severities and their correlation . . .	29
53	3.3.4	Classification of periodontitis severities by random forest models . . .	30
54	3.4	Discussion	51
55	4	Metagenomic signature analysis of Korean colorectal cancer	55
56	4.1	Introduction	55
57	4.2	Materials and methods	57
58	4.2.1	Study participants enrollment	57
59	4.2.2	DNA extraction procedure	57
60	4.2.3	Bioinformatics analysis	57
61	4.2.4	Data and code availability	58
62	4.3	Results	59
63	4.3.1	Summary of clinical characteristics	59
64	4.3.2	Gut microbiome compositions	59
65	4.3.3	Diversity indices	59
66	4.3.4	DAT selection	59
67	4.3.5	Pathway prediction	59
68	4.4	Discussion	63
69	5	Conclusion	64
70	References		65
71	Acknowledgments		80

72

List of Figures

73	1	DAT volcano plot	14
74	2	Salivary microbiome compositions over DAT	15
75	3	Random forest-based PTB prediction model	16
76	4	Diversity indices	17
77	5	PROM-related DAT	18
78	6	Validation of random forest-based PTB prediction model	19
79	7	Diversity indices	37
80	8	Differentially abundant taxa (DAT)	38
81	9	Correlation heatmap	39
82	10	Random forest classification metrics	40
83	11	Random forest classification metrics from external datasets	41
84	12	Rarefaction curves for alpha-diversity indices	42
85	13	Salivary microbiome compositions in the different periodontal statuses	43
86	14	Correlation plots for differentially abundant taxa	44
87	15	Clinical measurements by the periodontitis statuses	45
88	16	Number of read counts by the periodontitis statuses	46
89	17	Proportion of DAT	47

90	18	Random forest classification metrics with the full microbiome compositions and ANCOM-selected DAT compositions	48
91			
92	19	Alpha-diversity indices account for evenness	49
93	20	Gradient Boosting classification metrics	50
94	21	Gut microbiome compositions in genus level	61
95	22	Gut microbiome compositions in species level	62

List of Tables

97	1	Confusion matrix	6
98	2	Standard clinical information of study participants	13
99	3	Clinical characteristics of the study participants	32
100	4	Feature combinations and their evaluations	33
101	5	List of DAT among the periodontally healthy and periodontitis stages	34
102	6	Feature the importance of taxa in the classification of different periodontal statuses.	35
103	7	Beta-diversity pairwise comparisons on the periodontitis statuses	36
104	8	Clinical characteristics of CRC study participants	60

105

List of Abbreviations

106 **ACC** Accuracy

107 **ACE** Abundance-based coverage estimator

108 **ASV** Amplicon sequence variant

109 **AUC** Area-under-curve

110 **BA** Balanced accuracy

111 **C-section** Cesarean section

112 **DAT** Differentially abundant taxa

113 **F1** F1 score

114 **Faith PD** Faith's phylogenetic diversity

115 **FTB** Full-term birth

116 **GA** Gestational age

117 **MSI** Microsatellite instability

118 **MSS** Microsatellite stable

119 **MWU test** Mann-Whitney U-test

120 **OS** Overall survival

121 **PRE** Precision

122 **PROM** Prelabor rupture of membrane

123 **PTB** Preterm birth

124 **ROC curve** Receiver-operating characteristics curve

125 **rRNA** Ribosomal RNA

126 **SD** Standard deviation

127 **SEN** Sensitivity

128 **SPE** Specificity

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

130 **1 Introduction**

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

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

166 16S ribosomal RNA (rRNA) gene sequencing is one of the most extensively applied methods for
167 characterizing microbial communities by targeting the conserved 16S rRNA gene, which contains both

168 highly conserved and variable regions in bacteria (Tringe & Hugenholtz, 2008; Janda & Abbott, 2007).
169 The conserved regions enable universal primer binding, while the variable regions provide the specificity
170 needed to differentiate microbial taxa. Among these regions, the V3-V4 region is frequently selected for
171 sequencing due to its balance between phylogenetic resolution and sequencing efficiency (Johnson et al.,
172 2019; López-Aladid et al., 2023). Therefore, the V3-V4 region offers sufficient variability to classify a
173 wide range of bacteria taxa while maintaining compatibility with widely used sequencing platforms.

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

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

197 Differentially abundant taxa (DAT) detection is a key analytical approach in microbiome study to
198 identify microbial taxa that significantly differ in abundance between distinct study participant groups.
199 This DAT detection method is particularly valuable for understanding how microbial communities vary
200 across different conditions, such as disease states, environmental factors, and/or experimental treatments.
201 Various statistical and computational techniques, *e.g.* LEfSe (Segata et al., 2011), DESeq2 (Love, Huber,
202 & Anders, 2014), ANCOM (Lin & Peddada, 2020), and ANCOM-BC (Lin, Eggesbø, & Peddada,
203 2022; Lin & Peddada, 2024), are commonly used to assess differential abundance while accounting for
204 compositional and sparsity-related challenges in microbiome composition data (Swift, Cresswell, Johnson,
205 Stilianoudakis, & Wei, 2023; Cappellato, Baruzzo, & Di Camillo, 2022). Thus, identifying DAT can
206 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

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

252 This dissertation present a comprehensive, multi-disease human microbiome analysis, bridging the
253 association between preterm birth (PTB) (Section 2), periodontitis (Section 3), and colorectal cancer
254 (CRC) (Section 4) through a unified metagenomic approach. While previous studies have examined the
255 role and characteristics of human microbiome in these diseases individually, this dissertation uniquely
256 integrates human microbiome-driven insights across these diseases to identify shared and disease-specific
257 microbial signatures. By applying high-throughput metagenomic sequencing, microbial diversity analysis,
258 and advanced bioinformatics techniques, this dissertation aims to uncover novel microbiome-based
259 biomarkers and mechanistic insights into how microbial communities influence these conditions. These
260 findings contribute to a broader understanding of microbiome-mediated disease interactions and pave the
261 way for personalized medicine strategies, including microbiome-targeted diagnostics and therapeutics.

Table 1: Confusion matrix

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

262

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

263

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

264

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

265

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

266

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

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

267 **2 Predicting preterm birth using random forest classifier in salivary mi-**
268 **crobiome**

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

270 Hong, Y. M., **Lee, Jaewoong**, Cho, D. H., Jeon, J. H., Kang, J., Kim, M. G., ... & Kim, J. K. (2023).
271 Predicting preterm birth using machine learning techniques in oral microbiome. *Scientific Reports*, 13(1),
272 21105.

273 **2.1 Introduction**

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

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

291 Preterm prelabor rupture of membranes (PROM) brought on by gestational inflammation and infection
292 contribute to about 70% of PTB cases (Romero, Dey, & Fisher, 2014). Nevertheless, as antibiotics and
293 anti-inflammatory therapeutic strategies were ineffective to decrease PTB occurrence rates, the pathology
294 of PTB has not been entirely elucidated by inflammatory and infectious pathways (Romero, Hassan, et al.,
295 2014). Recent researches on maternal microbiomes were beginning to examine unidentified connections
296 of PTB as a consequence of developmental processes in molecular biological technology (Fettweis et al.,
297 2019).

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

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

306 Multiple unfavorable birth outcomes, including PROM and PTB, have been linked to periodontitis
307 as an independence risk factor, according to numerous epidemiological researches (Offenbacher et al.,
308 1996). It is expected that the oral microbiome will be able to explain additional hematogenous pathways
309 in light of these precedents; however, the oral microbiome composition of fetuses is limited understood.

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

313 **2.2 Materials and methods**

314 **2.2.1 Study design and study participants**

315 Between 2019 and 2021, singleton pregnant women who received treatment to Jeonbuk National University Hospital for childbirth were the participants of this study. This study was conducted according to the
316 Declaration of Helsinki (Goodyear, Krleza-Jeric, & Lemmens, 2007). The Institutional Review Board
317 authorized this study (IRB file No. 2019-01-024). Participants who were admitted for elective cesarean
318 sections (C-sections) or induction births, as well as those who had written informed consent obtained
319 with premature labor or PROM, were eligible.
320

321 **2.2.2 Clinical data collection and grouping**

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

- 324 • maternal age at delivery
- 325 • diabetes mellitus
- 326 • hypertension
- 327 • overweight and obesity
- 328 • C-section
- 329 • history PROM or PTB
- 330 • gestational week on delivery
- 331 • birth weight
- 332 • sex

333 **2.2.3 Salivary microbiome sample collection**

334 Salivary microbiome samples were collected 24 hours before to delivery using mouthwash. The standard
335 methods of sterilizing were performed. Medical experts oversaw each stage of the sample collecting
336 procedure. Participants received instruction not to eat, drink, or brush their teeth for 30 minutes before
337 sampling salivary microbiome. Saliva samples were gathered by washing the mouth for 30 seconds with
338 12 mL of a mouthwash solution (E-zен Gargle, JN Pharm, Pyeongtaek, Gyeonggi, Korea). The samples
339 were tagged with the anonymous ID for each participant and kept at 4 °C until they underwent further
340 processing. Genomic DNA was extracted using an ExgeneTM Clinic SV kit (GeneAll Biotechnology,
341 Seoul, Korea) following with the manufacturer instructions and store at -20 °C.

342 **2.2.4 16s rRNA gene sequencing**

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

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

349 **2.2.5 Bioinformatics analysis**

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

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

361 Taxonomic assignment were implemented with HOMD (version 15.22) (T. Chen et al., 2010).
362 Afterward, DESeq2 was implemented to identify differentially abundant taxa (DAT) that could dis-
363 tinguish between salivary microbiome from PTB and FTB participants (Love et al., 2014). Taxa with
364 $|\log_2 \text{FoldChange}| > 1$ and $p < 0.05$ were considered as statistically significant.

365 The taxa for predicting PTB using salivary microbiome data were determined using a random forest
366 classifier (Breiman, 2001). Through stratified *k*-fold cross-validation (*k* = 5) that preserves the existence
367 rate of PTB and FTB participants, consistency and trustworthy classification were ensured (Wong & Yeh,
368 2019).

369 **2.2.6 Data and code availability**

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

375 **2.3 Results**

376 **2.3.1 Overview of clinical information**

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

383 **2.3.2 Comparison of salivary microbiomes composition**

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

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

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

395 **2.3.3 Random forest classification to predict PTB risk**

396 To classify PTB according to DAT, random forest classifiers were constructed. The nine most significant
397 DAT were used to obtain the best BA (0.765 ± 0.071 ; Figure 3a). Moreover, random forest classification
398 model determined each DAT's importance (Figure 3b). We conducted a validation procedure on nine
399 twin pregnancies that were excluded in the initial study design in order to confirm the reliability and
400 dependability of our random forest-based PTB prediction model (Figure 6). Comparable to the PTB
401 prediction model on the 59 initial singleton study participants, the validation classification on PTB risk of
402 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

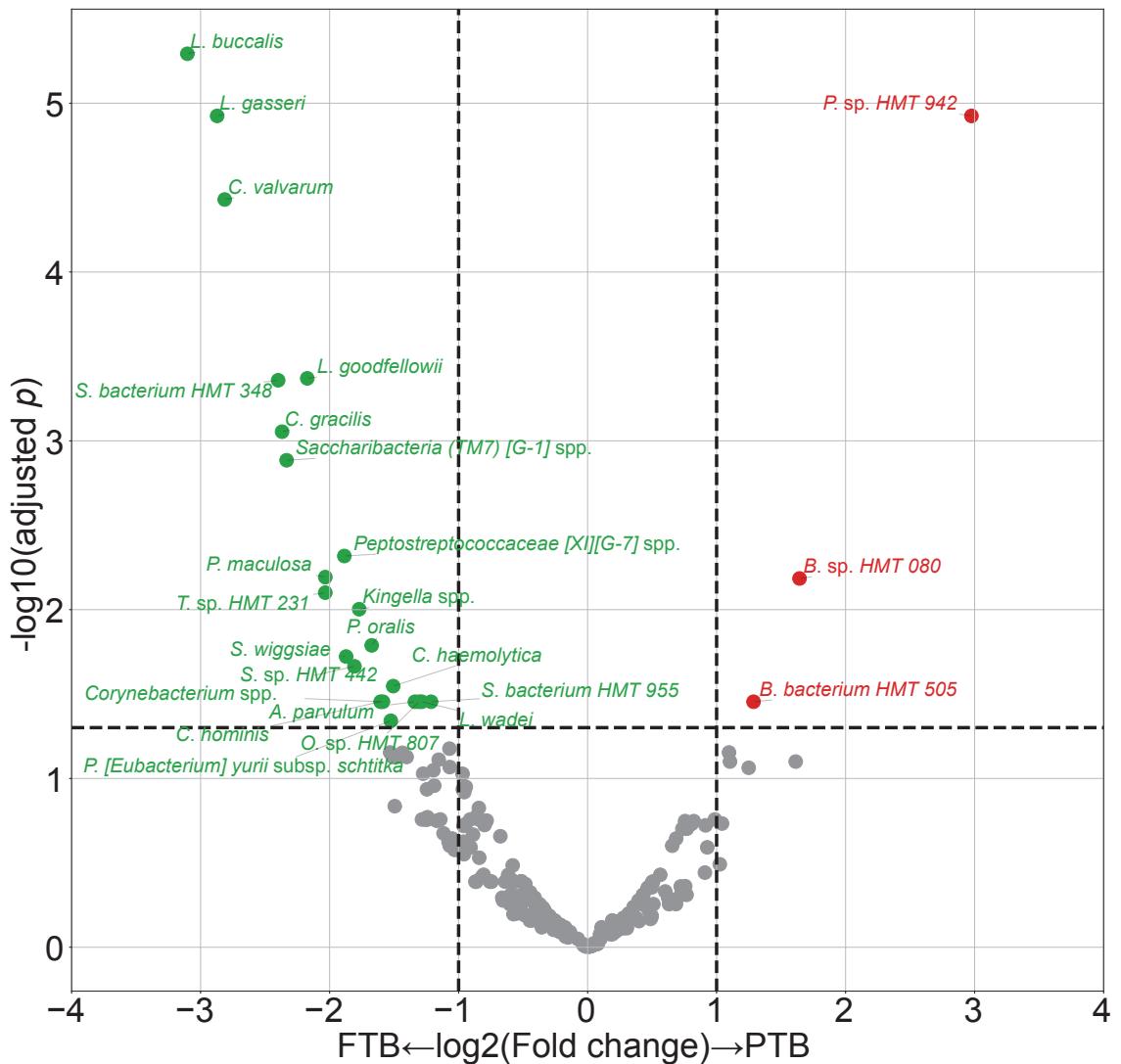


Figure 1: DAT volcano plot.

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

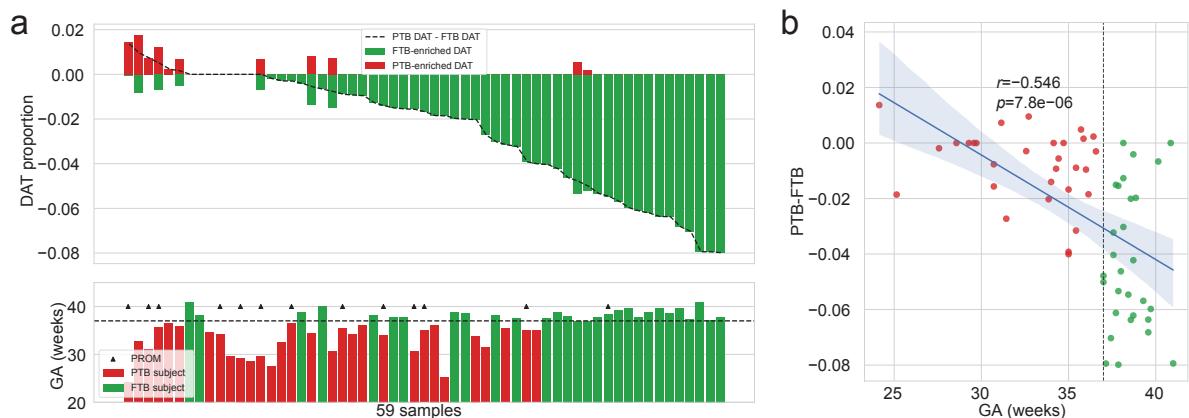


Figure 2: **Salivary microbiome compositions over DAT.**

(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.

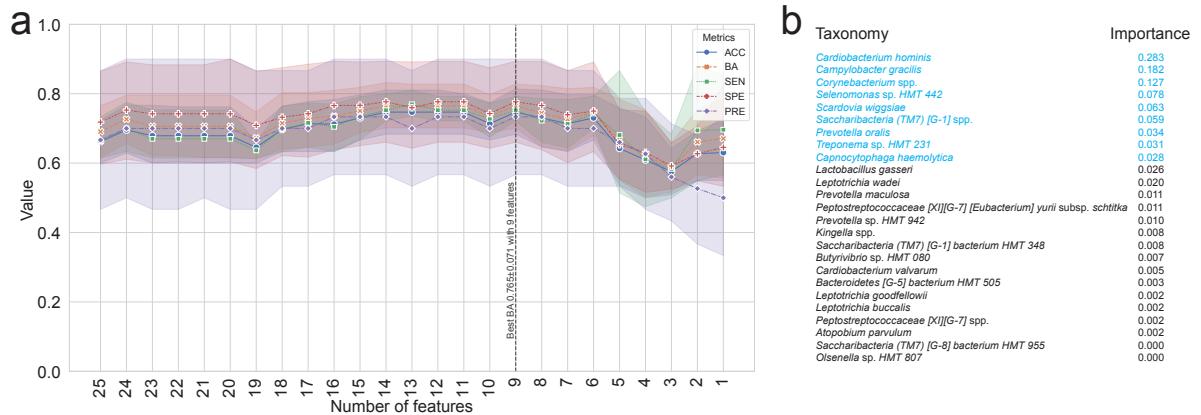


Figure 3: **Random forest-based PTB prediction model.**

(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.

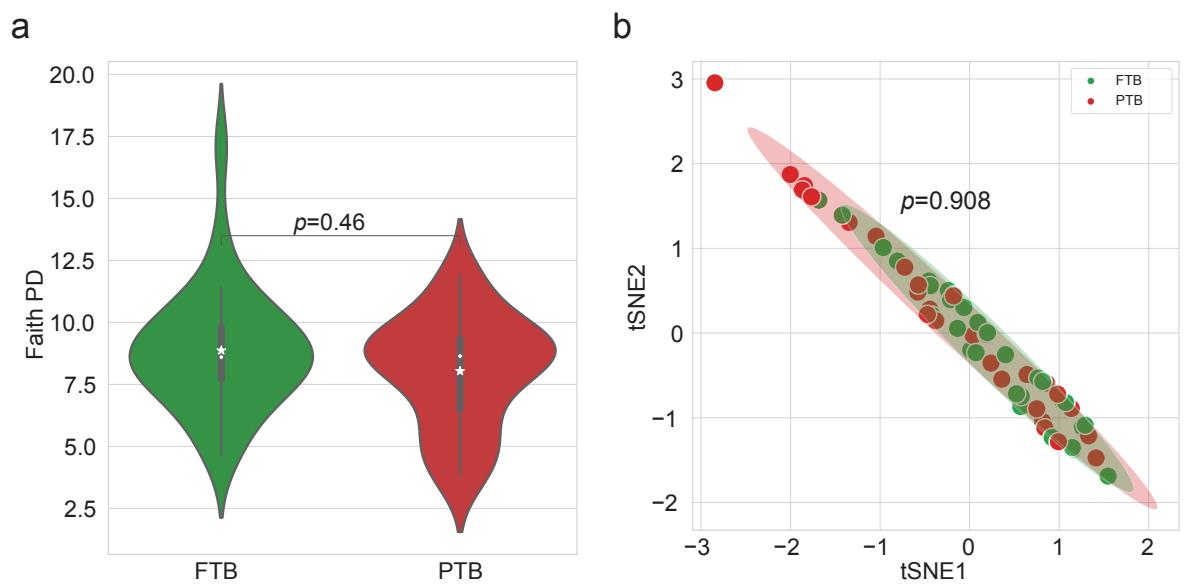


Figure 4: **Diversity indices.**

(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 $p = 0.908$)

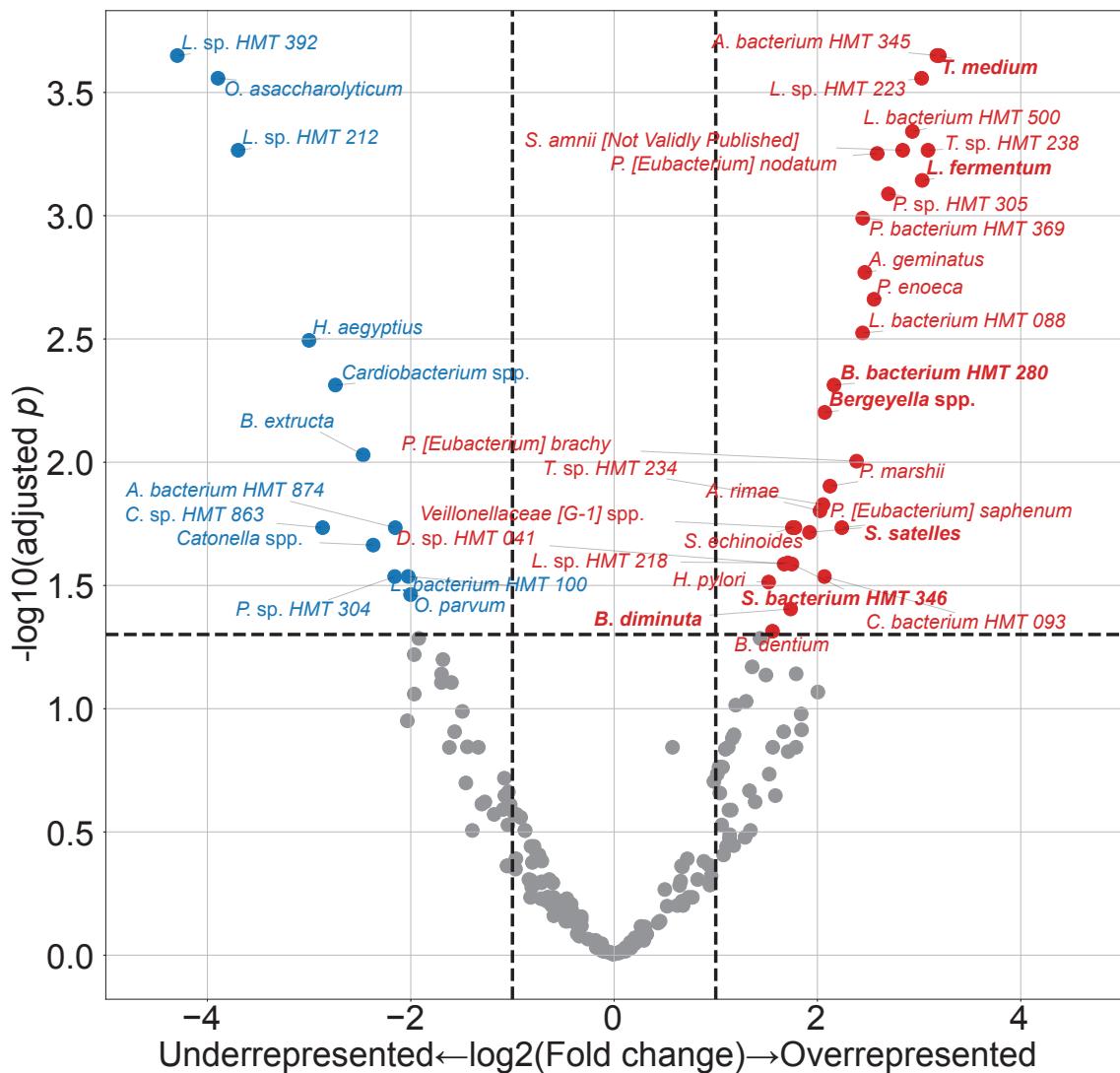


Figure 5: PROM-related DAT.

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.

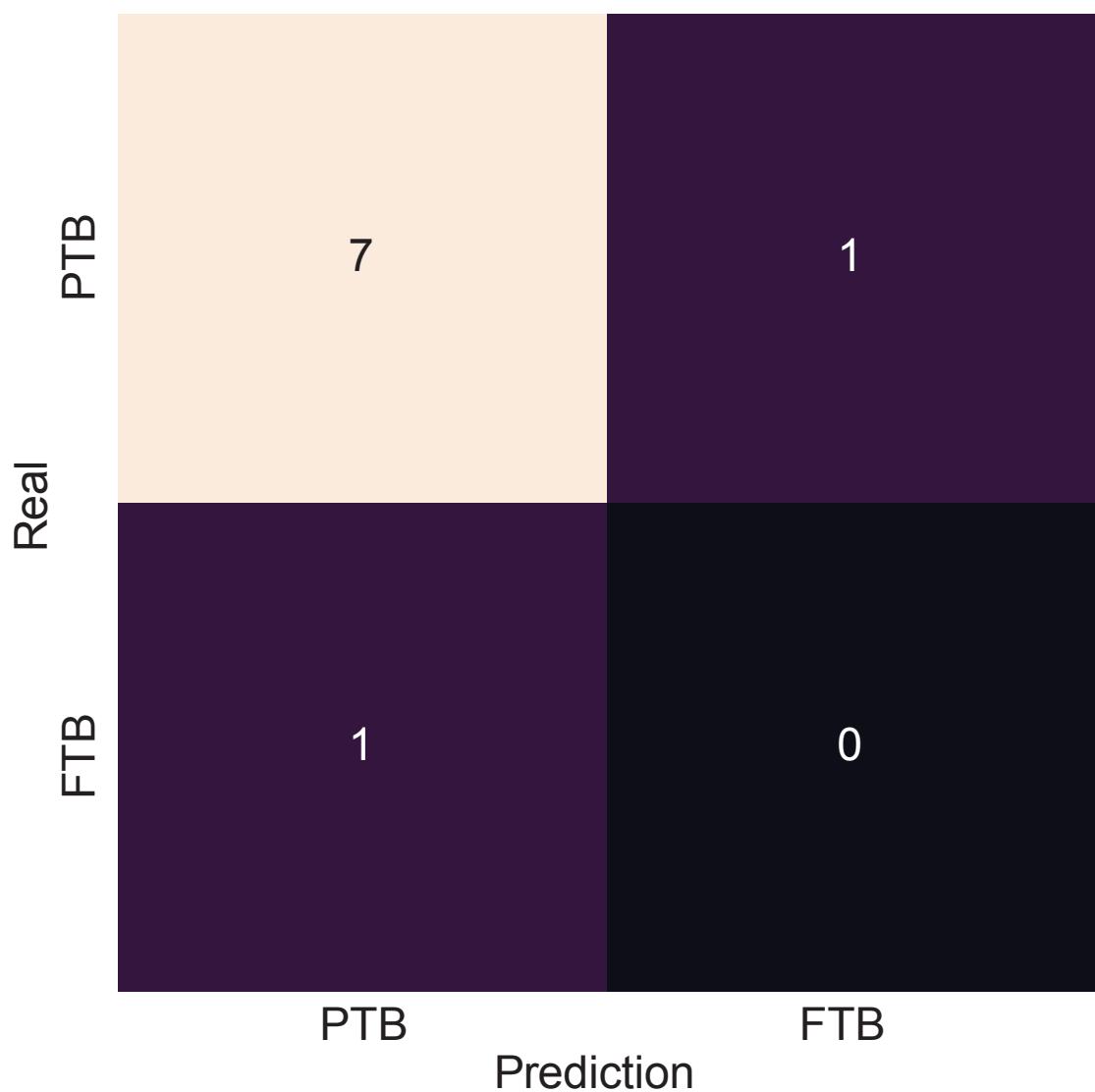


Figure 6: Validation of random forest-based PTB prediction model.

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)

403 **2.4 Discussion**

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

411 One of the most common salivary taxa that has been examined is *Fusobacterium nucleatum* (Han,
412 2015; Brennan & Garrett, 2019; Bolstad, Jensen, & Bakken, 1996), that is a Gram-negative, anaerobic, and
413 filamentous bacteria. *Fusobacterium nucleatum* can be separated from not only the salivary microbiome
414 but also the vaginal microbiome (Vander Haar, So, Gyamfi-Bannerman, & Han, 2018; Witkin, 2019). In
415 both animal and human investigation, *Fusobacterium nucleatum* infection has been linked to risk of PTB
416 (Doyle et al., 2014). According to recent researches, the placenta women who give birth prematurely may
417 include additional salivary microbiome dysbiosis, such as *Bergeyella* spp. and *Porphyromonas gingivalis*
418 (León et al., 2007; Katz, Chegini, Shiverick, & Lamont, 2009). Although *Bergeyella* spp. were one of the
419 PROM-overrepresented DAT (Figure 5), it was excluded in the final 25 PTB-related DAT. Furthermore,
420 *Porphyromonas gingivalis* and *Campylobacter gracilis* were pathogens of periodontitis in sub-gingival
421 microbiome (Yang et al., 2022). *Lactobacillus gasseri* was also one of the FTB-enriched DAT (Figure
422 1), and it is well established that early PTB risk can be reduced by *Lactobacillus gasseri* in the vaginal
423 microbiome (Basavaprabhu, Sonu, & Prabha, 2020; Payne et al., 2021).

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

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

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

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

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

455 **3 Random forest prediction model for periodontitis statuses based on the**
456 **salivary microbiomes**

457 **3.1 Introduction**

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

474 Due to the development of salivaomics, in this manner, the examination of saliva has emerged as
475 a significant alternative to the conventional ways of identifying periodontitis (Altingöz et al., 2021;
476 Melguizo-Rodríguez, Costela-Ruiz, Manzano-Moreno, Ruiz, & Illescas-Montes, 2020). Given that saliva
477 sampling is non-invasive, painless, and accessible to non-specialists, it may be a valuable instrument for
478 diagnosing periodontitis (Zhang et al., 2016). Furthermore, much research has suggested that periodontitis
479 could be a trigger in the development and exacerbation of metabolic syndrome (Morita et al., 2010; Nesbitt
480 et al., 2010). Consequently, alteration in these levels of salivary microbiome markers may serve as high
481 effective diagnostic, prognostic, and therapeutic indicators for periodontitis and other systemic diseases
482 (Miller, Ding, Dawson III, & Ebersole, 2021; Čižmárová et al., 2022). The pathogenesis of periodontitis
483 typically comprises qualitative as well as quantitative alterations in the salivary microbial community,
484 despite that it is a complex disease impacted by a number of contributing factors including age, smoking
485 status, stress, and nourishment (Abusleme, Hoare, Hong, & Diaz, 2021; Lafaurie et al., 2022). Depending
486 on the severity of periodontitis, the salivary microbial community's diversity and characteristics vary
487 (Abusleme et al., 2021), indicating that a new etiological diagnostic standards might be microbial
488 community profiling based on clinical diagnostic criteria. As a consequence, salivary microbiome
489 compositions have been characterized in numerous research in connection with periodontitis. High-
490 throughput sequencing, including 16S rRNA gene sequencing, has recently used in multiple studies to
491 identify variations in the bacterial composition of sub-gingival plaque collections from periodontal healthy

492 individuals and patients with periodontitis (Altabtbaei et al., 2021; Iniesta et al., 2023; Nemoto et al., 2021).
493 This realization has rendered clear that alterations in the salivary microbial community—especially, shifts to
494 dysbiosis—are significant contributors to the pathogenesis and development of periodontitis (Lamont, Koo,
495 & Hajishengallis, 2018). Yet most of these research either focused only on the microbiome alterations in
496 sub-gingival plaque collection, comprised a limited number of periodontitis study participants, or did not
497 account for the impact of multiple severities of periodontitis.

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

506 Recently, we employed multiplex quantitative-PCR and machine learning-based classification model
507 to predict the severity of periodontitis based on the amount of nine pathogens of periodontitis from
508 saliva collections (E.-H. Kim et al., 2020). On the other hand, the fact that we focused merely at nine
509 pathogens for periodontitis and neglected the variety bacterial species associated to the various severities
510 of periodontitis constrained the breadth of our investigation. By developing a machine learning model
511 that could classify multiple severities of periodontitis based on the salivary microbiome composition,
512 this study aims to fill these knowledge gaps and produce more accurate and therapeutically useful
513 guidance to evaluate progression of periodontitis. Hence, in order to examine the salivary microbiome
514 composition of both healthy controls and patients with periodontitis in multiple stages, we applied
515 16S rRNA gene sequencing. Furthermore, employing the 2018 classification criteria, we sought to find
516 biomarkers (species) for the precise prediction of periodontitis severities (Papapanou et al., 2018; Chapple
517 et al., 2018).

518 **3.2 Materials and methods**

519 **3.2.1 Study participants enrollment**

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

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

537 **3.2.2 Periodontal clinical parameter diagnosis**

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

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

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

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

555 0. Normal gingiva: without inflammation nor discoloration.

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

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

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

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

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

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

566 • Healthy:

567 1. Bleeding sites < 10%

568 2. Probing depth: \leq 3 mm

569 • Stage I:

570 1. No tooth loss because of periodontitis.

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

572 3. Radiographic bone loss: < 15%

573 • Stage II:

574 1. No tooth loss because of periodontitis.

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

576 3. Radiographic bone loss: 15-33%

577 • Stage III:

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

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

580 3. Radiographic bone loss: > 33%

581 **3.2.3 Saliva sampling and DNA extraction procedure**

582 All study participants received instructions to avoid eating, drinking, brushing, and using mouthwash for
583 at least an hour prior to the saliva sample collection process. These collections were conducted between
584 09:00 and 11:00. Mouth rinse was collected by rinsing the mouth for 30 seconds with 12 mL of a solution
585 (E-zen Gargle, JN Pharm, Korea). All saliva samples were tagged with anonymous ID and stored at -4 °C.

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

- 590 • Forward: 5' -TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'
591 • Reverse: 5' -GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'

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

- 594 1. Heat activation for 30 seconds at 95 °C.
595 2. 25 cycles for 30 seconds at 95 °C.
596 3. 30 seconds at 55 °C.
597 4. 30 seconds at 72 °C.

598 NexteraXT Indexed Primer was applied to amplification 10 µL of the purified initial PCR products for
599 the final library creation. The second PCR used the same conditions as the first PCR conditions but with
600 10 cycles. 16S rRNA gene sequencing was performed via 2×300 bp paired-end sequencing at Macrogen
601 Inc. (Macrogen, Seoul, Korea) using Illumina MiSeq platform (Illumina, San Diego, CA, USA).

602 **3.2.4 Bioinformatics analysis**

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

- 607 • Abundance-based Coverage Estimator (ACE) (Chao & Lee, 1992)
608 • Chao1 (Chao, 1984)
609 • Fisher (Fisher, Corbet, & Williams, 1943)
610 • Margalef (Magurran, 2021)
611 • Observed ASVs (DeSantis et al., 2006)
612 • Berger-Parker d (Berger & Parker, 1970)
613 • Gini index (Gini, 1912)

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

614 Aitchison index for a beta-diversity index was calculated using QIIME2 (version 2020.8) (Aitchison,
615 Barceló-Vidal, Martín-Fernández, & Pawlowsky-Glahn, 2000; Bolyen et al., 2019). We employed the
616 t-SNE algorithm to illustrate multi-dimensional data from the beta-diversity index computation (Van der
617 Maaten & Hinton, 2008). The beta-diversity index was compared using the PERMANOVA test (Anderson,
618 2014; Kelly et al., 2015) and MWU test.

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

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

631 As previously stated (E.-H. Kim et al., 2020), we used stratified k -fold cross-validation ($k = 10$)
632 by severity of periodontitis to achieve consistent and trustworthy classification results (Wong & Yeh,
633 2019). Additionally, we utilized various features with confusion matrices and their derivations to evaluate
634 the classification outcomes in order to identify which features optimize classification evaluations and
635 decrease sequencing efforts. Using the DAT discovered by ANCOM, we iteratively removed the least
636 significant taxa from the input features (taxa) of the random forest (Breiman, 2001) and gradient boosting
637 (Friedman, 2002) classification models using the backward elimination method. Random forest classifier
638 builds multiple decision trees independently using bootstrapped samples and aggregates their predictions,
639 enhancing stability and reducing overfitting problems. In contrast, Gradient boosting constructs trees
640 sequentially, where each new tree improves the errors of the previous ones using gradient descent, leading
641 to higher classification evaluations.

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

646 3.2.5 Data and code availability

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

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

654 **3.3 Results**

655 **3.3.1 Summary of clinical information and sequencing data**

656 Among clinical information of the study participants, clinical attachment level, probing depth, plaque
657 index, and gingival index, were significantly increased with periodontitis severity (Kruskal-Wallis test
658 $p < 0.001$), while sex were observed no significant difference (Table 2). Notably, clinical attachment level
659 and probing depth have significant differences among the periodontitis severities (MWU test $p < 0.01$;
660 Figure 15). Additionally, 71461.00 ± 11792.30 and 45909.78 ± 11404.65 reads per sample were obtained
661 before and after filtering low-quality reads and trimming extra-long tails, respectively (Figure 16). In 250
662 study subjects, we have found a total of 425 bacterial taxa (Figure 13).

663 **3.3.2 Diversity indices reveal differences among the periodontitis severities**

664 Rarefaction curves showed that the sequencing depth was sufficient (Figure 12). Alpha-diversity in-
665 dices indicated significant differences between the healthy and the periodontitis stages (MWU test
666 $p < 0.01$; Figure 7a-e); however, there were no significant differences between the periodontitis stages.
667 This emphasizes how essential it is to classify the salivary microbiome compositions and distinguish
668 between the stages of periodontitis using machine learning approaches.

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

674 **3.3.3 DAT among multiple periodontitis severities and their correlation**

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

- 678 • Group 1
 - 679 1. *Treponema* spp.
 - 680 2. *Prevotella* sp. HMT 304
 - 681 3. *Prevotella* sp. HMT 526
 - 682 4. *Peptostreptococcaceae [XI][G-5]* saphenum
 - 683 5. *Treponema* sp. HMT 260
 - 684 6. *Mycoplasma faecium*
 - 685 7. *Peptostreptococcaceae [XI][G-9]* brachy
 - 686 8. *Lachnospiraceae [G-8]* bacterium HMT 500
 - 687 9. *Peptostreptococcaceae [XI][G-6]* nodatum
 - 688 10. *Fretibacterium* spp.

- 689 • Group 2
- 690 1. *Porphyromonas gingivalis*
- 691 2. *Campylobacter showae*
- 692 3. *Filifactor alocis*
- 693 4. *Treponema putidum*
- 694 5. *Tannerella forsythia*
- 695 6. *Prevotella intermedia*
- 696 7. *Porphyromonas* sp. HMT 285

- 697 • Group 3
- 698 1. *Actinomyces* spp.
- 699 2. *Corynebacterium durum*
- 700 3. *Actinomyces graevenitzii*

701 Ten DAT that were significant enriched in stage II and stage III, but deficient in healthy formed Group
 702 1 (Figure 8). Furthermore, in comparison to the healthy, the seven DAT of Group 2 were significantly
 703 enriched in each of the stages of periodontitis. On the other hand, three DAT in Group 3 were deficient in
 704 stage II and stage III, but significantly enriched in healthy. The relative proportions of the DAT further
 705 supported these findings (Figure 8b), suggesting that the DAT is primarily linked to periodontitis rather
 706 than other salivary bacteria.

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

709 3.3.4 Classification of periodontitis severities by random forest models

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

714 Based on the proportion of DAT, random forest classifier were trained to classify the periodontitis
 715 severities (Table 6). We conducted multi-label classification for the multiple periodontitis severities,
 716 namely healthy, stage I, stage II, and stage III. In this setting, we classified multiple periodontitis
 717 severities with the highest BA of 0.779 ± 0.029 (Table 4). AUC ranged between 0.81 and 0.94 (Figure
 718 10b).

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

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

725 perform a multi-label classification.

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

732 Finally, to confirm the reliability and consistency of our random forest classifier, we validated our
733 classification model using openly accessible 16S rRNA gene sequencing from Spanish participants
734 (Iniesta et al., 2023) and Portuguese participants (Relvas et al., 2021) (Figure 11). Although some
735 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 spp.</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

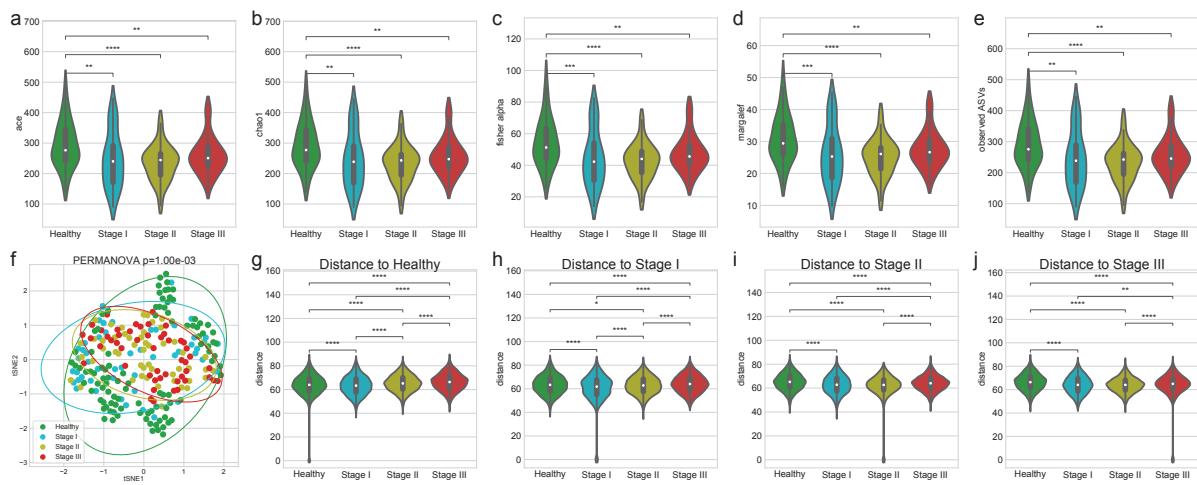


Figure 7: **Diversity indices.**

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

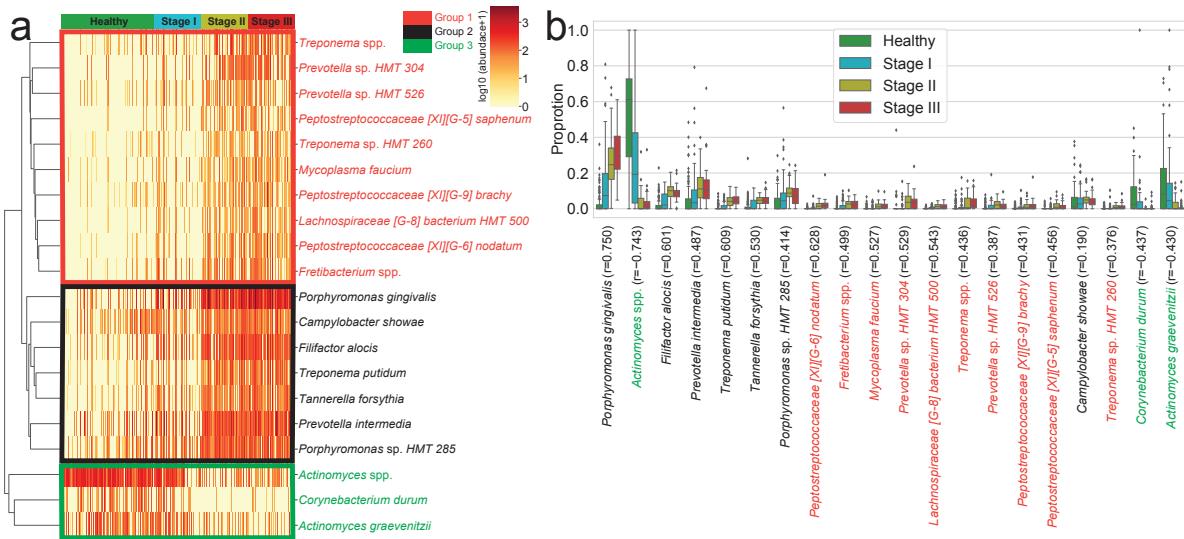


Figure 8: **Differentially abundant taxa (DAT).**

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.

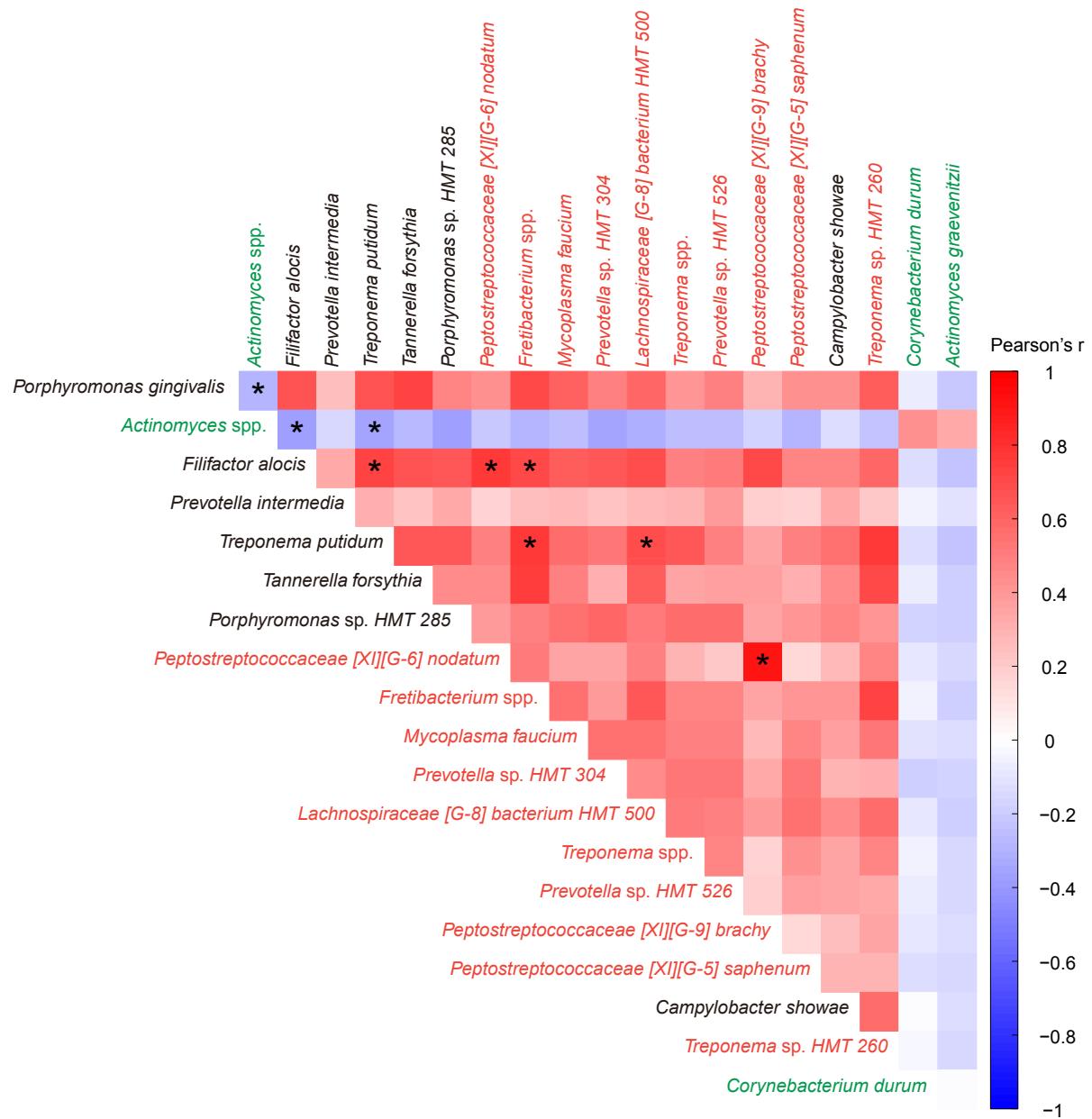


Figure 9: Correlation heatmap.

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

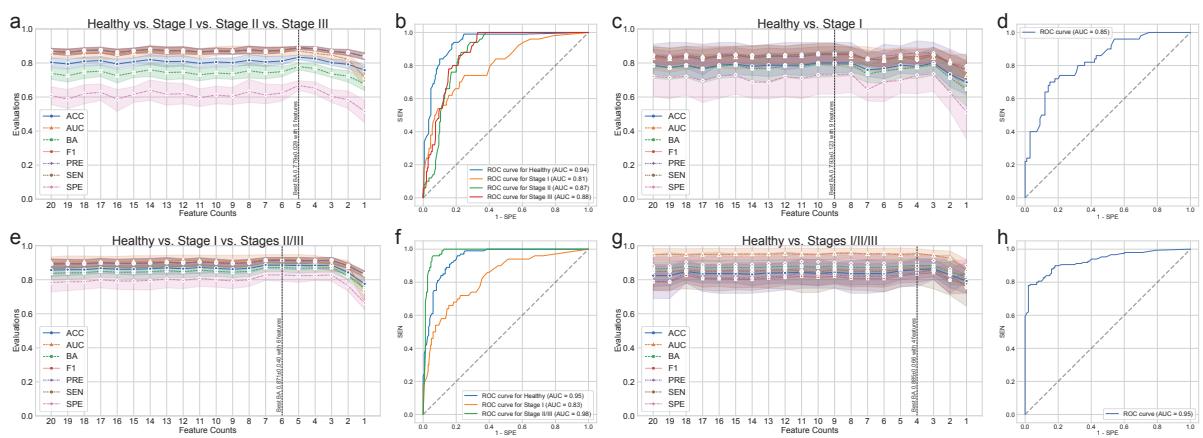


Figure 10: Random forest classification metrics.

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).

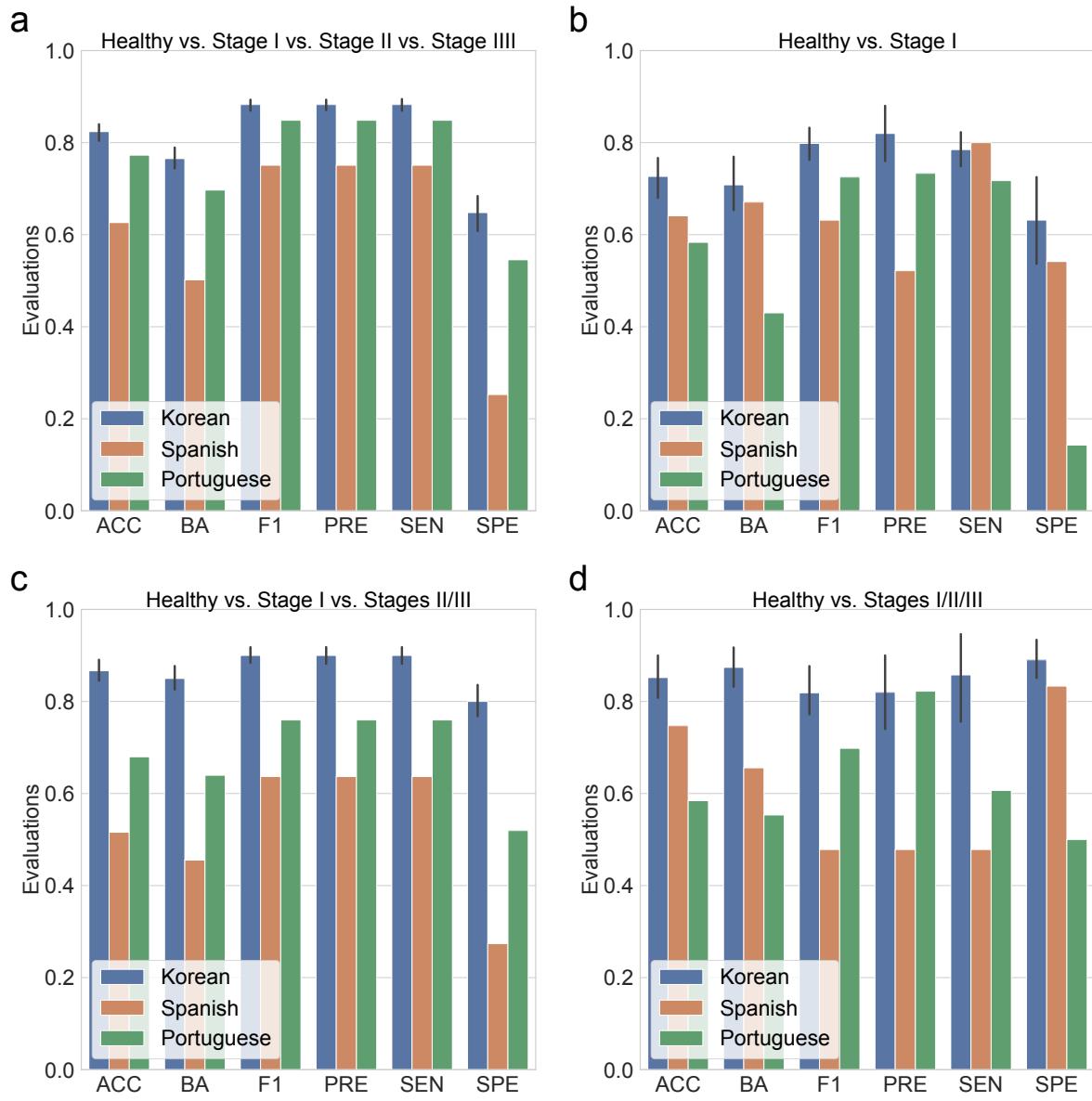


Figure 11: **Random forest classification metrics from external datasets.**

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.

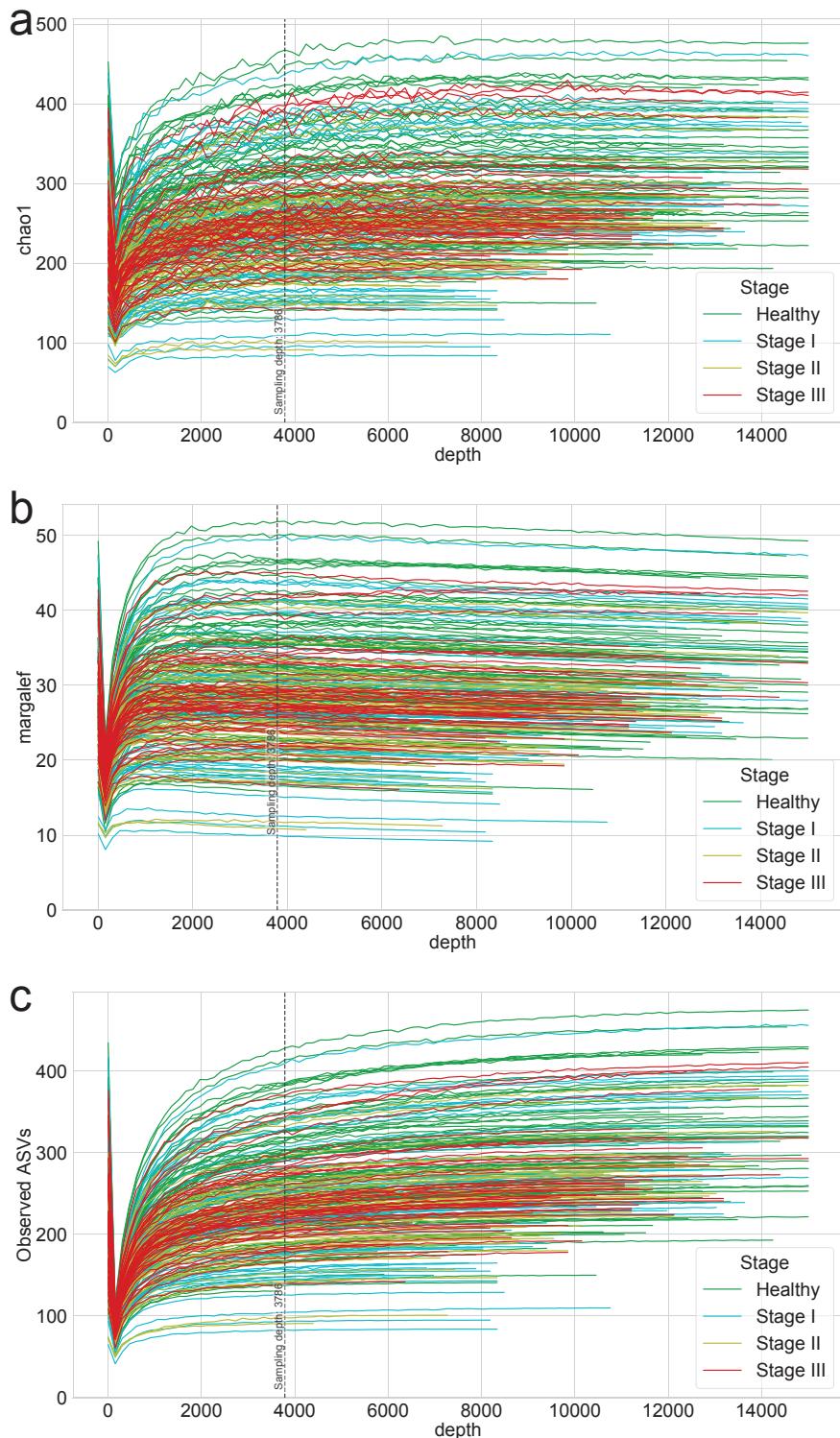


Figure 12: Rarefaction curves for alpha-diversity indices.

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

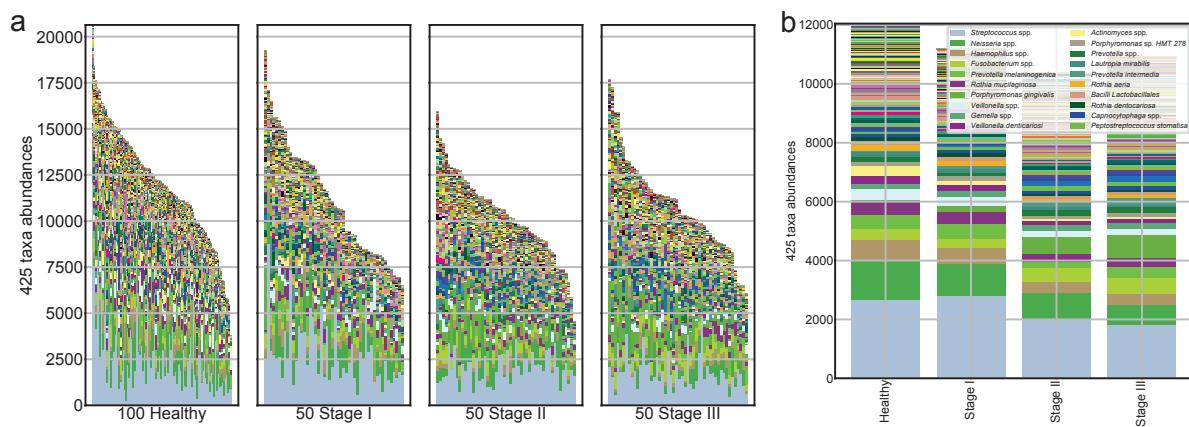


Figure 13: Salivary microbiome compositions in the different periodontal statuses.

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**).

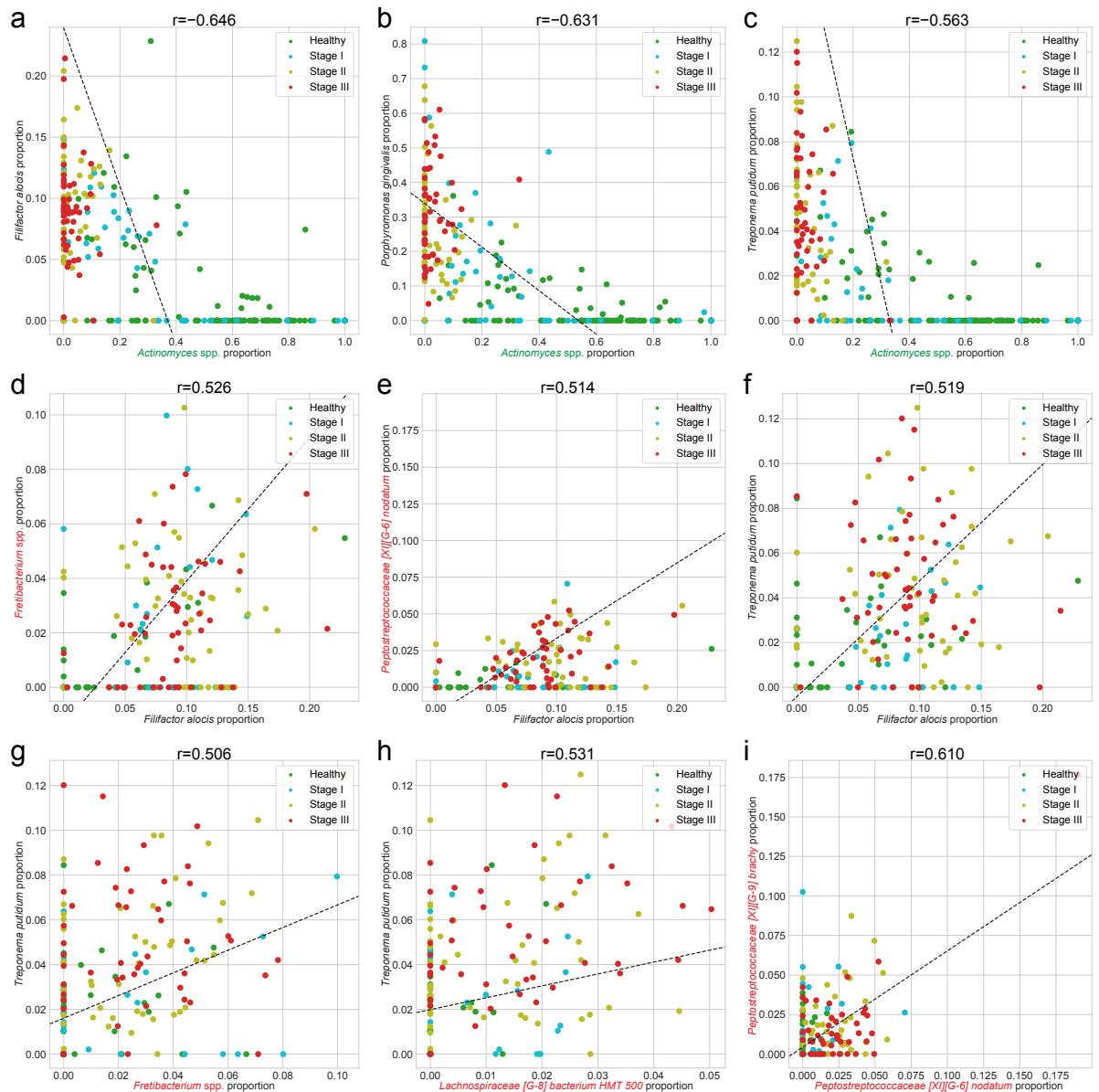


Figure 14: Correlation plots for differentially abundant taxa.

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).

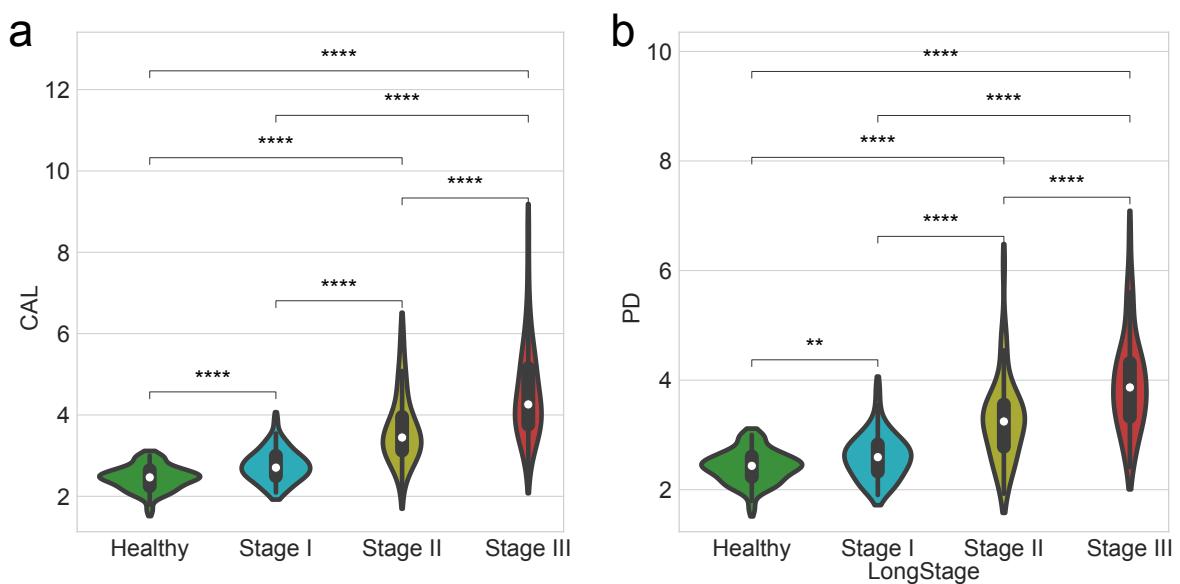


Figure 15: **Clinical measurements by the periodontitis statuses.**

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

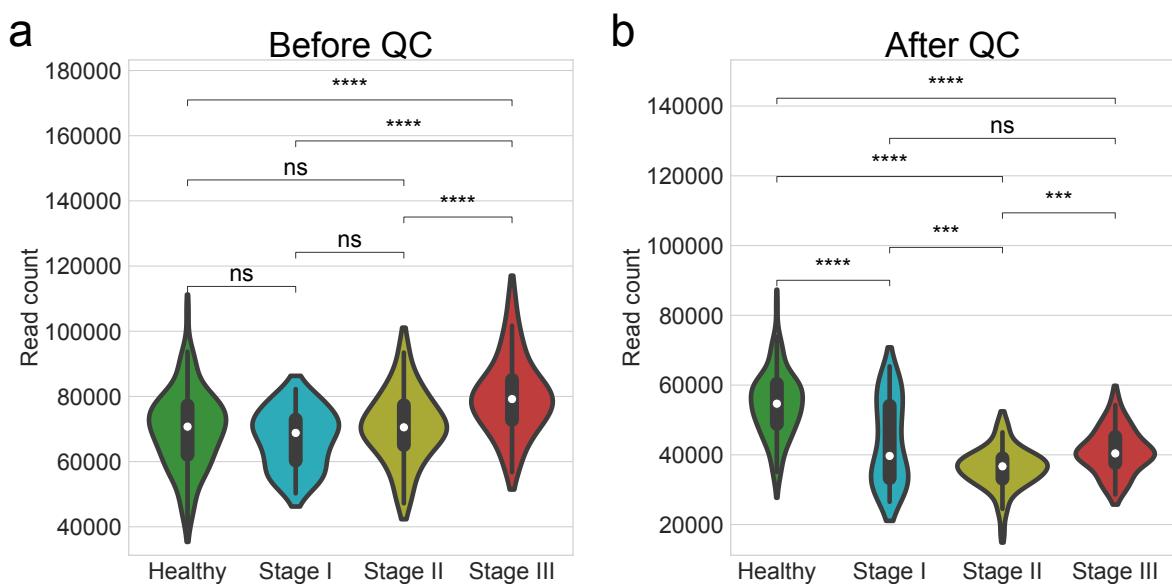


Figure 16: **Number of read counts by the periodontitis statuses.**

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

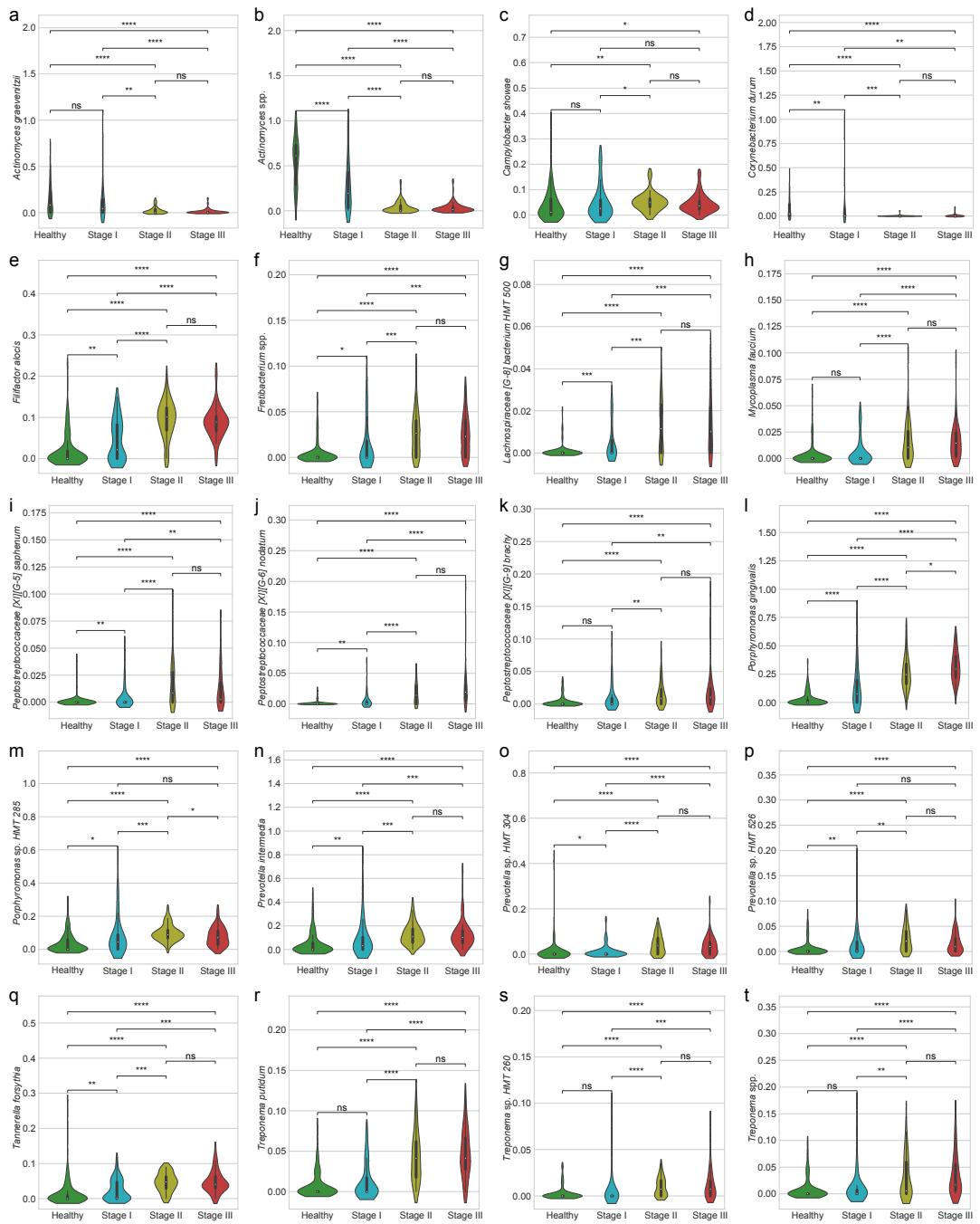


Figure 17: Proportion of DAT.

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

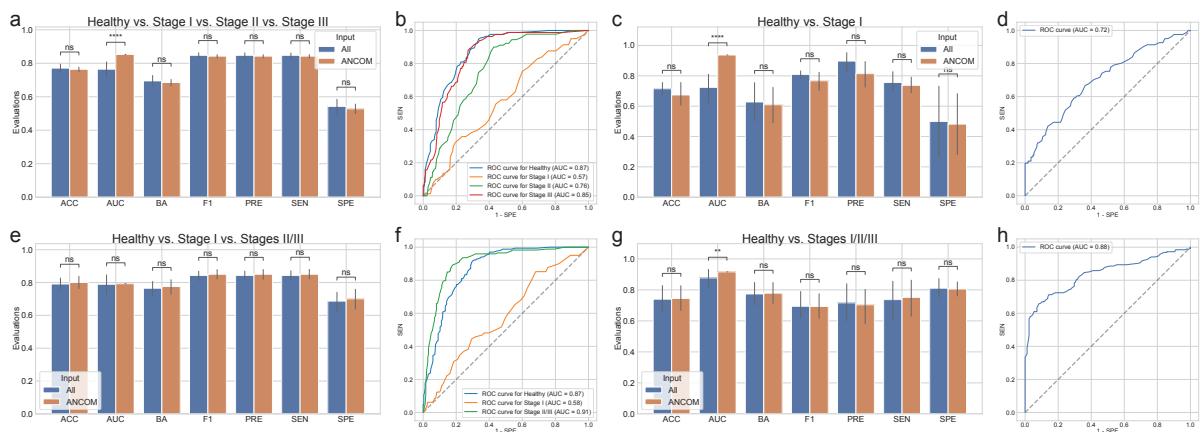


Figure 18: Random forest classification metrics with the full microbiome compositions and ANCOM-selected DAT compositions.

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

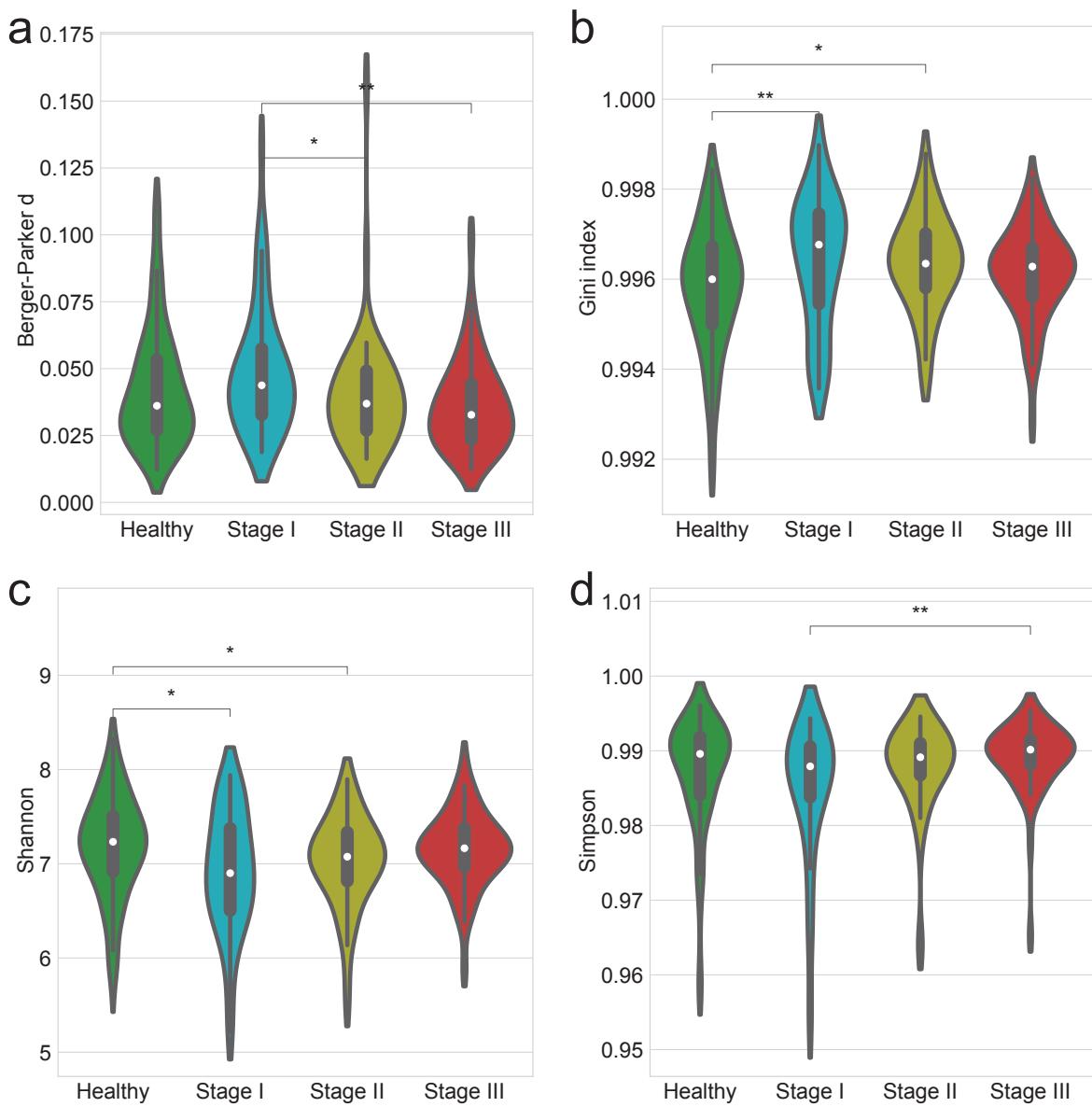


Figure 19: **Alpha-diversity indices account for evenness.**

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 $p \leq 0.01$ (**)

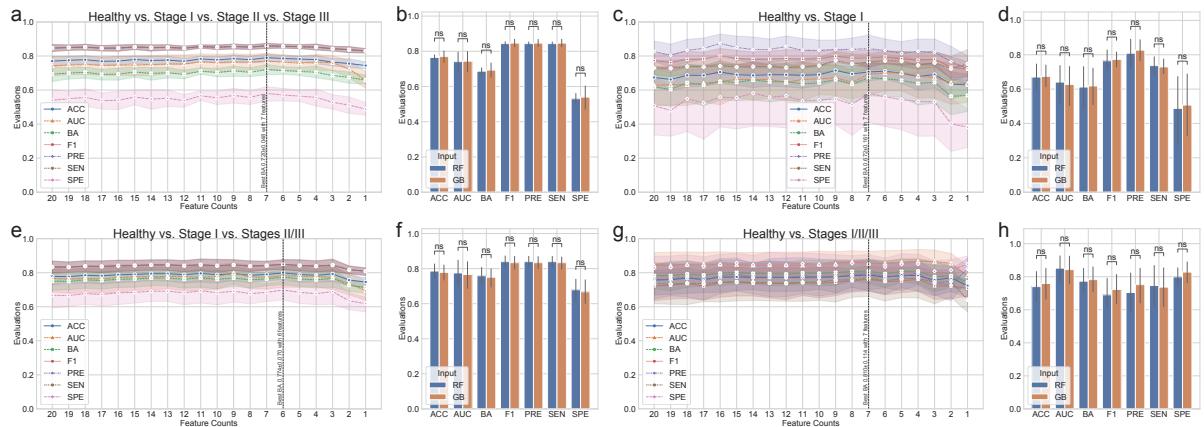


Figure 20: Gradient Boosting classification metrics.

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.

736 **3.4 Discussion**

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

745 Previous research identified the red complex as the primary pathogens of periodontitis (Listgarten,
746 1986): *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. Other studies, however,
747 have shown that periodontal pathogens communicate with other bacteria in the salivary microbiome
748 networks to generate dental plaque prior to the pathogenesis and development of periodontitis (Lamont &
749 Jenkinson, 2000; Rosan & Lamont, 2000; Yoshimura, Murakami, Nishikawa, Hasegawa, & Kawaminami,
750 2009).

751 Using subgingival plaque collections, recent researches have suggested a connection between the
752 periodontitis severity and the salivary microbiome compositions (Altabtbaei et al., 2021; Iniesta et al.,
753 2023; Nemoto et al., 2021). Therefore, we have examined the salivary microbiome compositions of
754 patients with multiple severities of periodontitis and periodontally healthy controls, extending on earlier
755 studies.

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

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

783 Therefore, we calculated beta-diversity indices to analyze the differences between the study partici-
784 pants. The distances for the multiple stages of periodontitis, including stage I, stage II, and stage III, as
785 well as healthy controls (Figure 4g-j and Table 7), suggesting notable differences among the multiple
786 periodontitis severities. In other words, the composition of the salivary microbiome compositions varies
787 depending on the periodontitis stages, so that supporting the findings from a previous study (Iniesta et al.,
788 2023). Taken together that it is nearly impossible to fully restore the attachment level after it has been lost
789 due to the progression and development of periodontitis, the ability to rapidly screen for periodontitis in
790 its early phases using saliva collections would be highly beneficial for effective disease management and
791 treatment.

792 Of the total of 425 taxa in the salivary microbiome composition that have been identified (Figure 13),
793 ANCOM was applied to select 20 taxa as the DAT that indicated notable abundance variation among
794 the periodontitis severities (Figure 8 and Table 5). Three sub-groups were formed from the DAT using
795 hierarchical clustering (Figure 8a). Surprisingly, two of the red complex pathogens (Rôças, Siqueira Jr,
796 Santos, Coelho, & de Janeiro, 2001), *Porphyromonas gingivalis* and *Tannerella forsythia*, were classified
797 in Group 2 and were more prevalent in stage II and stage II periodontitis compared to healthy controls.
798 *Campylobacter showae* was additionally placed in Group 2 of the orange complex pathogens (Gambin et
799 al., 2021). Furthermore, some of the DAT in Group 2 have reported their crucial roles in pathogenesis
800 and development of periodontitis: *Filifactor alocis* (Aruni et al., 2015), *Treponema putidum* (Wyss et
801 al., 2004), *Tannerella forsythia* (Stafford, Roy, Honma, & Sharma, 2012; W. Zhu & Lee, 2016), and
802 *Prevotella intermedia* (Karched, Bhardwaj, Qudeimat, Al-Khabbaz, & Ellepolo, 2022). Taken together,
803 this indicates that DAT in Group 2 is essential to periodontitis. The portion of some Group 1 DAT,
804 including *Peptostreptococcaceae[XI][G-5] saphenum*, *Peptostreptococcaceae[XI][G-6] nodatum*, and
805 *Peptostreptococcaceae[XI][G-9] brachy*, in healthy controls and patients with periodontitis significantly
806 differed, according to earlier research (Lafaurie et al., 2022). These outcomes support our research,
807 implying that Group 1 DAT are also essential to the etiology and progression of periodontitis. However,
808 in contrast to patients with periodontitis, Group 3 DAT, namely *Corynebacterium durum* and *Actinomyces*
809 *graevenitzii*, were enriched in healthy controls, which is consistent with earlier research (Redanz et al.,
810 2021; Nibali et al., 2020).

811 In our correlation analysis (Figure 9), we have discovered strongly negative correlations (coefficient \leq
812 -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%

- 852 • BA: 84%
853 • SEN: 94%
854 • SPE: 74%
- 855 By separating patients with periodontitis from healthy controls using only four DAT, *e.g.* *Actinomyces*
856 *graevenitzii*, *Actinomyces* spp., *Corynebacterium durum*, and *Porphyromonas gingivalis*, our machine
857 learning model performed better than previously published models (Figure 10, Table 4, and Table 6):
- 858 • AUC: $95.3\% \pm 4.9\%$
859 • BA: $88.5\% \pm 6.6\%$
860 • SEN: $86.4\% \pm 15.7\%$
861 • SPE: $90.5\% \pm 7.0\%$
- 862 This result showed that by detecting Group 3 bacteria that were substantially abundant in health
863 controls than patients with periodontitis, our study increased BA by at least 5% and SPE by at least 17%.
- 864 Furthermore, we have validated our machine-learning prediction model using openly accessible 16S
865 gene rRNA sequencing data from Portuguese (Iniesta et al., 2023) and Spanish participants (Relvas et
866 al., 2021) in order to ensure the consistency of our random forest classification model (Figure 11). Our
867 classification models employed in this study were primarily developed and assessed on Korean study par-
868 ticipants, which may limit their generalizability to other ethnic groups with different salivary microbiome
869 compositions (Premaraj et al., 2020; Renson et al., 2019). Therefore, the evaluations of this periodonti-
870 tis classification models can be affected by ethnic-specific variances and differences, highlighting the
871 necessity for additional validation and adjustment across a spectrum of ethnic backgrounds.
- 872 Regarding the clinical characteristics and potential confounders influencing the analysis of salivary
873 microbiome compositions connected with periodontitis severity, this study had a number of limitations
874 that were pointed out. We did not offer clinical information, such as the percentage of teeth, the percentage
875 of bleeding on probing, nor dental furcation involvement, even though we did gather information on
876 attachment level, probing depth, plaque index, and gingival index (Renvert & Persson, 2002); this might
877 have it challenging to present thorough and in-depth data about periodontal health. Moreover, the broad age
878 range may make it tougher to evaluate the relationship between age and periodontitis statuses, providing
879 the necessity for future studies to consider into account more comprehensive clinical characteristics
880 associated with periodontitis. Additionally, potential confounders—*e.g.* body mass index (Bombin, Yan,
881 Bombin, Mosley, & Ferguson, 2022) and e-cigarette use (Suzuki, Nakano, Yoneda, Hirofumi, & Hanioka,
882 2022)—which might have affected dental health and salivary microbiome composition were disregarding
883 consideration in addition to smoking status and systemic diseases. Thus, future research incorporating
884 these components would offer a more thorough knowledge of how lifestyle factors interact and affect the
885 salivary microbiome composition and periodontal health. Throughout, resolving these limitations will
886 advance our understanding in pathogenesis and development of periodontitis, offering significant novel
887 insights on the causal connection between systemic diseases and the salivary microbiome compositions.

888 **4 Metagenomic signature analysis of Korean colorectal cancer**

889 **4.1 Introduction**

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

903 The gut microbiome plays a fundamental role in maintaining host health by helping digestion
904 (Joscelyn & Kasper, 2014; Cerqueira, Photenhauer, Pollet, Brown, & Koropatkin, 2020), regulating
905 metabolism (Dabke, Hendrick, Devkota, et al., 2019; Utzschneider, Kratz, Damman, & Hullarg, 2016;
906 Magnúsdóttir & Thiele, 2018), adjusting immune function (Kau, Ahern, Griffin, Goodman, & Gordon,
907 2011; Shi, Li, Duan, & Niu, 2017; Broom & Kogut, 2018), and even coordinating neurological processes
908 by the brain-gut axis (Martin et al., 2018; Aziz & Thompson, 1998; R. Li et al., 2024). Comprising
909 these gut microbiota, including, archaea, bacteria, fungi, and viruses, the gut microbiome contributes
910 to the synthesis of essential vitamins, and production of fatty acids, which influence intestinal integrity
911 and immune responses. Thus, well-balanced gut microbiome composition modulates systemic immune
912 function by interacting with gut-associated lymphoid tissue, shaping immune tolerance and response
913 to infections. Hence, emerging evidence suggests that dysbiosis in the gut microbiome composition are
914 associated not only a narrow range of diseases, e.g. diarrhea and enteritis (Paganini & Zimmermann,
915 2017; Gao, Yin, Xu, Li, & Yin, 2019) but also a wide range of diseases, e.g. obesity, diabetes, and cancers
916 (Barlow et al., 2015; Hartstra et al., 2015; Helmink et al., 2019; Cullin et al., 2021).

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

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

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

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

948 **4.2 Materials and methods**

949 **4.2.1 Study participants enrollment**

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

965 **4.2.2 DNA extraction procedure**

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

975 **4.2.3 Bioinformatics analysis**

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

983 including alpha-diversity to evaluate single-sample diversity and beta-diversity to compare microbial
984 composition between the tumor tissues and their corresponding adjacent normal tissues. Differentially
985 abundant taxa (DAT) were identified using statistical method, (DESeq2 (Love et al., 2014), ANCOM
986 (Lin & Peddada, 2020)), adjusting for sequencing depth and potential confounders to highlight taxa
987 significantly associated with CRC. To explore functional implications, microbial pathway prediction was
988 performed using (PICRUSt3, HUMAnN3), linking microbial composition to metabolic and functional
989 pathways relevant to carcinogenesis and progression of CRC. This multi-layered bioinformatics approach
990 enabled a comprehensive investigation of gut microbiome alteration in CRC, facilitating the identification
991 of potential microbial biomarkers for diagnosis and prognosis of CRC.

992 **4.2.4 Data and code availability**

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

998 **4.3 Results**

999 **4.3.1 Summary of clinical characteristics**

1000 **4.3.2 Gut microbiome compositions**

1001 **4.3.3 Diversity indices**

1002 **4.3.4 DAT selection**

1003 **4.3.5 Pathway prediction**

Table 8: Clinical characteristics of CRC study participants

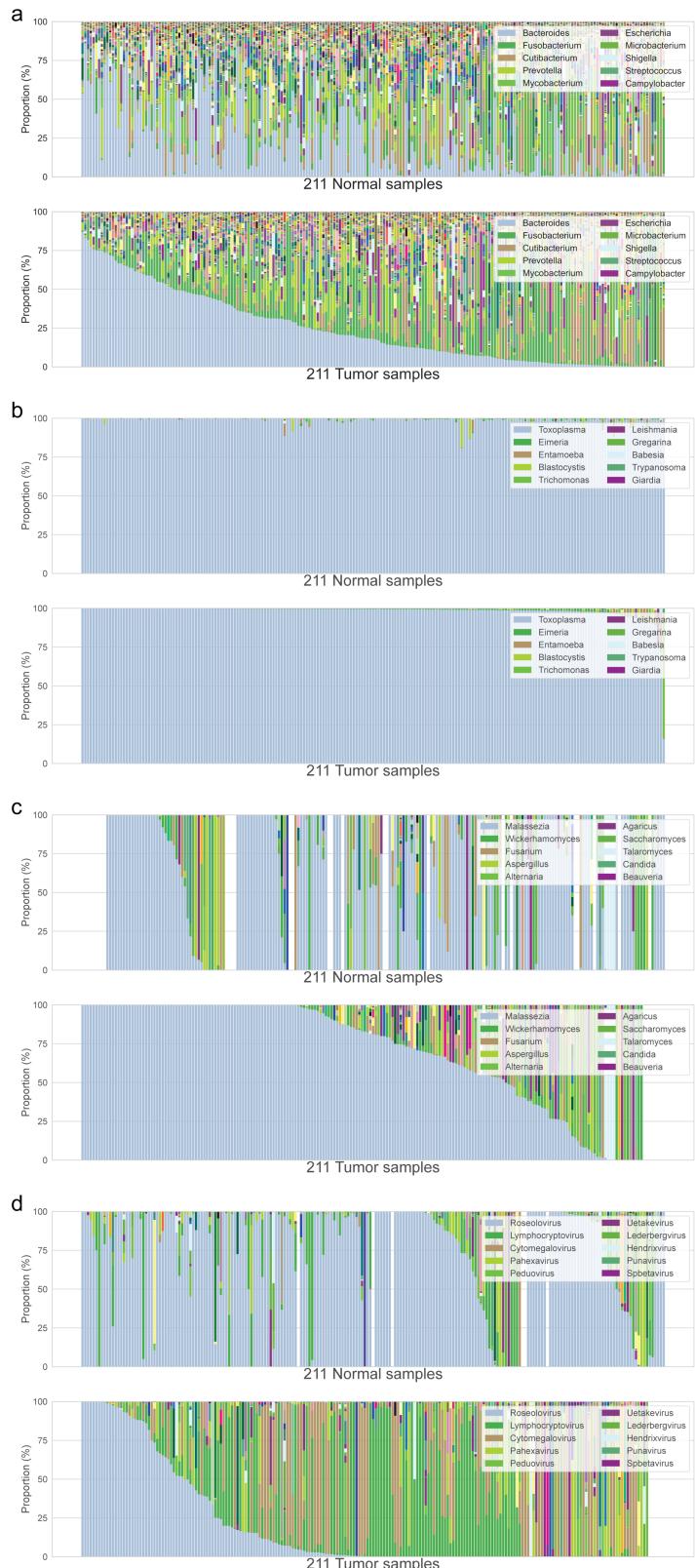


Figure 21: Gut microbiome compositions in genus level.

Taxa were sorted from the most prevalent taxon to the least prevalent taxon. CRC patients were sorted by the most prevalent taxon in descending order. **(a)** Bacteria kingdom **(b)** Eukaryota kingdom **(c)** Fungi kingdom **(d)** Viruses kingdom

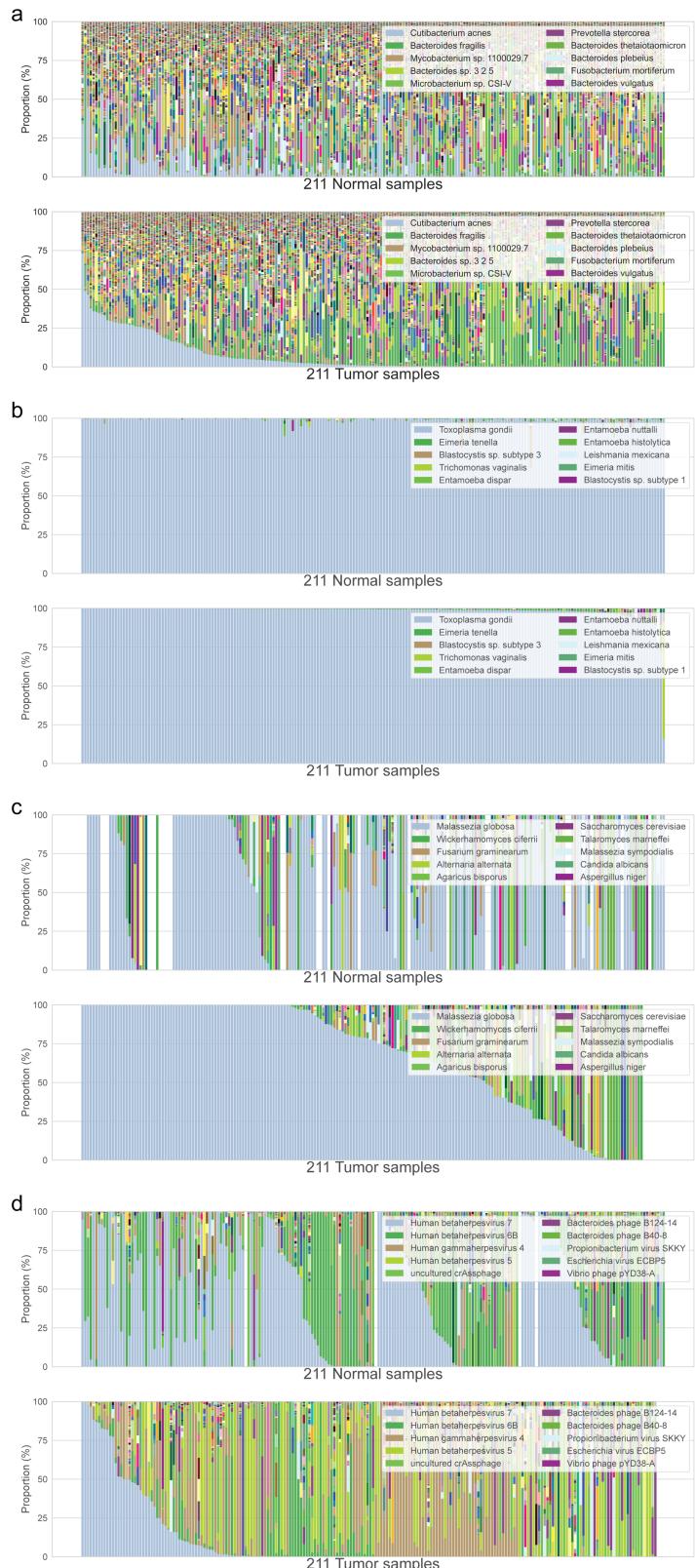


Figure 22: Gut microbiome compositions in species level.

Taxa were sorted from the most prevalent taxon to the least prevalent taxon. CRC patients were sorted by the most prevalent taxon in descending order. **(a)** Bacteria kingdom **(b)** Eukaryota kingdom **(c)** Fungi kingdom **(d)** Viruses kingdom

1004 **4.4 Discussion**

1005 **5 Conclusion**

1006 In conclusion, the research described in this doctoral dissertation was conducted to identify significant ...

1007 In the section 2, I show that

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