

¹

Doctoral Thesis

²

Microbiota in Human Diseases

³

Jaewoong Lee

⁴

Department of Biomedical Engineering

⁵

Ulsan National Institute of Science and Technology

⁶

2025

⁷

Microbiota in Human Diseases

⁸

Jaewoong Lee

⁹

Department of Biomedical Engineering

¹⁰

Ulsan National Institute of Science and Technology



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



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

13

Abstract

14 (Microbiome)

15 (PTB) Section 2 introduces...

16 (Periodontitis) Section 3 describes...

17 (Colon) Setion 4...

18 (Conclusion)

19

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

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

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 **ASV** Amplicon sequence variant

108 **AUC** Area-under-curve

109 **BA** Balanced accuracy

110 **C-section** Cesarean section

111 **DAT** Differentially abundant taxa

112 **F1** F1 score

113 **Faith PD** Faith's phylogenetic diversity

114 **FTB** Full-term birth

115 **GA** Gestational age

116 **MSI** Microsatellite instability

117 **MSs** Microsatellite stable

118 **MWU test** Mann-Whitney U-test

119 **OS** Overall survival

120 **PRE** Precision

121 **PROM** Prelabor rupture of membrane

122 **PTB** Preterm birth

123 **ROC curve** Receiver-operating characteristics curve

124 **rRNA** Ribosomal RNA

125 **SD** Standard deviation

126 **SEN** Sensitivity

127 **SPE** Specificity

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

129 **1 Introduction**

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

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

165 16S ribosomal RNA (rRNA) gene sequencing is one of the most extensively applied methods for
166 characterizing microbial communities by targeting the conserved 16S rRNA gene, which contains both

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

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

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

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

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

251 (Limitation & Novelty)

Table 1: Confusion matrix

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

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

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

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

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

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

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

IP + FP

257 **2 Predicting preterm birth using random forest classifier in salivary mi-**
258 **crobiome**

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

260 Hong, Y. M., **Lee, Jaewoong**, Cho, D. H., Jeon, J. H., Kang, J., Kim, M. G., ... & Kim, J. K. (2023).
261 Predicting preterm birth using machine learning techniques in oral microbiome. *Scientific Reports*, 13(1),
262 21105.

263 **2.1 Introduction**

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

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

281 Preterm prelabor rupture of membranes (PROM) brought on by gestational inflammation and infection
282 contribute to about 70% of PTB cases (Romero, Dey, & Fisher, 2014). Nevertheless, as antibiotics and
283 anti-inflammatory therapeutic strategies were ineffective to decrease PTB occurrence rates, the pathology
284 of PTB has not been entirely elucidated by inflammatory and infectious pathways (Romero, Hassan, et al.,
285 2014). Recent researches on maternal microbiomes were beginning to examine unidentified connections
286 of PTB as a consequence of developmental processes in molecular biological technology (Fettweis et al.,
287 2019).

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

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

296 Multiple unfavorable birth outcomes, including PROM and PTB, have been linked to periodontitis
297 as an independence risk factor, according to numerous epidemiological researches (Offenbacher et al.,
298 1996). It is expected that the oral microbiome will be able to explain additional hematogenous pathways
299 in light of these precedents; however, the oral microbiome composition of fetuses is limited understood.

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

303 **2.2 Materials and methods**

304 **2.2.1 Study design and study participants**

305 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
306 Declaration of Helsinki (Goodyear, Krleza-Jeric, & Lemmens, 2007). The Institutional Review Board
307 authorized this study (IRB file No. 2019-01-024). Participants who were admitted for elective cesarean
308 sections (C-sections) or induction births, as well as those who had written informed consent obtained
309 with premature labor or PROM, were eligible.
310

311 **2.2.2 Clinical data collection and grouping**

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

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

323 **2.2.3 Salivary microbiome sample collection**

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

332 **2.2.4 16s rRNA gene sequencing**

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

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

339 **2.2.5 Bioinformatics analysis**

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

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

351 Taxonomic assignment were implemented with HOMD (version 15.22) (T. Chen et al., 2010).
352 Afterward, DESeq2 was implemented to identify differentially abundant taxa (DAT) that could dis-
353 tinguish between salivary microbiome from PTB and FTB participants (Love et al., 2014). Taxa with
354 $|\log_2 \text{FoldChange}| > 1$ and $p < 0.05$ were considered as statistically significant.

355 The taxa for predicting PTB using salivary microbiome data were determined using a random forest
356 classifier (Breiman, 2001). Through stratified *k*-fold cross-validation (*k* = 5) that preserves the existence
357 rate of PTB and FTB participants, consistency and trustworthy classification were ensured (Wong & Yeh,
358 2019).

359 **2.2.6 Data and code availability**

360 All sequences from the 59 study participants have been published to the Sequence Read Archives
361 (project ID PRJNA985119): <https://dataview.ncbi.nlm.nih.gov/object/PRJNA985119>. Docker
362 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.

365 **2.3 Results**

366 **2.3.1 Overview of clinical information**

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

373 **2.3.2 Comparison of salivary microbiomes composition**

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

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

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

385 **2.3.3 Random forest classification to predict PTB risk**

386 To classify PTB according to DAT, random forest classifiers were constructed. The nine most significant
387 DAT were used to obtain the best BA (0.765 ± 0.071 ; Figure 3a). Moreover, random forest classification
388 model determined each DAT's importance (Figure 3b). We conducted a validation procedure on nine
389 twin pregnancies that were excluded in the initial study design in order to confirm the reliability and
390 dependability of our random forest-based PTB prediction model (Figure 6). Comparable to the PTB
391 prediction model on the 59 initial singleton study participants, the validation classification on PTB risk of
392 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

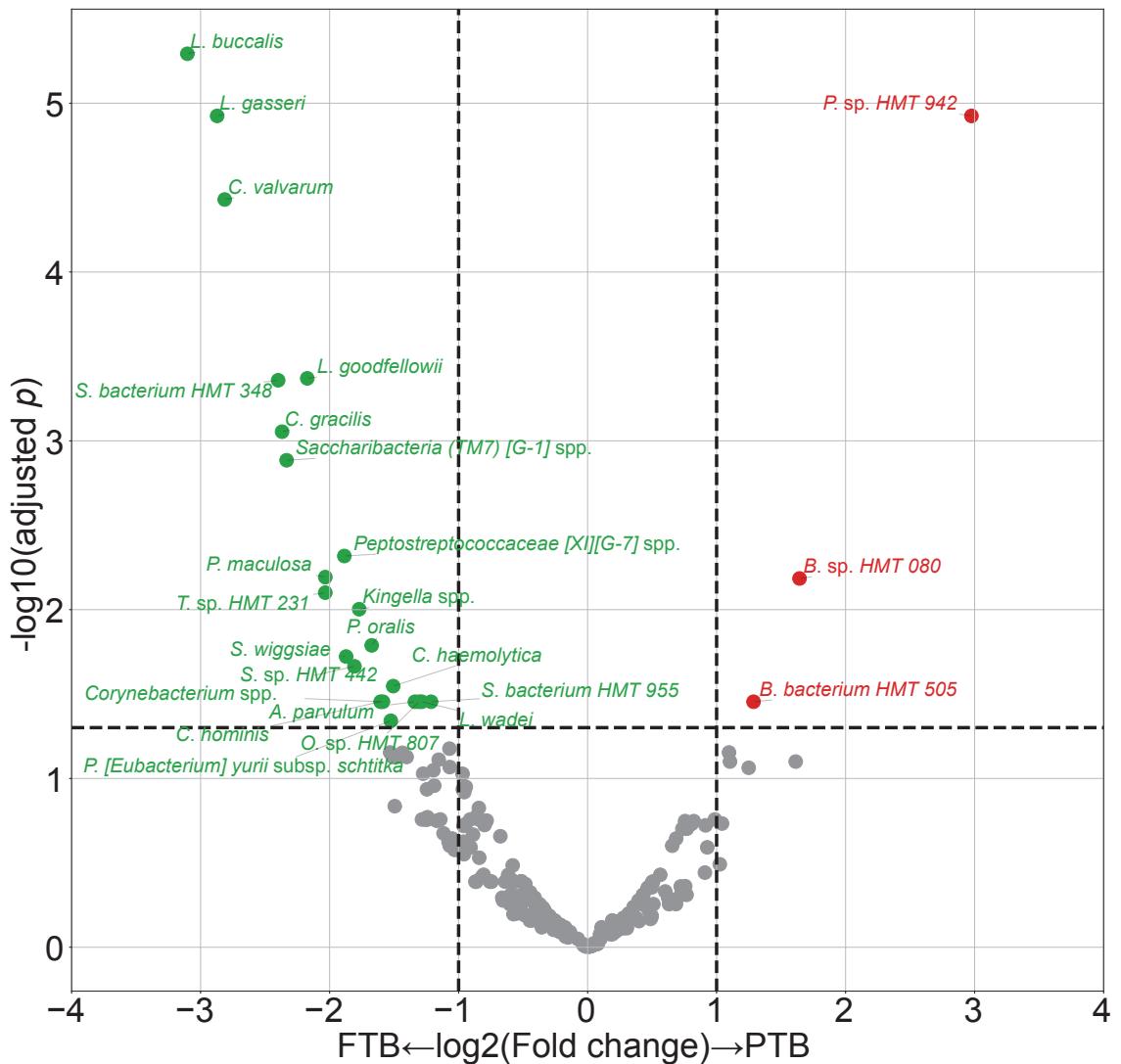


Figure 1: DAT volcano plot.

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

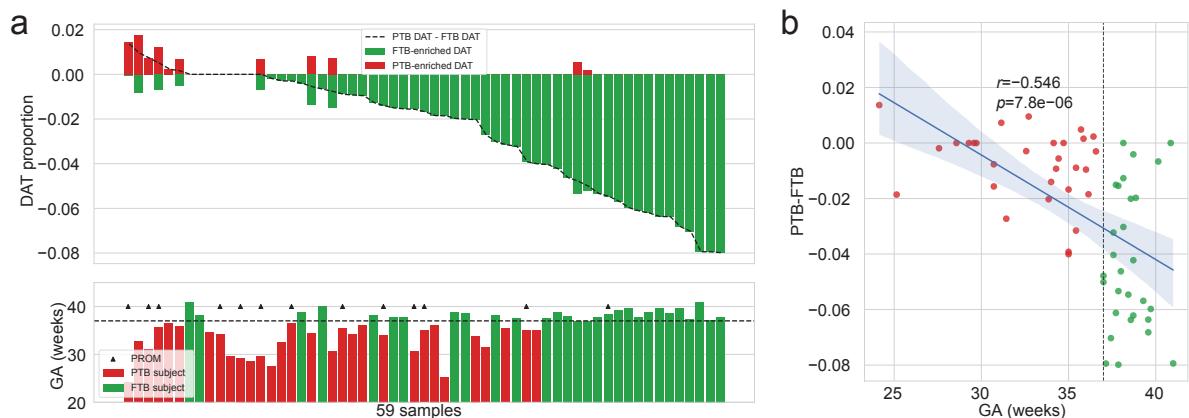


Figure 2: **Salivary microbiome compositions over DAT.**

(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.

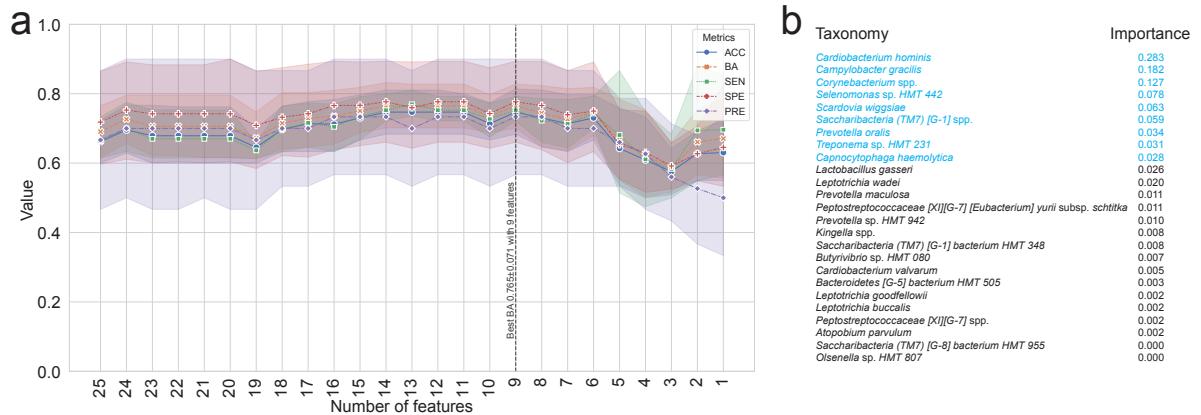


Figure 3: **Random forest-based PTB prediction model.**

(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.

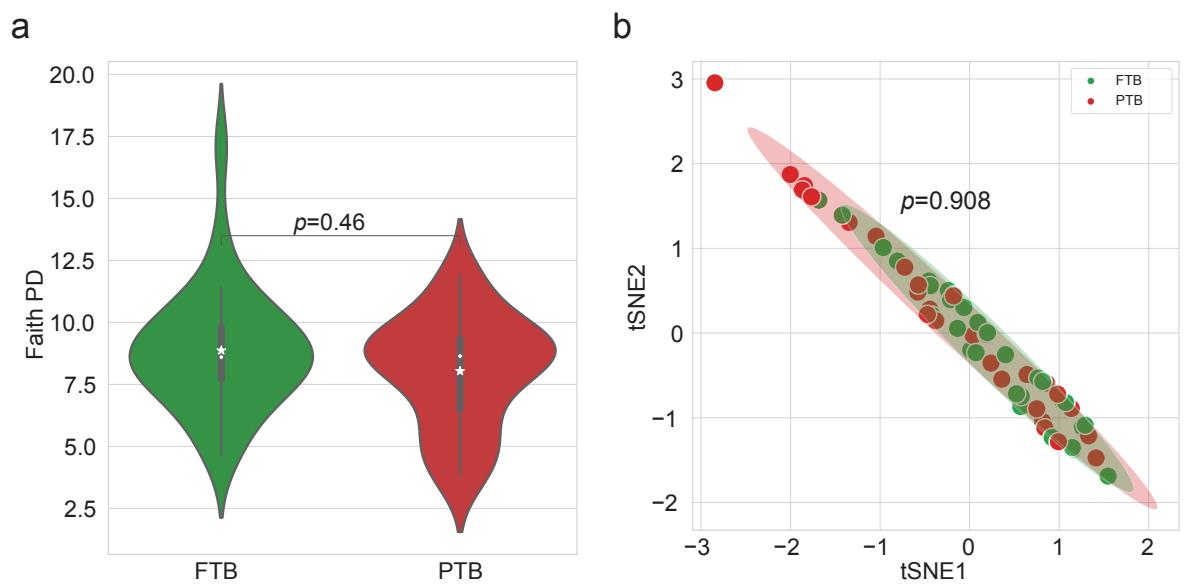


Figure 4: **Diversity indices.**

(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 $p = 0.908$)

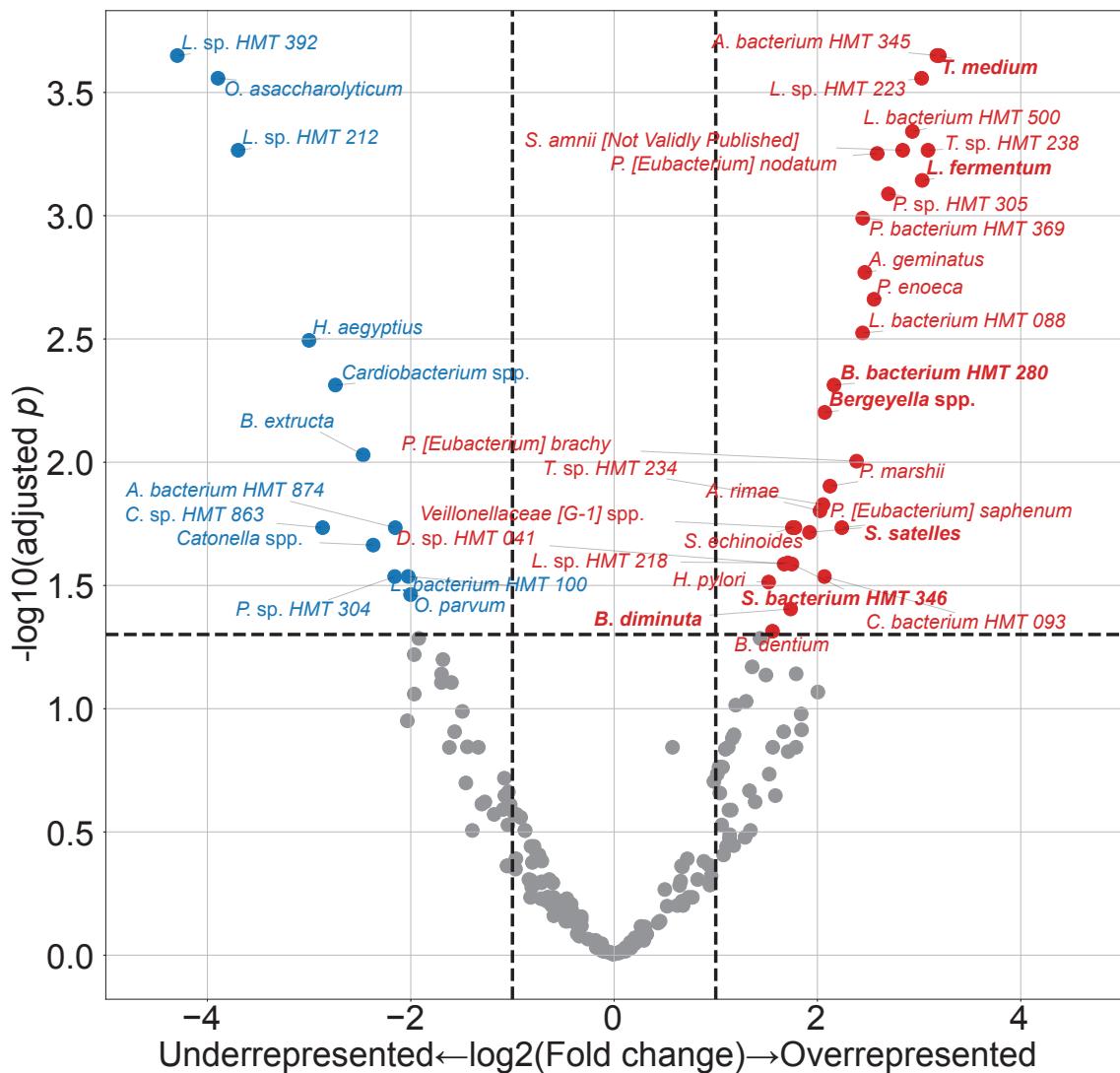


Figure 5: PROM-related DAT.

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.

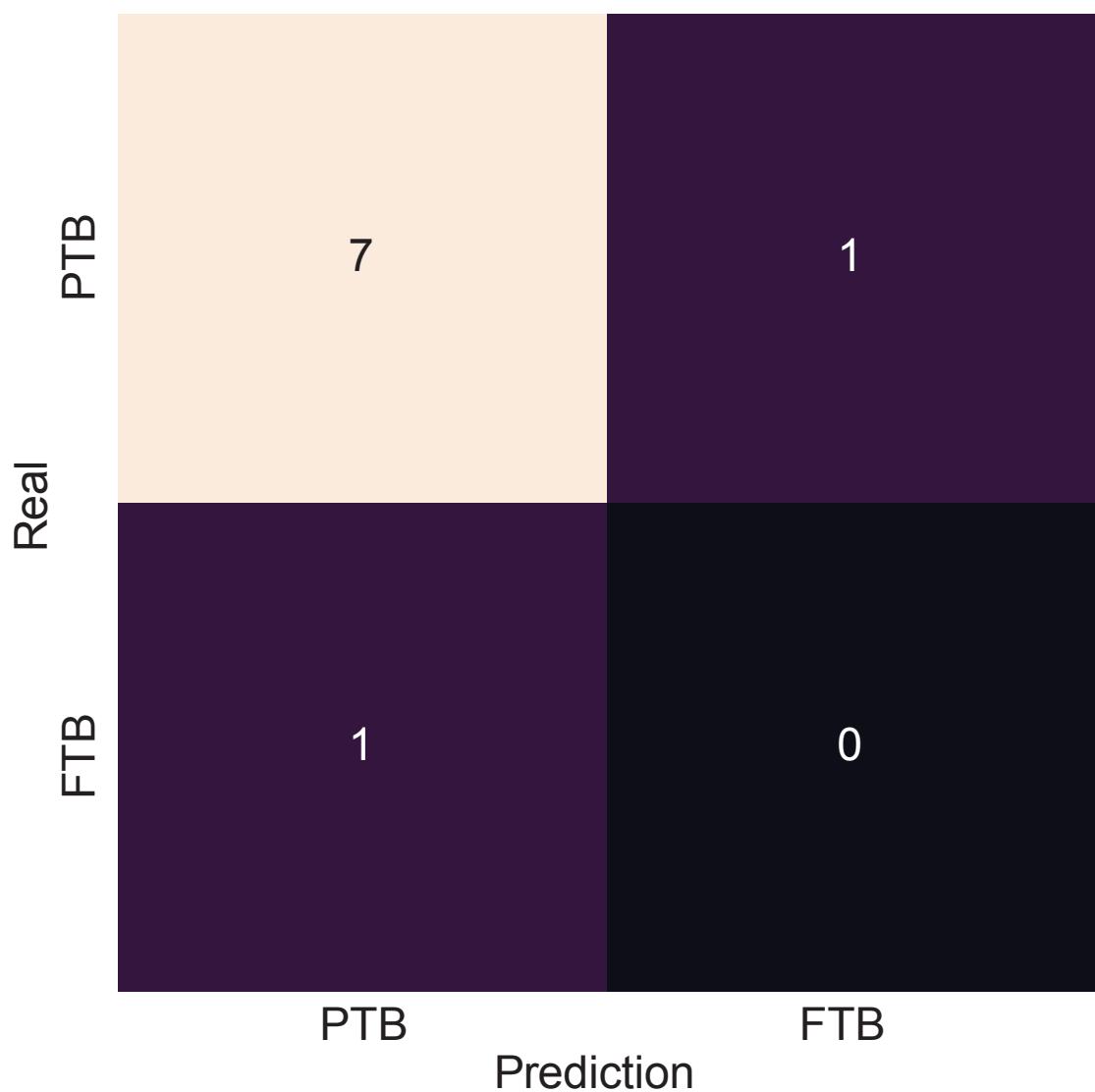


Figure 6: Validation of random forest-based PTB prediction model.

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)

393 **2.4 Discussion**

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

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

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

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

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

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

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

445 **3 Random forest prediction model for periodontitis statuses based on the**
446 **salivary microbiomes**

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

448

449 **3.1 Introduction**

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

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

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

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

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

510 **3.2 Materials and methods**

511 **3.2.1 Study participants enrollment**

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

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

529 **3.2.2 Periodontal clinical parameter diagnosis**

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

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

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

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

547 0. Normal gingiva: without inflammation nor discoloration.

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

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

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

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

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

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

558 • Healthy:

559 1. Bleeding sites < 10%

560 2. Probing depth: \leq 3 mm

561 • Stage I:

562 1. No tooth loss because of periodontitis.

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

564 3. Radiographic bone loss: < 15%

565 • Stage II:

566 1. No tooth loss because of periodontitis.

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

568 3. Radiographic bone loss: 15-33%

569 • Stage III:

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

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

572 3. Radiographic bone loss: > 33%

573 **3.2.3 Saliva sampling and DNA extraction procedure**

574 All study participants received instructions to avoid eating, drinking, brushing, and using mouthwash for
575 at least an hour prior to the saliva sample collection process. These collections were conducted between
576 09:00 and 11:00. Mouth rinse was collected by rinsing the mouth for 30 seconds with 12 mL of a solution
577 (E-zen Gargle, JN Pharm, Korea). All saliva samples were tagged with anonymous ID and stored at -4 °C.

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

- 582 • Forward: 5' -TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNNGCWGCAG-3'
583 • Reverse: 5' -GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'

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

- 586 1. Heat activation for 30 seconds at 95 °C.
587 2. 25 cycles for 30 seconds at 95 °C.
588 3. 30 seconds at 55 °C.
589 4. 30 seconds at 72 °C.

590 NexteraXT Indexed Primer was applied to amplification 10 µL of the purified initial PCR products for
591 the final library creation. The second PCR used the same conditions as the first PCR conditions but with
592 10 cycles. 16S rRNA gene sequencing was performed via 2×300 bp paired-end sequencing at Macrogen
593 Inc. (Macrogen, Seoul, Korea) using Illumina MiSeq platform (Illumina, San Diego, CA, USA).

594 **3.2.4 Bioinformatics analysis**

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

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

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

606 Aitchison index for a beta-diversity index was calculated using QIIME2 (version 2020.8) (Aitchison,
607 Barceló-Vidal, Martín-Fernández, & Pawlowsky-Glahn, 2000; Bolyen et al., 2019). We employed the
608 t-SNE algorithm to illustrate multi-dimensional data from the beta-diversity index computation (Van der
609 Maaten & Hinton, 2008). The beta-diversity index was compared using the PERMANOVA test (Anderson,
610 2014; Kelly et al., 2015) and MWU test.

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

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

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

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

640 3.2.5 Data and code availability

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

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

646 **3.3 Results**

647 **3.3.1 Summary of clinical information and sequencing data**

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

655 **3.3.2 Diversity indices reveal differences among the periodontitis severities**

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

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

666 **3.3.3 DAT among multiple periodontitis severities and their correlation**

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

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

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

- 689 • Group 3
- 690 1. *Actinomyces* spp.
- 691 2. *Corynebacterium durum*
- 692 3. *Actinomyces graevenitzii*

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

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

701 3.3.4 Classification of periodontitis severities by random forest models

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

706 Based on the proportion of DAT, random forest classifier were trained to classify the periodontitis
707 severities (Table 6). We conducted multi-label classification for the multiple periodontitis severities,
708 namely healthy, stage I, stage II, and stage III. In this setting, we classified multiple periodontitis
709 severities with the highest BA of 0.779 ± 0.029 (Table 4). AUC ranged between 0.81 and 0.94 (Figure
710 10b).

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

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

717 perform a multi-label classification.

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

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

Table 3: Clinical characteristics of the study participants.

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

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

Table 4: Feature combinations and their evaluations

Classification performance with the most important taxon, the two most important taxa, and taxa with the best-balanced accuracy. *P.gingivalis* and *Act.* are *Porphyromonas gingivalis* and *Actinomyces* spp., respectively.

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

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

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

Table 6: Feature the importance of taxa in the classification of different periodontal statuses
 Taxa are ranked in descending order of importance; from most important to least important.

Condition	Healthy vs. Stage I vs. Stage II vs. Stage III			Healthy vs. Stage I			Healthy vs. Stage I vs. Stage II/III			Healthy vs. Stage I/II/III		
	Rank	Taxa	Importance	Taxa	Importance	Taxa	Importance	Taxa	Importance	Taxa	Importance	
1	<i>Porphyromonas gingivalis</i>	0.297	<i>Actinomyces spp.</i>	0.195	<i>Porphyromonas gingivalis</i>	0.360	<i>Porphyromonas gingivalis</i>	0.426	<i>Porphyromonas gingivalis</i>	0.461		
2	<i>Actinomyces spp.</i>	0.195	<i>Actinomyces graevenitzii</i>	0.054	<i>Actinomyces spp.</i>	0.125	<i>Actinomyces spp.</i>	0.244	<i>Actinomyces spp.</i>	0.257		
3	<i>Prevotella intermedia</i>	0.054	<i>Actinomyces graevenitzii</i>	0.052	<i>Porphyromonas sp. HMT 285</i>	0.055	<i>Actinomyces graevenitzii</i>	0.049	<i>Actinomyces graevenitzii</i>	0.059		
4	<i>Actinomyces graevenitzii</i>	0.052	<i>Lachnospiraceae (G-8) bacterium HMT 500</i>	0.050	<i>Porphyromonas sp. HMT 285</i>	0.062	<i>Corynebacterium durum</i>	0.046	<i>Corynebacterium durum</i>	0.035		
5	<i>Filifactor alocis</i>	0.050	<i>Campylobacter showae</i>	0.042	<i>Campylobacter showae</i>	0.052	<i>Filifactor alocis</i>	0.036	<i>Filifactor alocis</i>	0.032		
6	<i>Campylobacter showae</i>	0.042	<i>Porphyromonas sp. HMT 285</i>	0.040	<i>Corynebacterium durum</i>	0.052	<i>Prevotella intermedia</i>	0.033	<i>Campylobacter showae</i>	0.023		
7	<i>Porphyromonas sp. HMT 285</i>	0.040	<i>Treponema spp.</i>	0.032	<i>Treponema spp.</i>	0.038	<i>Tannerella forsythia</i>	0.025	<i>Porphyromonas sp. HMT 285</i>	0.022		
8	<i>Corynebacterium durum</i>	0.032	<i>Tannerella forsythia</i>	0.026	<i>Tannerella forsythia</i>	0.037	<i>Prevotella intermedia</i>	0.023	<i>Prevotella intermedia</i>	0.022		
9	<i>Treponema spp.</i>	0.032	<i>Prevotella intermedia</i>	0.025	<i>Prevotella intermedia</i>	0.029	<i>Treponema spp.</i>	0.021	<i>Treponema spp.</i>	0.022		
10	<i>Tannerella forsythia</i>	0.026	<i>Prevotella intermedia</i>	0.025	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.026	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.018	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.015		
11	<i>Treponema putidum</i>	0.025	<i>Freibacterium spp.</i>	0.023	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.018	<i>Lachnospiraceae (G-8) bacterium HMT 500</i>	0.014	<i>Lachnospiraceae (G-8) bacterium HMT 500</i>	0.010		
12	<i>Freibacterium spp.</i>	0.023	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.021	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.018	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.011	<i>Tannerella forsythia</i>	0.009		
13	<i>Peptostreptococcaceae (XII)(G-9) brachy</i>	0.021	<i>Treponema putidum</i>	0.019	<i>Treponema putidum</i>	0.014	<i>Treponema putidum</i>	0.010	<i>Freibacterium spp.</i>	0.009		
14	<i>Treponema sp. HMT 260</i>	0.019	<i>Prevotella sp. HMT 526</i>	0.018	<i>Prevotella sp. HMT 526</i>	0.011	<i>Prevotella sp. HMT 526</i>	0.009	<i>Prevotella sp. HMT 526</i>	0.006		
15	<i>Prevotella sp. HMT 526</i>	0.018	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.018	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.008	<i>Freibacterium spp.</i>	0.008	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.004		
16	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.018	<i>Prevotella sp. HMT 304</i>	0.017	<i>Peptostreptococcaceae (XII)(G-6) nodatum</i>	0.008	<i>Treponema sp. HMT 260</i>	0.008	<i>Treponema sp. HMT 260</i>	0.004		
17	<i>Prevotella sp. HMT 304</i>	0.017	<i>Mycoplasma faecium</i>	0.014	<i>Mycoplasma faecium</i>	0.004	<i>Prevotella sp. HMT 304</i>	0.005	<i>Mycoplasma faecium</i>	0.003		
18	<i>Mycoplasma faecium</i>	0.014	<i>Prevotella sp. HMT 304</i>	0.014	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.003	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.005	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.002		
19	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.014	<i>Lachnospiraceae (G-8) bacterium HMT 500</i>	0.013	<i>Peptostreptococcaceae (XII)(G-5) saphenum</i>	0.003	<i>Prevotella sp. HMT 304</i>	0.004	<i>Prevotella sp. HMT 304</i>	0.001		
20	<i>Lachnospiraceae (G-8) bacterium HMT 500</i>	0.013										

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

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

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

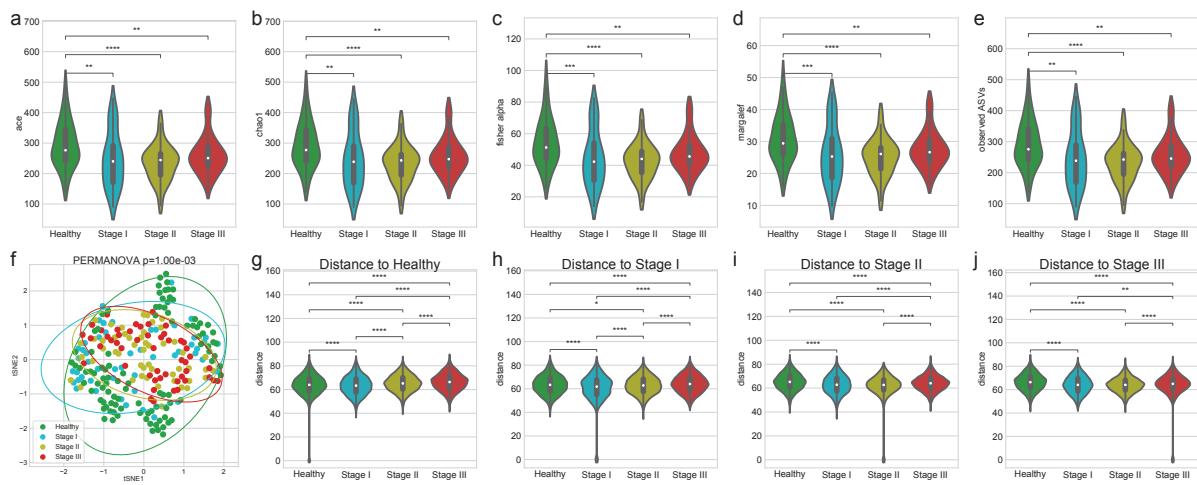


Figure 7: Diversity indices.

Alpha-diversity indices (**a-e**) indicate that healthy controls have increased heterogeneity than periodontitis stages as measured by: (**a**) ace (**b**) chao1 (**c**) Fisher alpha (**d**) Margalef, and (**e**) observed ASVs. (**f**) The beta-diversity index (weighted UniFrac) was visualized using a tSNE-transformed plot. The confidence ellipses are shown to display the distribution of each periodontitis stage. The distance to each stage demonstrated that each periodontitis stage was distinguished from the other periodontitis stages: (**g**) distance to Healthy (**h**) distance to Stage I (**i**) distance to Stage II, and (**j**) distance to Stage III. Statistical significance determined by the MWU test and the PERMANOVA test: $p \leq 0.01$ (**) and $p \leq 0.0001$ (****).

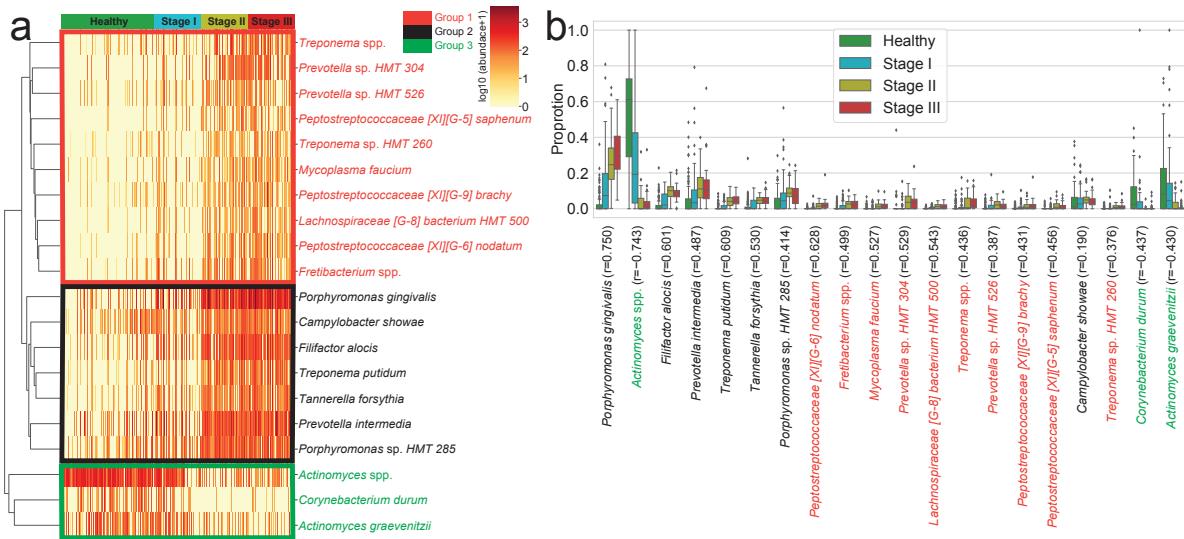


Figure 8: **Differentially abundant taxa (DAT).**

DAT that were identified by ANCOM. **(a)** Heatmap of clustered DAT with similar distribution among subjects. Group 1, Group 2, and Group 3 are marked in red, black, and green, respectively. **(b)** Box plots showing the proportions of DAT. Taxa were sorted by their importance according to ANCOM.

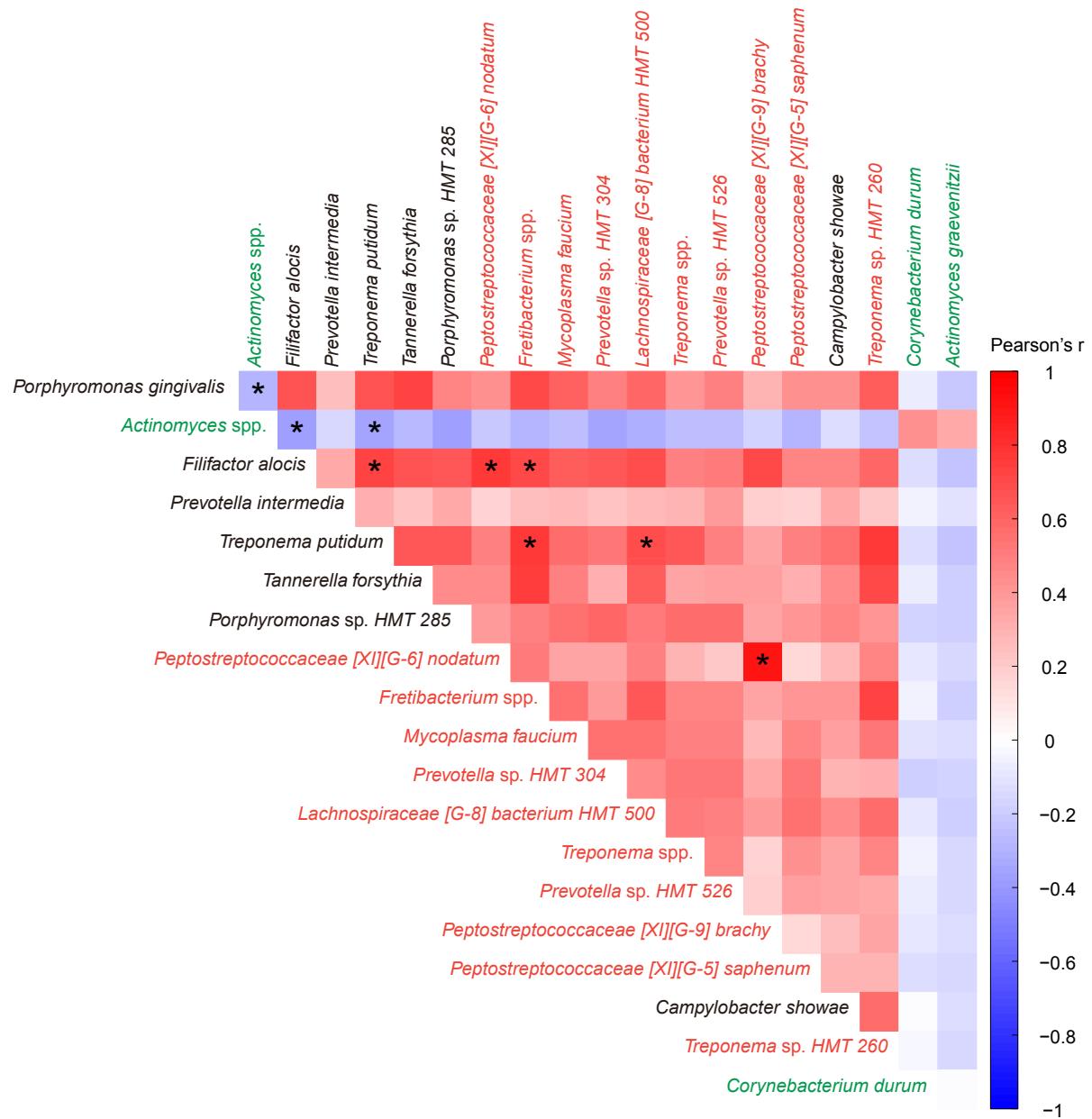


Figure 9: Correlation heatmap.

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

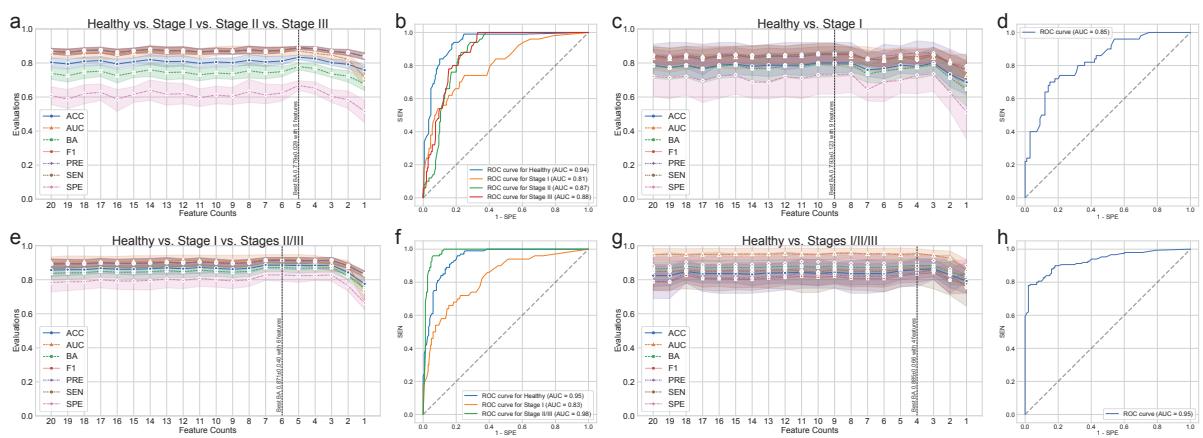


Figure 10: Random forest classification metrics.

The classification metrics in the random forest classifications were as follows: ACC, AUC, BA, F1, PRE, SEN, and SPE. **(a)** Classification performance for healthy vs. stage I vs. stage II vs. stage III. **(b)** ROC curve for the highest BA of (a). **(c)** Classification performance for healthy vs. stage I. **(d)** ROC curve on the highest BA of (c). **(e)** Classification performance for healthy vs. stage I vs. stages II/III. **(f)** ROC curve for the highest BA of (e). **(g)** Classification performance for healthy vs. stages I/II/III. **(h)** ROC curve for the highest BA of (h).

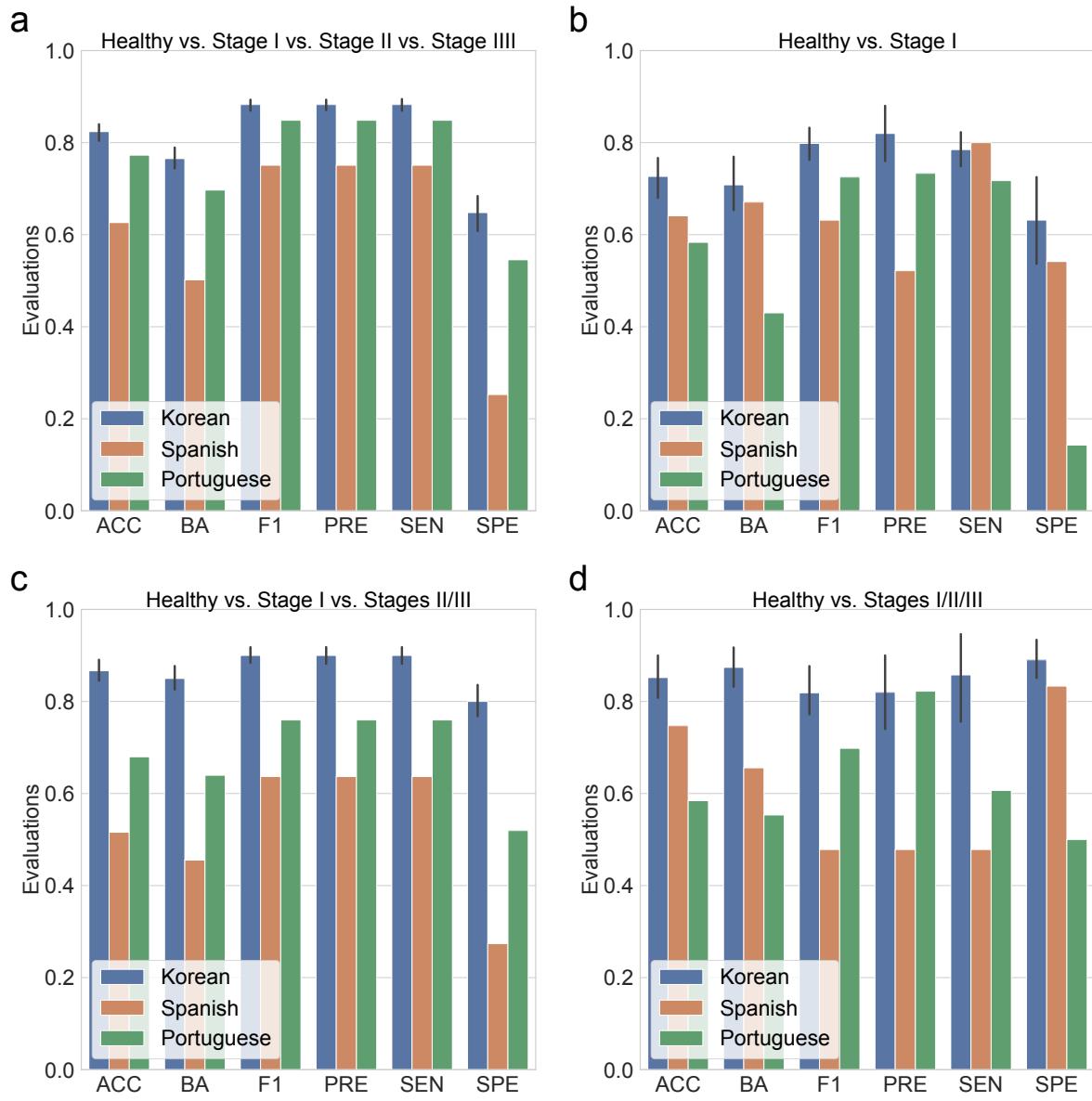


Figure 11: **Random forest classification metrics from external datasets.**

The classification metrics in the random forest classifications were as follows: ACC, AUC, BA, F1, PRE, SEN, and SPE. **(a)** Classification performance for healthy vs. stage I vs. stage II vs. stage III. **(b)** Classification performance for healthy vs. stage I. **(c)** Classification performance for healthy vs. stage I vs. stages II/III. **(d)** Classification performance for healthy vs. stages I/II/III.

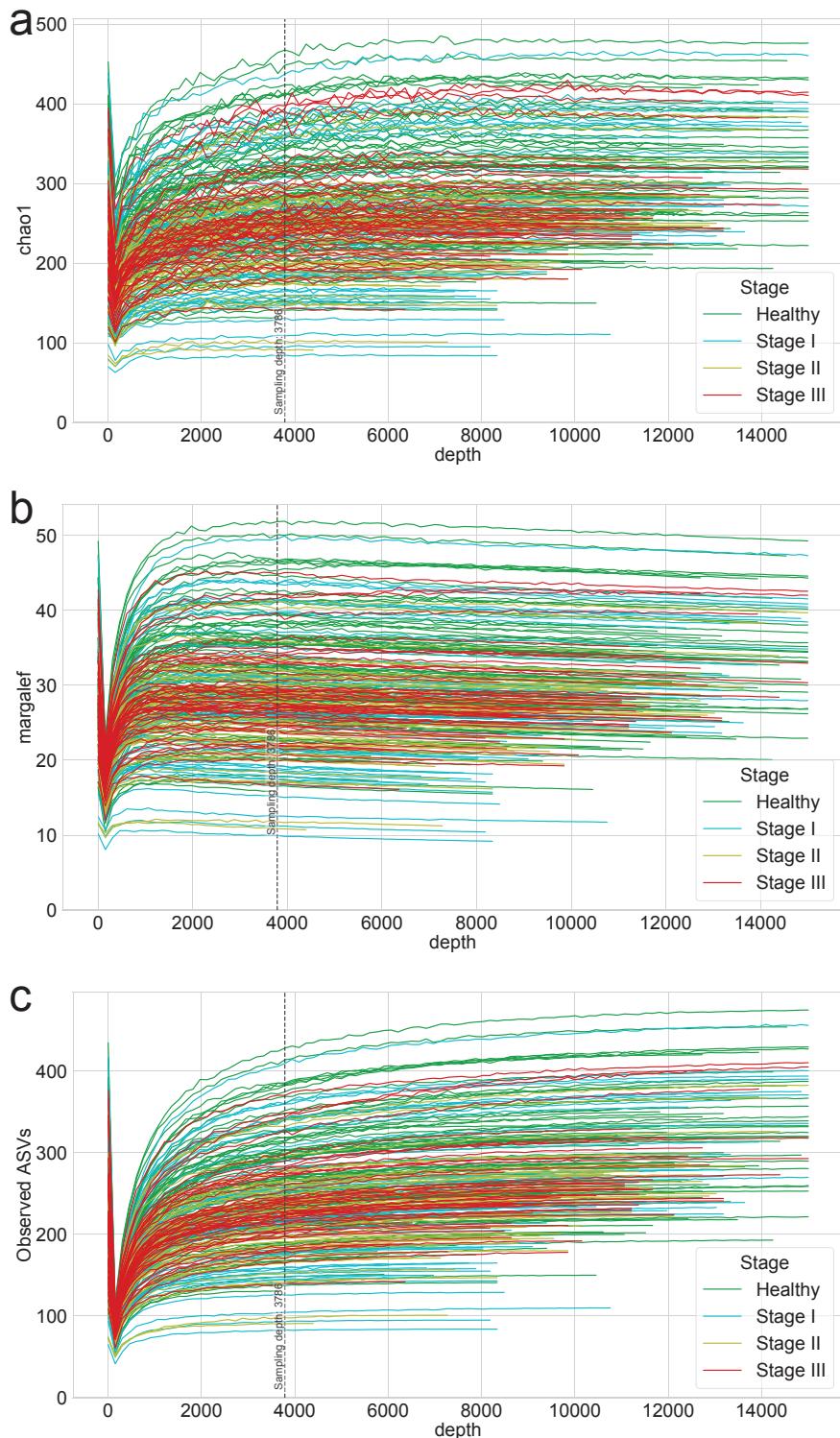


Figure 12: Rarefaction curves for alpha-diversity indices.

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

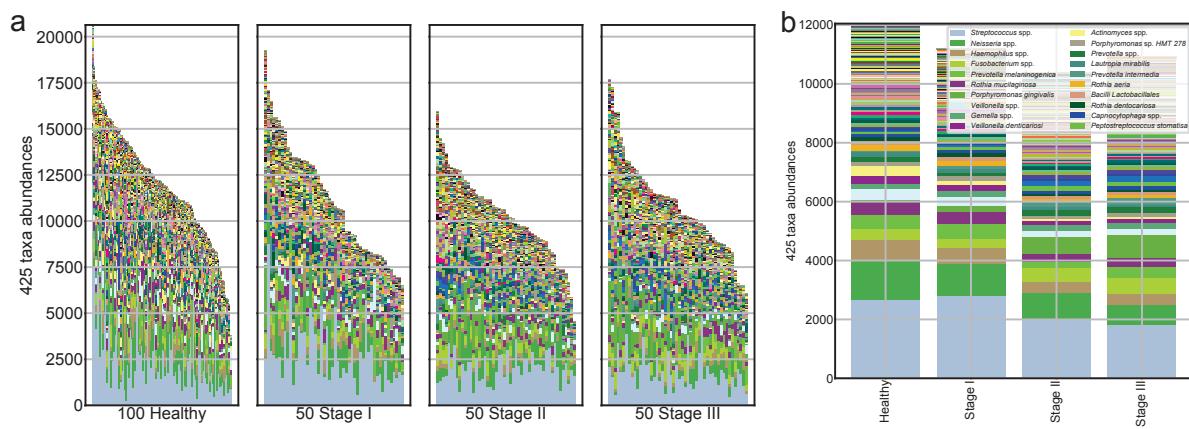


Figure 13: Salivary microbiome compositions in the different periodontal statuses.

Stacked bar plot of the absolute abundance of bacterial species for all samples (**a**) and the mean absolute abundance of bacterial species in the healthy, stage I, stage II, and stage III groups (**b**).

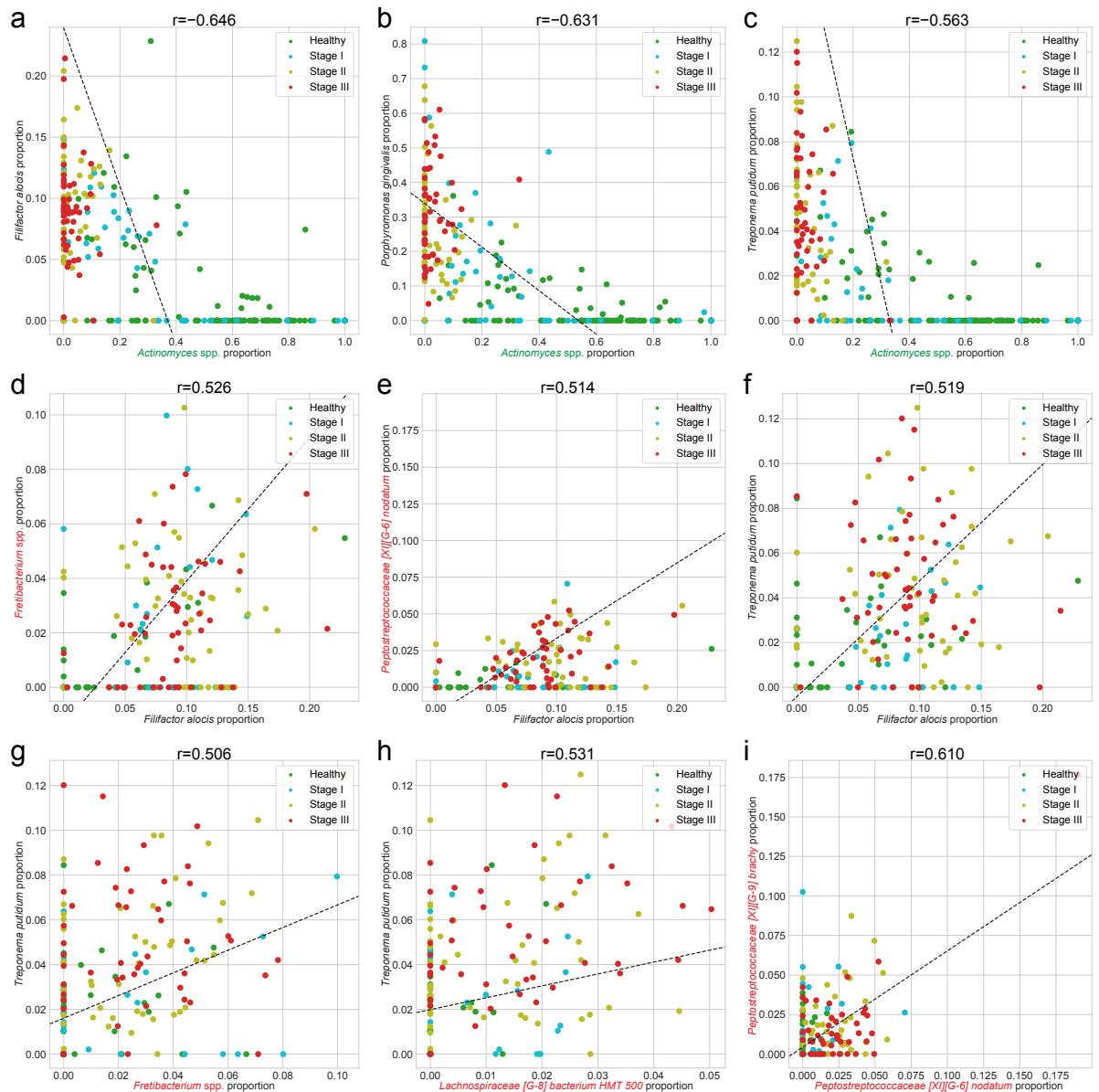


Figure 14: Correlation plots for differentially abundant taxa.

We selected the combinations of DAT with absolute Spearman correlation coefficients greater than 0.5. The color represents periodontal healthy periodontal statuses (green: healthy, cyan: stage I, yellow: stage II, and red: stage III).

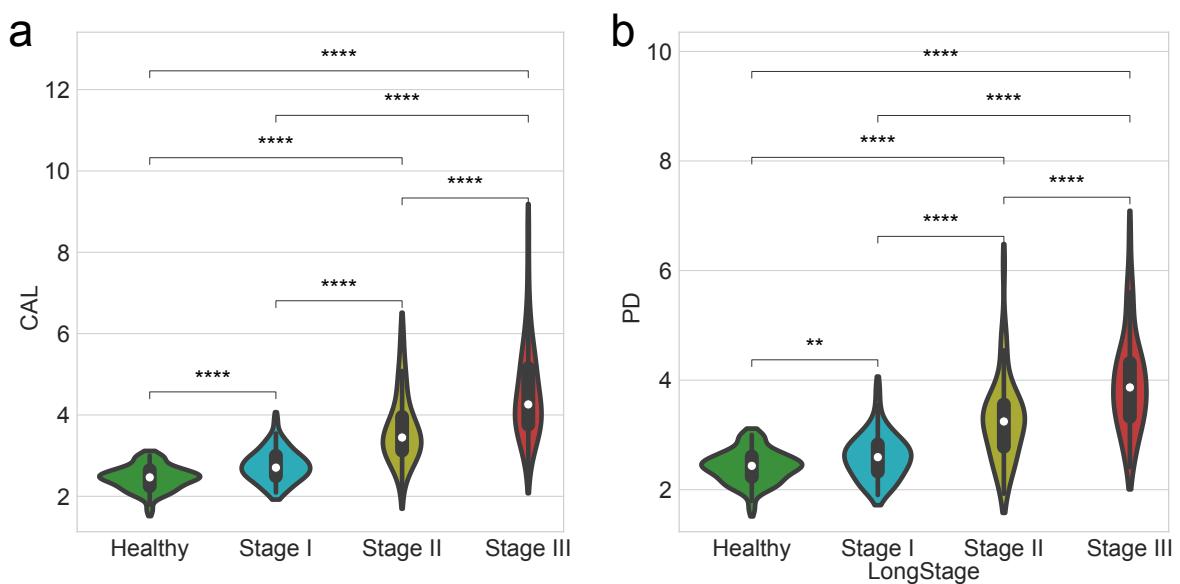


Figure 15: Clinical measurements by the periodontitis statuses.

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

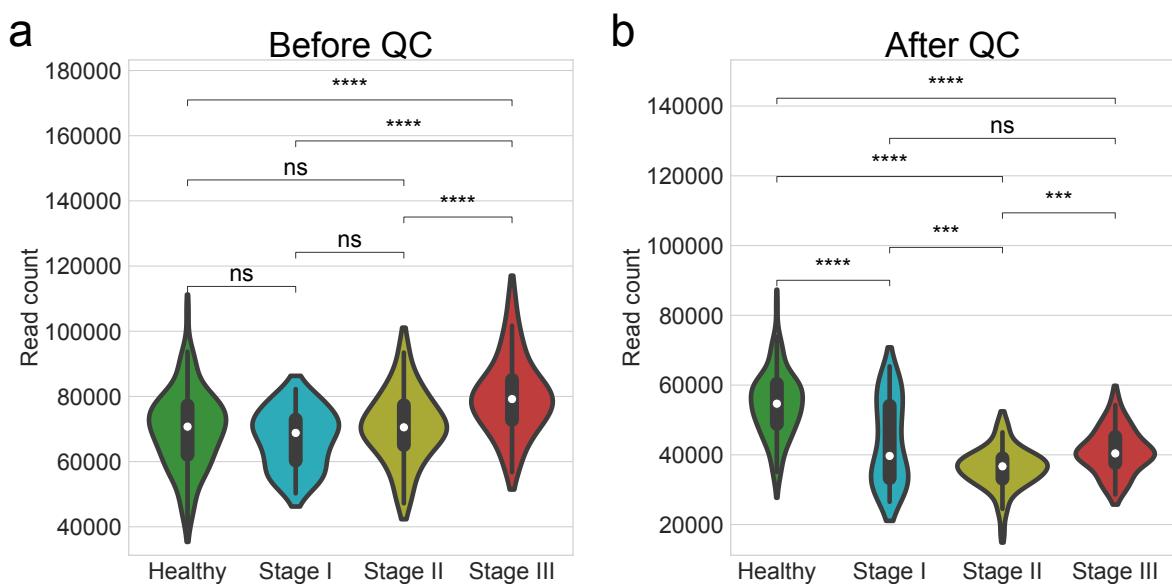


Figure 16: **Number of read counts by the periodontitis statuses.**

Comparisons of the number of read counts among healthy controls and patients with various periodontitis stages. **(a)** Before quality check **(b)** After quality check. Statistical significance determined by the MWU test: $p > 0.05$ (ns), $p \leq 0.001$ (***) , and $p \leq 0.0001$ (****).

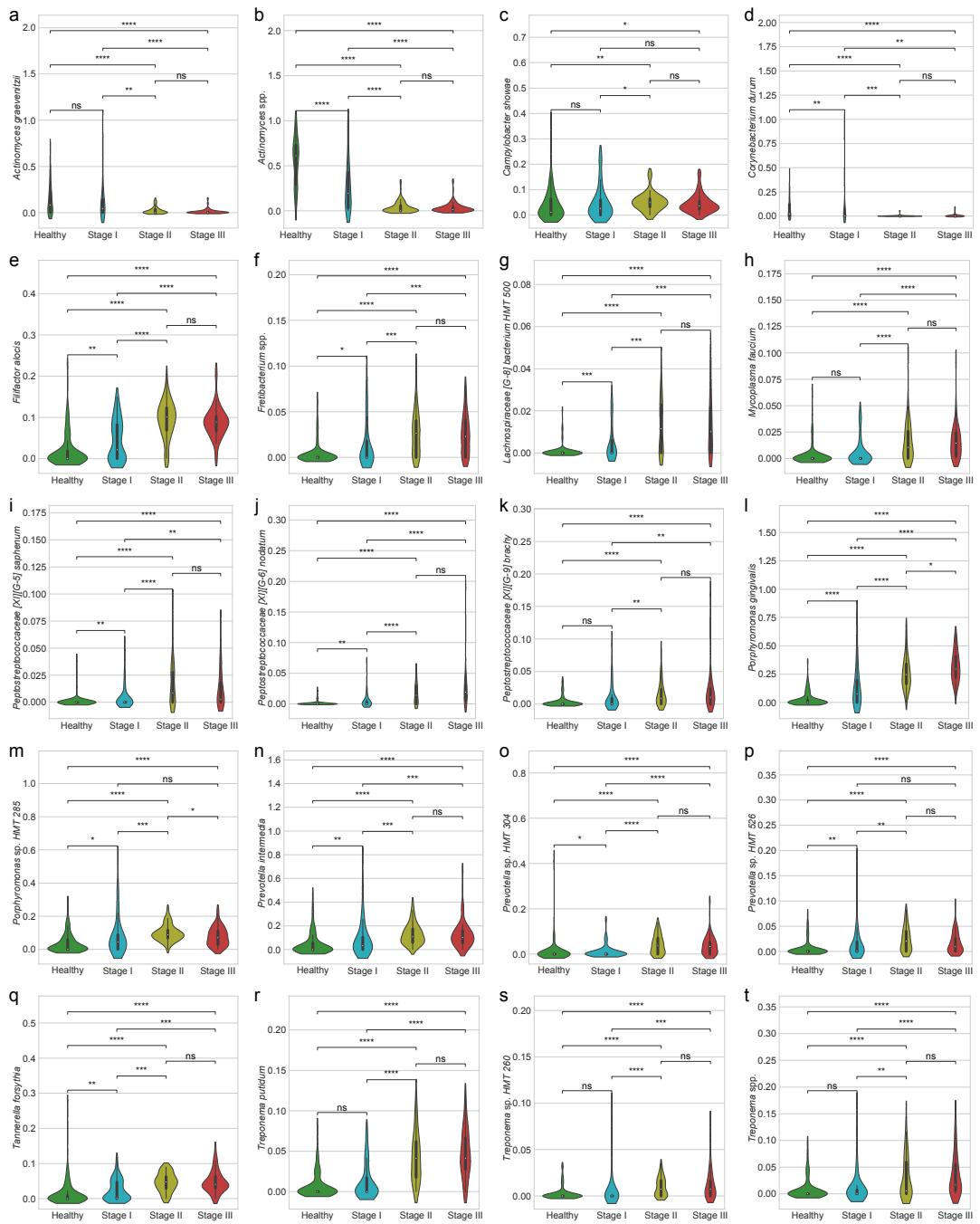


Figure 17: Proportion of DAT.

(a) *Actinomyces graevenitzii* (b) *Actinomyces* spp. (c) *Campylobacter showae* (d) *Corynebacterium durum* (e) *Filifactor alocis* (f) *Fretibacterium* spp. (g) *Lachnospiraceae [G-8] bacterium HMT 500* (h) *Mycoplasma faecium* (i) *Peptostreptococcaceae [XI][G-5] saphenum* (j) *Peptostreptococcaceae [XI][G-6] nodatum* (k) *Peptostreptococcaceae [XI][G-9] brachy* (l) *Porphyromonas gingivalis* (m) *Porphyromonas* sp. HMT 285 (n) *Prevotella intermedia* (o) *Prevotella* sp. HMT 304 (p) *Prevotella* sp. HMT 526 (q) *Tannerella forsythia* (r) *Treponema putidum* (s) *Treponema* sp. HMT 260 (t) *Treponema* spp. Statistical significance determined by the MWU test: $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), and $p \leq 0.0001$ (****).

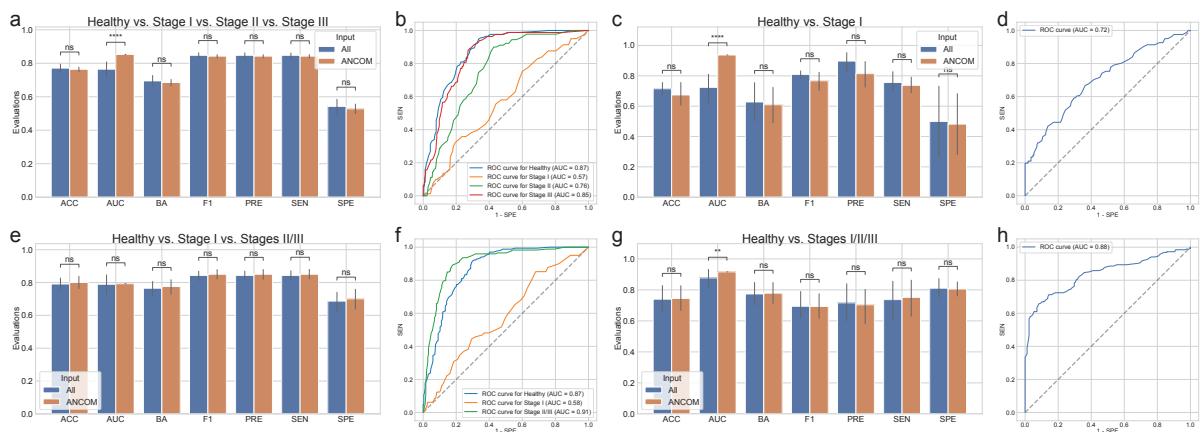


Figure 18: Random forest classification metrics with the full microbiome compositions and ANCOM-selected DAT compositions.

The classification metrics in the random forest classifications were as follows: ACC, AUC, BA, F1, PRE, SEN, and SPE. **(a)** Classification performance for healthy vs. stage I vs. stage II vs. stage III. **(b)** ROC curve for the highest BA of (a). **(c)** Classification performance for healthy vs. stage I. **(d)** ROC curve on the highest BA of (c). **(e)** Classification performance for healthy vs. stage I vs. stages II/III. **(f)** ROC curve for the highest BA of (e). **(g)** Classification performance for healthy vs. stages I/II/III. **(h)** ROC curve for the highest BA of (g). Statistical significance determined by the MWU test: $p > 0.05$ (ns), $p \leq 0.01$ (**), and $p \leq 0.0001$ (****).

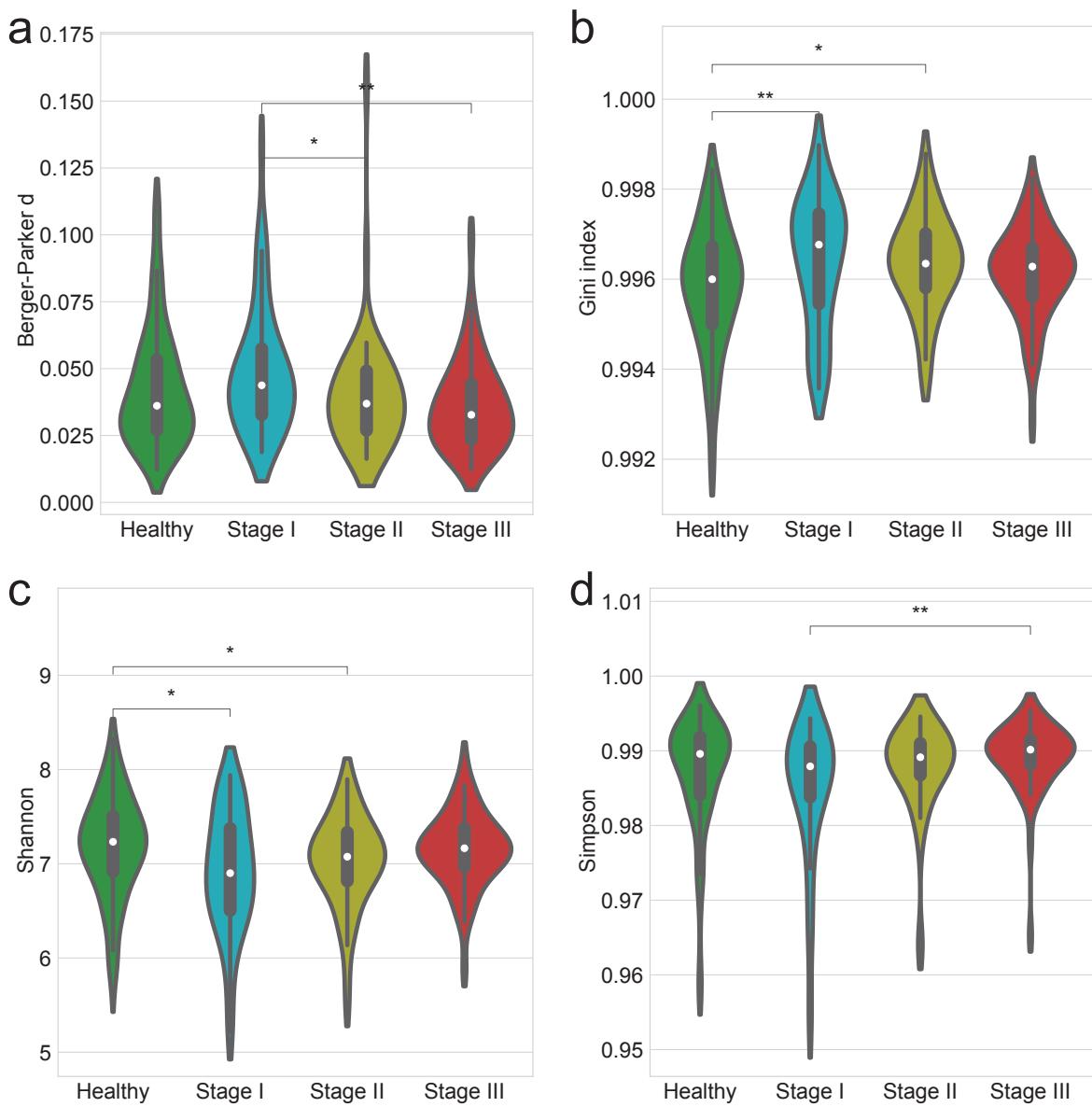


Figure 19: **Alpha-diversity indices account for evenness.**

Alpha-diversity indices (**a-d**) indicate that the heterogeneity between the periodontitis stages as measured by: **(a)** Berger-Parker *d* **(b)** Gini **(c)** Shannon **(d)** Simpson. Statistical significance determined by the MWU test: $p \leq 0.05$ (*) and $p \leq 0.01$ (**)

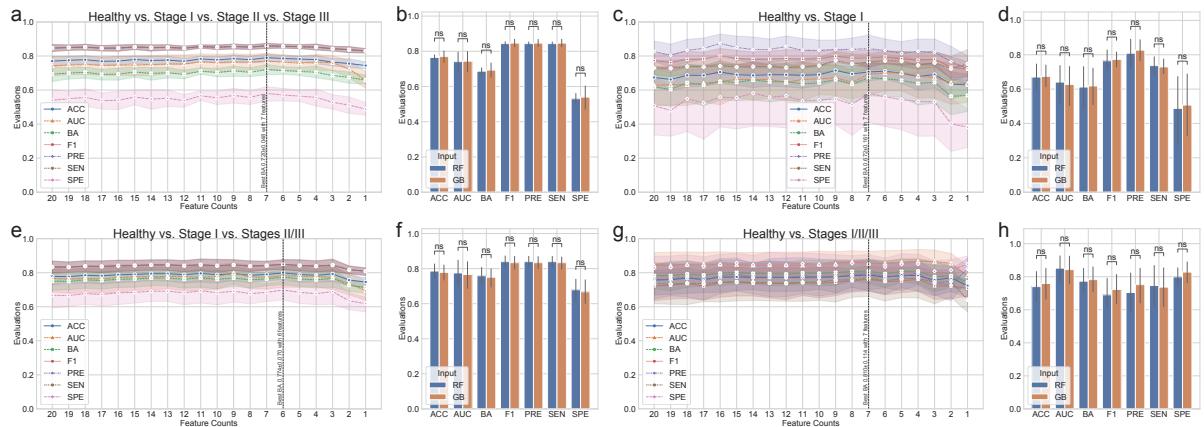


Figure 20: Gradient Boosting classification metrics.

The classification metrics in the random forest classifications were as follows: ACC, AUC, BA, F1, PRE, SEN, and SPE. The feature counts mean that the classification model trained on the most important n features as the Table 5. **(a)** Comparison of Random forest (RF) and Gradient boosting (GB) for healthy vs. stage I vs. stage II vs. stage III. **(b)** Comparison of RF and GB for the highest BA of (a). **(c)** Classification performance for healthy vs. stage I. **(d)** Comparison of RF and GB for healthy vs. stage I vs. stages II/III. **(e)** Comparison of RF and GB for the highest BA of (d). **(f)** Comparison of RF and GB for Healthy vs. Stage I vs. Stages II/III. **(g)** Classification performance for healthy vs. stages I/II/III. **(h)** Comparison of RF and GB for Healthy vs. Stages I/II/III.

728 **3.4 Discussion**

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

737 Previous research identified the red complex as the primary pathogens of periodontitis (Listgarten,
738 1986): *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. Other studies, however,
739 have shown that periodontal pathogens communicate with other bacteria in the salivary microbiome
740 networks to generate dental plaque prior to the pathogenesis and development of periodontitis (Lamont &
741 Jenkinson, 2000; Rosan & Lamont, 2000; Yoshimura, Murakami, Nishikawa, Hasegawa, & Kawaminami,
742 2009).

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

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

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

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

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

803 In our correlation analysis (Figure 9), we have discovered strongly negative correlations (coefficient \leq
804 -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%

- 844 • BA: 84%
845 • SEN: 94%
846 • SPE: 74%

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

- 850 • AUC: $95.3\% \pm 4.9\%$
851 • BA: $88.5\% \pm 6.6\%$
852 • SEN: $86.4\% \pm 15.7\%$
853 • SPE: $90.5\% \pm 7.0\%$

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

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

864 Regarding the clinical characteristics and potential confounders influencing the analysis of salivary
865 microbiome compositions connected with periodontitis severity, this study had a number of limitations
866 that were pointed out. We did not offer clinical information, such as the percentage of teeth, the percentage
867 of bleeding on probing, nor dental furcation involvement, even though we did gather information on
868 attachment level, probing depth, plaque index, and gingival index (Renvert & Persson, 2002); this might
869 have it challenging to present thorough and in-depth data about periodontal health. Moreover, the broad age
870 range may make it tougher to evaluate the relationship between age and periodontitis statuses, providing
871 the necessity for future studies to consider into account more comprehensive clinical characteristics
872 associated with periodontitis. Additionally, potential confounders—*e.g.* body mass index (Bombin, Yan,
873 Bombin, Mosley, & Ferguson, 2022) and e-cigarette use (Suzuki, Nakano, Yoneda, Hirofumi, & Hanioka,
874 2022)—which might have affected dental health and salivary microbiome composition were disregarding
875 consideration in addition to smoking status and systemic diseases. Thus, future research incorporating
876 these components would offer a more thorough knowledge of how lifestyle factors interact and affect the
877 salivary microbiome composition and periodontal health. Throughout, resolving these limitations will
878 advance our understanding in pathogenesis and development of periodontitis, offering significant novel
879 insights on the causal connection between systemic diseases and the salivary microbiome compositions.

880 4 Metagenomic signature analysis of Korean colorectal cancer

881 4.1 Introduction

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

895 The gut microbiome plays a fundamental role in maintaining host health by helping digestion
896 (Joscelyn & Kasper, 2014; Cerqueira, Photenhauer, Pollet, Brown, & Koropatkin, 2020), regulating
897 metabolism (Dabke, Hendrick, Devkota, et al., 2019; Utzschneider, Kratz, Damman, & Hullarg, 2016;
898 Magnúsdóttir & Thiele, 2018), adjusting immune function (Kau, Ahern, Griffin, Goodman, & Gordon,
899 2011; Shi, Li, Duan, & Niu, 2017; Broom & Kogut, 2018), and even coordinating neurological processes
900 by the brain-gut axis (Martin et al., 2018; Aziz & Thompson, 1998; R. Li et al., 2024). Comprising
901 these gut microbiota, including, archaea, bacteria, fungi, and viruses, the gut microbiome contributes
902 to the synthesis of essential vitamins, and production of fatty acids, which influence intestinal integrity
903 and immune responses. Thus, well-balanced gut microbiome composition modulates systemic immune
904 function by interacting with gut-associated lymphoid tissue, shaping immune tolerance and response
905 to infections. Hence, emerging evidence suggests that dysbiosis in the gut microbiome composition are
906 associated not only a narrow range of diseases, e.g. diarrhea and enteritis (Paganini & Zimmermann,
907 2017; Gao, Yin, Xu, Li, & Yin, 2019) but also a wide range of diseases, e.g. obesity, diabetes, and cancers
908 (Barlow et al., 2015; Hartstra et al., 2015; Helmink et al., 2019; Cullin et al., 2021).

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

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

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

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

940 **4.2 Materials and methods**

941 **4.2.1 Study participants enrollment**

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

957 **4.2.2 DNA extraction procedure**

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

967 **4.2.3 Bioinformatics analysis**

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

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

984 **4.2.4 Data and code availability**

985 All sequences from the 211 study participants have been published to the Korea Bioinformation Center
986 (data ID KGD10008857): <https://kbds.re.kr/KGD10008857>. Docker image that employed through-
987 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>.

990 **4.3 Results**

991 **4.3.1 Summary of clinical characteristics**

992 **4.3.2 Gut microbiome compositions**

993 **4.3.3 Diversity indices**

994 **4.3.4 DAT selection**

995 **4.3.5 Pathway prediction**

Table 8: Clinical characteristics of CRC study participants

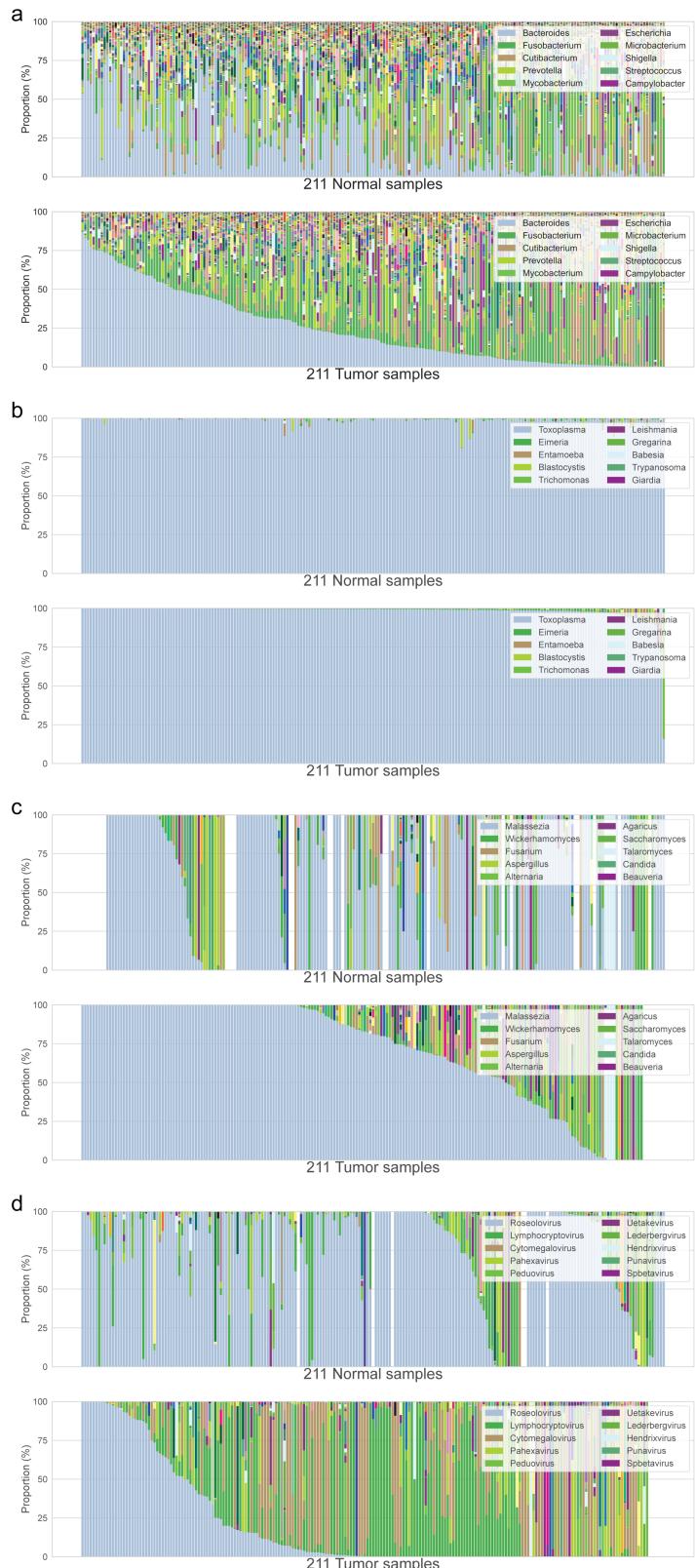


Figure 21: Gut microbiome compositions in genus level.

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

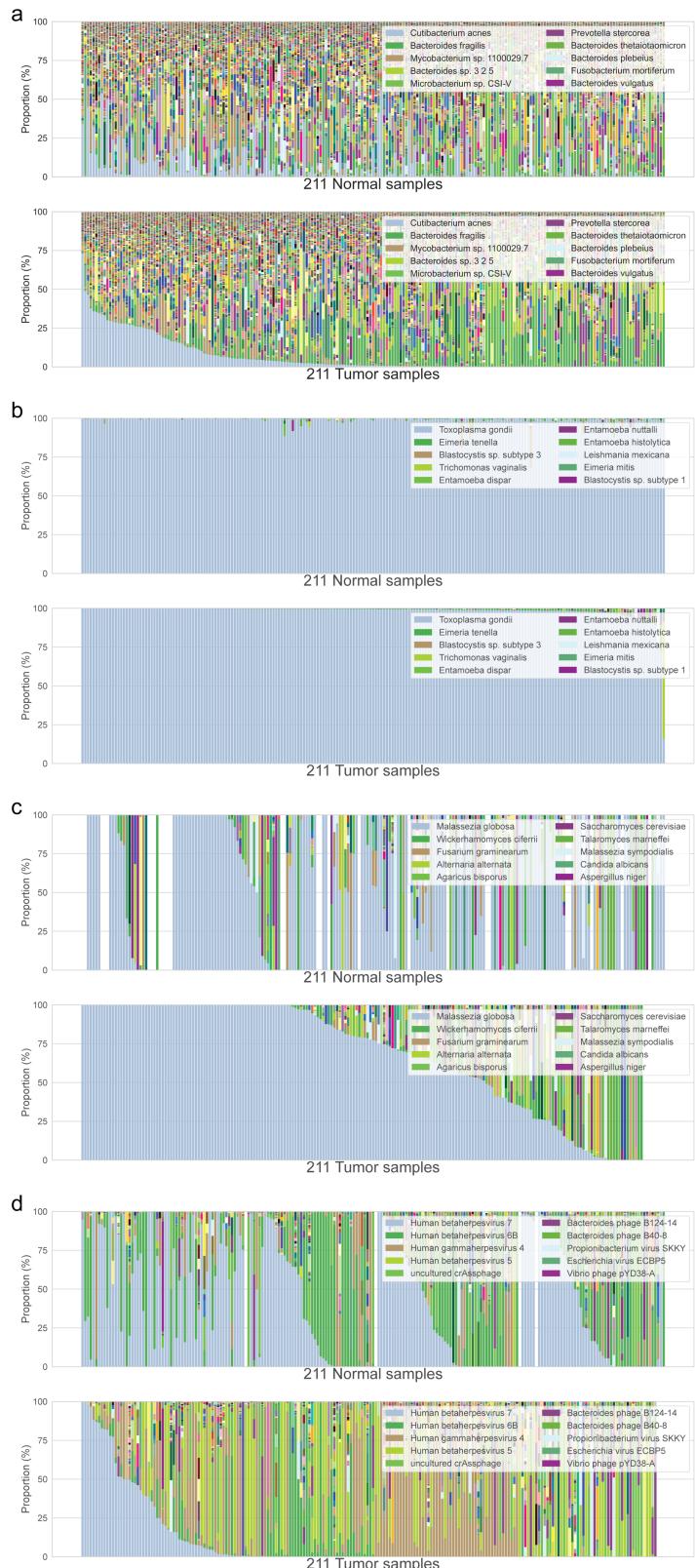


Figure 22: Gut microbiome compositions in species level.

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

996 **4.4 Discussion**

⁹⁹⁷ **5 Conclusion**

⁹⁹⁸ In conclusion, the research described in this doctoral dissertation was conducted to identify significant ...
⁹⁹⁹ In the section 2, I show that

1000 References

- 1001 Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., & Versalovic, J. (2014). The placenta harbors
1002 a unique microbiome. *Science translational medicine*, 6(237), 237ra65–237ra65.
- 1003 Abu-Ghazaleh, N., Chua, W. J., & Gopalan, V. (2021). Intestinal microbiota and its association with
1004 colon cancer and red/processed meat consumption. *Journal of gastroenterology and hepatology*,
1005 36(1), 75–88.
- 1006 Abusleme, L., Hoare, A., Hong, B.-Y., & Diaz, P. I. (2021). Microbial signatures of health, gingivitis,
1007 and periodontitis. *Periodontology 2000*, 86(1), 57–78.
- 1008 Aitchison, J., Barceló-Vidal, C., Martín-Fernández, J. A., & Pawlowsky-Glahn, V. (2000). Logratio
1009 analysis and compositional distance. *Mathematical geology*, 32, 271–275.
- 1010 Aja, E., Mangar, M., Fletcher, H., & Mishra, A. (2021). Filifactor alocis: recent insights and advances.
1011 *Journal of dental research*, 100(8), 790–797.
- 1012 Alelyani, S. (2021). Stable bagging feature selection on medical data. *Journal of Big Data*, 8(1), 11.
- 1013 Altabtbaei, K., Maney, P., Ganesan, S. M., Dabdoub, S. M., Nagaraja, H. N., & Kumar, P. S. (2021). Anna
1014 karenina and the subgingival microbiome associated with periodontitis. *Microbiome*, 9, 1–15.
- 1015 Altingöz, S. M., Kurgan, Ş., Önder, C., Serdar, M. A., Ünlütürk, U., Uyanık, M., ... Günhan, M.
1016 (2021). Salivary and serum oxidative stress biomarkers and advanced glycation end products in
1017 periodontitis patients with or without diabetes: A cross-sectional study. *Journal of periodontology*,
1018 92(9), 1274–1285.
- 1019 Alverdy, J., Hyoju, S., Weigerinck, M., & Gilbert, J. (2017). The gut microbiome and the mechanism of
1020 surgical infection. *Journal of British Surgery*, 104(2), e14–e23.
- 1021 An, S., & Park, S. (2022). Association of physical activity and sedentary behavior with the risk of
1022 colorectal cancer. *Journal of Korean Medical Science*, 37(19).
- 1023 Anderson, M. J. (2014). Permutational multivariate analysis of variance (permanova). *Wiley statsref:
1024 statistics reference online*, 1–15.
- 1025 Aruni, A. W., Mishra, A., Dou, Y., Chioma, O., Hamilton, B. N., & Fletcher, H. M. (2015). Filifactor
1026 alocis—a new emerging periodontal pathogen. *Microbes and infection*, 17(7), 517–530.
- 1027 Aziz, Q., & Thompson, D. G. (1998). Brain-gut axis in health and disease. *Gastroenterology*, 114(3),
1028 559–578.
- 1029 Bai, X., Wei, H., Liu, W., Coker, O. O., Gou, H., Liu, C., ... others (2022). Cigarette smoke promotes
1030 colorectal cancer through modulation of gut microbiota and related metabolites. *Gut*, 71(12),

- 1031 2439–2450.
- 1032 Baldelli, V., Scaldaferrri, F., Putignani, L., & Del Chierico, F. (2021). The role of enterobacteriaceae in
1033 gut microbiota dysbiosis in inflammatory bowel diseases. *Microorganisms*, 9(4), 697.
- 1034 Bardou, M., Rouland, A., Martel, M., Loffroy, R., Barkun, A. N., & Chapelle, N. (2022). Obesity and
1035 colorectal cancer. *Alimentary Pharmacology & Therapeutics*, 56(3), 407–418.
- 1036 Barlow, G. M., Yu, A., & Mathur, R. (2015). Role of the gut microbiome in obesity and diabetes mellitus.
1037 *Nutrition in clinical practice*, 30(6), 787–797.
- 1038 Basavaprabhu, H., Sonu, K., & Prabha, R. (2020). Mechanistic insights into the action of probiotics
1039 against bacterial vaginosis and its mediated preterm birth: An overview. *Microbial pathogenesis*,
1040 141, 104029.
- 1041 Belstrøm, D., Constancias, F., Drautz-Moses, D. I., Schuster, S. C., Veleba, M., Mahé, F., & Givskov, M.
1042 (2021). Periodontitis associates with species-specific gene expression of the oral microbiota. *npj
1043 Biofilms and Microbiomes*, 7(1), 76.
- 1044 Berger, W. H., & Parker, F. L. (1970). Diversity of planktonic foraminifera in deep-sea sediments.
1045 *Science*, 168(3937), 1345–1347.
- 1046 Berghella, V. (2012). Universal cervical length screening for prediction and prevention of preterm birth.
1047 *Obstetrical & gynecological survey*, 67(10), 653–657.
- 1048 Blencowe, H., Cousens, S., Oestergaard, M. Z., Chou, D., Moller, A.-B., Narwal, R., ... others (2012).
1049 National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends
1050 since 1990 for selected countries: a systematic analysis and implications. *The lancet*, 379(9832),
1051 2162–2172.
- 1052 Boland, C. R., & Goel, A. (2010). Microsatellite instability in colorectal cancer. *Gastroenterology*,
1053 138(6), 2073–2087.
- 1054 Boleij, A., Hechenbleikner, E. M., Goodwin, A. C., Badani, R., Stein, E. M., Lazarev, M. G., ... others
1055 (2015). The bacteroides fragilis toxin gene is prevalent in the colon mucosa of colorectal cancer
1056 patients. *Clinical Infectious Diseases*, 60(2), 208–215.
- 1057 Bolstad, A., Jensen, H. B., & Bakken, V. (1996). Taxonomy, biology, and periodontal aspects of
1058 fusobacterium nucleatum. *Clinical microbiology reviews*, 9(1), 55–71.
- 1059 Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., ... others
1060 (2019). Reproducible, interactive, scalable and extensible microbiome data science using qiime 2.
1061 *Nature biotechnology*, 37(8), 852–857.
- 1062 Bombin, A., Yan, S., Bombin, S., Mosley, J. D., & Ferguson, J. F. (2022). Obesity influences composition
1063 of salivary and fecal microbiota and impacts the interactions between bacterial taxa. *Physiological
1064 reports*, 10(7), e15254.
- 1065 Bonnet, M., Buc, E., Sauvanet, P., Darcha, C., Dubois, D., Pereira, B., ... Darfeuille-Michaud, A. (2014).
1066 Colonization of the human gut by e. coli and colorectal cancer risk. *Clinical Cancer Research*,
1067 20(4), 859–867.
- 1068 Breiman, L. (2001). Random forests. *Machine learning*, 45, 5–32.
- 1069 Brennan, C. A., & Garrett, W. S. (2019). Fusobacterium nucleatum—symbiont, opportunist and

- 1070 oncobacterium. *Nature Reviews Microbiology*, 17(3), 156–166.
- 1071 Broom, L. J., & Kogut, M. H. (2018). The role of the gut microbiome in shaping the immune system of
1072 chickens. *Veterinary immunology and immunopathology*, 204, 44–51.
- 1073 Bryll, R., Gutierrez-Osuna, R., & Quek, F. (2003). Attribute bagging: improving accuracy of classifier
1074 ensembles by using random feature subsets. *Pattern recognition*, 36(6), 1291–1302.
- 1075 Bullman, S., Pedamallu, C. S., Sicinska, E., Clancy, T. E., Zhang, X., Cai, D., ... others (2017). Analysis
1076 of fusobacterium persistence and antibiotic response in colorectal cancer. *Science*, 358(6369),
1077 1443–1448.
- 1078 Burt, R. W., Leppert, M. F., Slattery, M. L., Samowitz, W. S., Spirio, L. N., Kerber, R. A., ... others
1079 (2004). Genetic testing and phenotype in a large kindred with attenuated familial adenomatous
1080 polyposis. *Gastroenterology*, 127(2), 444–451.
- 1081 Cai, Y., Li, Y., Xiong, Y., Geng, X., Kang, Y., & Yang, Y. (2024). Diabetic foot exacerbates gut
1082 mycobiome dysbiosis in adult patients with type 2 diabetes mellitus: revealing diagnostic markers.
1083 *Nutrition & Diabetes*, 14(1), 71.
- 1084 Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016).
1085 Dada2: High-resolution sample inference from illumina amplicon data. *Nature methods*, 13(7),
1086 581–583.
- 1087 Canakci, V., & Canakci, C. F. (2007). Pain levels in patients during periodontal probing and mechanical
1088 non-surgical therapy. *Clinical oral investigations*, 11, 377–383.
- 1089 Cappellato, M., Baruzzo, G., & Di Camillo, B. (2022). Investigating differential abundance methods in
1090 microbiome data: A benchmark study. *PLoS computational biology*, 18(9), e1010467.
- 1091 Castaner, O., Goday, A., Park, Y.-M., Lee, S.-H., Magkos, F., Shiow, S.-A. T. E., & Schröder, H. (2018).
1092 The gut microbiome profile in obesity: a systematic review. *International journal of endocrinology*,
1093 2018(1), 4095789.
- 1094 Center, M. M., Jemal, A., Smith, R. A., & Ward, E. (2009). Worldwide variations in colorectal cancer.
1095 *CA: a cancer journal for clinicians*, 59(6), 366–378.
- 1096 Centor, R. M. (1991). Signal detectability: the use of roc curves and their analyses. *Medical decision
1097 making*, 11(2), 102–106.
- 1098 Cerqueira, F. M., Photenhauer, A. L., Pollet, R. M., Brown, H. A., & Koropatkin, N. M. (2020). Starch
1099 digestion by gut bacteria: crowdsourcing for carbs. *Trends in Microbiology*, 28(2), 95–108.
- 1100 Champagne, C., McNairn, H., Daneshfar, B., & Shang, J. (2014). A bootstrap method for assessing
1101 classification accuracy and confidence for agricultural land use mapping in canada. *International
1102 Journal of Applied Earth Observation and Geoinformation*, 29, 44–52.
- 1103 Chao, A. (1984). Nonparametric estimation of the number of classes in a population. *Scandinavian
1104 Journal of statistics*, 265–270.
- 1105 Chao, A., & Lee, S.-M. (1992). Estimating the number of classes via sample coverage. *Journal of the
1106 American statistical Association*, 87(417), 210–217.
- 1107 Chapple, I. L., Mealey, B. L., Van Dyke, T. E., Bartold, P. M., Dommisch, H., Eickholz, P., ... others
1108 (2018). Periodontal health and gingival diseases and conditions on an intact and a reduced

- periodontium: Consensus report of workgroup 1 of the 2017 world workshop on the classification of periodontal and peri-implant diseases and conditions. *Journal of periodontology*, 89, S74–S84.
- Chen, T., Marsh, P., & Al-Hebshi, N. (2022). Smdi: an index for measuring subgingival microbial dysbiosis. *Journal of dental research*, 101(3), 331–338.
- Chen, T., Yu, W.-H., Izard, J., Baranova, O. V., Lakshmanan, A., & Dewhirst, F. E. (2010). The human oral microbiome database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database*, 2010.
- Chen, X., D’Souza, R., & Hong, S.-T. (2013). The role of gut microbiota in the gut-brain axis: current challenges and perspectives. *Protein & cell*, 4, 403–414.
- Chen, X., Jansen, L., Guo, F., Hoffmeister, M., Chang-Claude, J., & Brenner, H. (2021). Smoking, genetic predisposition, and colorectal cancer risk. *Clinical and translational gastroenterology*, 12(3), e00317.
- Chen, X., Li, H., Guo, F., Hoffmeister, M., & Brenner, H. (2022). Alcohol consumption, polygenic risk score, and early-and late-onset colorectal cancer risk. *EClinicalMedicine*, 49.
- Chew, R. J. J., Tan, K. S., Chen, T., Al-Hebshi, N. N., & Goh, C. E. (2024). Quantifying periodontitis-associated oral dysbiosis in tongue and saliva microbiomes—an integrated data analysis. *Journal of Periodontology*.
- Čižmárová, B., Tomečková, V., Hubková, B., Hurajtová, A., Ohlasová, J., & Birková, A. (2022). Salivary redox homeostasis in human health and disease. *International Journal of Molecular Sciences*, 23(17), 10076.
- Cullin, N., Antunes, C. A., Straussman, R., Stein-Thoeringer, C. K., & Elinav, E. (2021). Microbiome and cancer. *Cancer Cell*, 39(10), 1317–1341.
- Dabke, K., Hendrick, G., Devkota, S., et al. (2019). The gut microbiome and metabolic syndrome. *The Journal of clinical investigation*, 129(10), 4050–4057.
- DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., … Andersen, G. L. (2006). Greengenes, a chimera-checked 16s rrna gene database and workbench compatible with arb. *Applied and environmental microbiology*, 72(7), 5069–5072.
- Doyle, R., Alber, D., Jones, H., Harris, K., Fitzgerald, F., Peebles, D., & Klein, N. (2014). Term and preterm labour are associated with distinct microbial community structures in placental membranes which are independent of mode of delivery. *Placenta*, 35(12), 1099–1101.
- Fahmy, C. A., Gamal-Eldeen, A. M., El-Hussieny, E. A., Raafat, B. M., Mehanna, N. S., Talaat, R. M., & Shaaban, M. T. (2019). Bifidobacterium longum suppresses murine colorectal cancer through the modulation of oncomirs and tumor suppressor mirnas. *Nutrition and cancer*, 71(4), 688–700.
- Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biological conservation*, 61(1), 1–10.
- Fettweis, J. M., Serrano, M. G., Brooks, J. P., Edwards, D. J., Girerd, P. H., Parikh, H. I., … others (2019). The vaginal microbiome and preterm birth. *Nature medicine*, 25(6), 1012–1021.
- Fisher, R. A., Corbet, A. S., & Williams, C. B. (1943). The relation between the number of species and the number of individuals in a random sample of an animal population. *The Journal of Animal*

- 1148 *Ecology*, 42–58.
- 1149 Flanagan, L., Schmid, J., Ebert, M., Soucek, P., Kunicka, T., Liska, V., ... others (2014). Fusobacterium
1150 nucleatum associates with stages of colorectal neoplasia development, colorectal cancer and disease
1151 outcome. *European journal of clinical microbiology & infectious diseases*, 33, 1381–1390.
- 1152 Fortenberry, J. D. (2013). The uses of race and ethnicity in human microbiome research. *Trends in
1153 microbiology*, 21(4), 165–166.
- 1154 Francescone, R., Hou, V., & Grivennikov, S. I. (2014). Microbiome, inflammation, and cancer. *The
1155 Cancer Journal*, 20(3), 181–189.
- 1156 Friedman, J. H. (2002). Stochastic gradient boosting. *Computational statistics & data analysis*, 38(4),
1157 367–378.
- 1158 Gambin, D. J., Vitali, F. C., De Carli, J. P., Mazzon, R. R., Gomes, B. P., Duque, T. M., & Trentin, M. S.
1159 (2021). Prevalence of red and orange microbial complexes in endodontic-periodontal lesions: a
1160 systematic review and meta-analysis. *Clinical Oral Investigations*, 1–14.
- 1161 Gao, J., Yin, J., Xu, K., Li, T., & Yin, Y. (2019). What is the impact of diet on nutritional diarrhea
1162 associated with gut microbiota in weaning piglets: a system review. *BioMed research international*,
1163 2019(1), 6916189.
- 1164 Ghanavati, R., Akbari, A., Mohammadi, F., Asadollahi, P., Javadi, A., Talebi, M., & Rohani, M. (2020).
1165 Lactobacillus species inhibitory effect on colorectal cancer progression through modulating the
1166 wnt/β-catenin signaling pathway. *Molecular and Cellular Biochemistry*, 470, 1–13.
- 1167 Ghorbani, E., Avan, A., Ryzhikov, M., Ferns, G., Khazaei, M., & Soleimanpour, S. (2022). Role of
1168 lactobacillus strains in the management of colorectal cancer: An overview of recent advances.
1169 *Nutrition*, 103, 111828.
- 1170 Gilbert, J. A., Blaser, M. J., Caporaso, J. G., Jansson, J. K., Lynch, S. V., & Knight, R. (2018). Current
1171 understanding of the human microbiome. *Nature medicine*, 24(4), 392–400.
- 1172 Gini, C. (1912). Variabilità e mutabilità (variability and mutability). *Tipografia di Paolo Cuppini,
1173 Bologna, Italy*, 156.
- 1174 Goldenberg, R. L., Culhane, J. F., Iams, J. D., & Romero, R. (2008). Epidemiology and causes of preterm
1175 birth. *The lancet*, 371(9606), 75–84.
- 1176 Gonçalves, L., Subtil, A., Oliveira, M. R., & de Zea Bermudez, P. (2014). Roc curve estimation: An
1177 overview. *REVSTAT-Statistical journal*, 12(1), 1–20.
- 1178 Goodyear, M. D., Krleza-Jeric, K., & Lemmens, T. (2007). *The declaration of helsinki* (Vol. 335) (No.
1179 7621). British Medical Journal Publishing Group.
- 1180 Haffajee, A., Teles, R., & Socransky, S. (2006). Association of eubacterium nodatum and treponema
1181 denticola with human periodontitis lesions. *Oral microbiology and immunology*, 21(5), 269–282.
- 1182 Hajishengallis, G. (2015). Periodontitis: from microbial immune subversion to systemic inflammation.
1183 *Nature reviews immunology*, 15(1), 30–44.
- 1184 Hamjane, N., Mechita, M. B., Nourouti, N. G., & Barakat, A. (2024). Gut microbiota dysbiosis-associated
1185 obesity and its involvement in cardiovascular diseases and type 2 diabetes. a systematic review.
1186 *Microvascular Research*, 151, 104601.

- 1187 Hamming, R. W. (1950). Error detecting and error correcting codes. *The Bell system technical journal*,
1188 29(2), 147–160.
- 1189 Hampel, H., Frankel, W. L., Martin, E., Arnold, M., Khanduja, K., Kuebler, P., ... others (2008).
1190 Feasibility of screening for lynch syndrome among patients with colorectal cancer. *Journal of
1191 Clinical Oncology*, 26(35), 5783–5788.
- 1192 Han, Y. W. (2015). Fusobacterium nucleatum: a commensal-turned pathogen. *Current opinion in
1193 microbiology*, 23, 141–147.
- 1194 Han, Y. W., & Wang, X. (2013). Mobile microbiome: oral bacteria in extra-oral infections and
1195 inflammation. *Journal of dental research*, 92(6), 485–491.
- 1196 Hand, D. J. (2012). Assessing the performance of classification methods. *International Statistical Review*,
1197 80(3), 400–414.
- 1198 Hartstra, A. V., Bouter, K. E., Bäckhed, F., & Nieuwdorp, M. (2015). Insights into the role of the
1199 microbiome in obesity and type 2 diabetes. *Diabetes care*, 38(1), 159–165.
- 1200 Hashemi Goradel, N., Heidarzadeh, S., Jahangiri, S., Farhood, B., Mortezaee, K., Khanlarkhani, N., &
1201 Negahdari, B. (2019). Fusobacterium nucleatum and colorectal cancer: A mechanistic overview.
1202 *Journal of Cellular Physiology*, 234(3), 2337–2344.
- 1203 Helmink, B. A., Khan, M. W., Hermann, A., Gopalakrishnan, V., & Wargo, J. A. (2019). The microbiome,
1204 cancer, and cancer therapy. *Nature medicine*, 25(3), 377–388.
- 1205 Hill, M. O. (1973). Diversity and evenness: a unifying notation and its consequences. *Ecology*, 54(2),
1206 427–432.
- 1207 Hiranmayi, K. V., Sirisha, K., Rao, M. R., & Sudhakar, P. (2017). Novel pathogens in periodontal
1208 microbiology. *Journal of Pharmacy and Bioallied Sciences*, 9(3), 155–163.
- 1209 Honda, K., & Littman, D. R. (2012). The microbiome in infectious disease and inflammation. *Annual
1210 review of immunology*, 30(1), 759–795.
- 1211 Honest, H., Forbes, C., Durée, K., Norman, G., Duffy, S., Tsourapas, A., ... others (2009). Screening to
1212 prevent spontaneous preterm birth: systematic reviews of accuracy and effectiveness literature with
1213 economic modelling. *Health Technol Assess*, 13(43), 1–627.
- 1214 Hong, Y. M., Lee, J., Cho, D. H., Jeon, J. H., Kang, J., Kim, M.-G., ... J. K. (2023). Predicting preterm
1215 birth using machine learning techniques in oral microbiome. *Scientific Reports*, 13(1), 21105.
- 1216 Hossin, M., & Sulaiman, M. N. (2015). A review on evaluation metrics for data classification evaluations.
1217 *International journal of data mining & knowledge management process*, 5(2), 1.
- 1218 Huang, R.-Y., Lin, C.-D., Lee, M.-S., Yeh, C.-L., Shen, E.-C., Chiang, C.-Y., ... Fu, E. (2007). Mandibular
1219 disto-lingual root: a consideration in periodontal therapy. *Journal of periodontology*, 78(8), 1485–
1220 1490.
- 1221 Iams, J. D., & Berghella, V. (2010). Care for women with prior preterm birth. *American journal of
1222 obstetrics and gynecology*, 203(2), 89–100.
- 1223 Ide, M., & Papapanou, P. N. (2013). Epidemiology of association between maternal periodontal
1224 disease and adverse pregnancy outcomes—systematic review. *Journal of clinical periodontology*,
1225 40, S181–S194.

- 1226 Iniesta, M., Chamorro, C., Ambrosio, N., Marín, M. J., Sanz, M., & Herrera, D. (2023). Subgingival
1227 microbiome in periodontal health, gingivitis and different stages of periodontitis. *Journal of*
1228 *Clinical Periodontology*, 50(7), 905–920.
- 1229 Inra, J. A., Steyerberg, E. W., Grover, S., McFarland, A., Syngal, S., & Kastrinos, F. (2015). Racial
1230 variation in frequency and phenotypes of apc and mutyh mutations in 6,169 individuals undergoing
1231 genetic testing. *Genetics in Medicine*, 17(10), 815–821.
- 1232 Janda, J. M., & Abbott, S. L. (2007). 16s rrna gene sequencing for bacterial identification in the diagnostic
1233 laboratory: pluses, perils, and pitfalls. *Journal of clinical microbiology*, 45(9), 2761–2764.
- 1234 Jiang, W., & Simon, R. (2007). A comparison of bootstrap methods and an adjusted bootstrap approach
1235 for estimating the prediction error in microarray classification. *Statistics in medicine*, 26(29),
1236 5320–5334.
- 1237 John, G. K., & Mullin, G. E. (2016). The gut microbiome and obesity. *Current oncology reports*, 18,
1238 1–7.
- 1239 Johnson, J. S., Spakowicz, D. J., Hong, B.-Y., Petersen, L. M., Demkowicz, P., Chen, L., . . . others (2019).
1240 Evaluation of 16s rrna gene sequencing for species and strain-level microbiome analysis. *Nature*
1241 *communications*, 10(1), 5029.
- 1242 Jorth, P., Turner, K. H., Gumus, P., Nizam, N., Buduneli, N., & Whiteley, M. (2014). Metatranscriptomics
1243 of the human oral microbiome during health and disease. *MBio*, 5(2), 10–1128.
- 1244 Joscelyn, J., & Kasper, L. H. (2014). Digesting the emerging role for the gut microbiome in central
1245 nervous system demyelination. *Multiple Sclerosis Journal*, 20(12), 1553–1559.
- 1246 Kang, Y., Kang, X., Yang, H., Liu, H., Yang, X., Liu, Q., . . . others (2022). Lactobacillus acidophilus ame-
1247 liorates obesity in mice through modulation of gut microbiota dysbiosis and intestinal permeability.
1248 *Pharmacological research*, 175, 106020.
- 1249 Karched, M., Bhardwaj, R. G., Qudeimat, M., Al-Khabbaz, A., & Ellepol, A. (2022). Proteomic analysis
1250 of the periodontal pathogen prevotella intermedia secretomes in biofilm and planktonic lifestyles.
1251 *Scientific Reports*, 12(1), 5636.
- 1252 Katz, J., Chegini, N., Shiverick, K., & Lamont, R. (2009). Localization of p. gingivalis in preterm delivery
1253 placenta. *Journal of dental research*, 88(6), 575–578.
- 1254 Kau, A. L., Ahern, P. P., Griffin, N. W., Goodman, A. L., & Gordon, J. I. (2011). Human nutrition, the
1255 gut microbiome and the immune system. *Nature*, 474(7351), 327–336.
- 1256 Kelly, B. J., Gross, R., Bittinger, K., Sherrill-Mix, S., Lewis, J. D., Collman, R. G., . . . Li, H. (2015).
1257 Power and sample-size estimation for microbiome studies using pairwise distances and permanova.
1258 *Bioinformatics*, 31(15), 2461–2468.
- 1259 Kennedy, J., Alexander, P., Taillie, L. S., & Jaacks, L. M. (2024). Estimated effects of reductions in
1260 processed meat consumption and unprocessed red meat consumption on occurrences of type 2
1261 diabetes, cardiovascular disease, colorectal cancer, and mortality in the usa: a microsimulation
1262 study. *The Lancet Planetary Health*, 8(7), e441–e451.
- 1263 Kim, B.-R., Shin, J., Guevarra, R. B., Lee, J. H., Kim, D. W., Seol, K.-H., . . . Isaacson, R. E. (2017).
1264 Deciphering diversity indices for a better understanding of microbial communities. *Journal of*

- 1265 *Microbiology and Biotechnology*, 27(12), 2089–2093.
- 1266 Kim, C. H. (2018). Immune regulation by microbiome metabolites. *Immunology*, 154(2), 220–229.
- 1267 Kim, E.-H., Kim, S., Kim, H.-J., Jeong, H.-o., Lee, J., Jang, J., ... others (2020). Prediction of chronic
1268 periodontitis severity using machine learning models based on salivary bacterial copy number.
1269 *Frontiers in Cellular and Infection Microbiology*, 10, 571515.
- 1270 Kim, J.-H. (2009). Estimating classification error rate: Repeated cross-validation, repeated hold-out and
1271 bootstrap. *Computational statistics & data analysis*, 53(11), 3735–3745.
- 1272 Kinane, D. F., Stathopoulou, P. G., & Papapanou, P. N. (2017). Periodontal diseases. *Nature reviews
1273 Disease primers*, 3(1), 1–14.
- 1274 Kindinger, L. M., Bennett, P. R., Lee, Y. S., Marchesi, J. R., Smith, A., Cacciato, S., ... MacIntyre,
1275 D. A. (2017). The interaction between vaginal microbiota, cervical length, and vaginal progesterone
1276 treatment for preterm birth risk. *Microbiome*, 5, 1–14.
- 1277 Kogut, M. H., Lee, A., & Santin, E. (2020). Microbiome and pathogen interaction with the immune
1278 system. *Poultry science*, 99(4), 1906–1913.
- 1279 Kostic, A. D., Ojesina, A. I., Pedamallu, C. S., Jung, J., Verhaak, R. G., Getz, G., & Meyerson, M. (2011).
1280 Pathseq: software to identify or discover microbes by deep sequencing of human tissue. *Nature
1281 biotechnology*, 29(5), 393–396.
- 1282 Kotsiantis, S. B., Zaharakis, I. D., & Pintelas, P. E. (2006). Machine learning: a review of classification
1283 and combining techniques. *Artificial Intelligence Review*, 26, 159–190.
- 1284 Kuipers, E. J., Grady, W. M., Lieberman, D., Seufferlein, T., Sung, J. J., Boelens, P. G., ... Watanabe, T.
1285 (2015). Colorectal cancer. *Nature reviews. Disease primers*, 1, 15065.
- 1286 Lafaurie, G. I., Neuta, Y., Ríos, R., Pacheco-Montealegre, M., Pianeta, R., Castillo, D. M., ... oth-
1287 ers (2022). Differences in the subgingival microbiome according to stage of periodontitis: A
1288 comparison of two geographic regions. *PLoS one*, 17(8), e0273523.
- 1289 Lamont, R. J., & Jenkinson, H. F. (2000). Subgingival colonization by porphyromonas gingivalis. *Oral
1290 Microbiology and Immunology: Mini-review*, 15(6), 341–349.
- 1291 Lamont, R. J., Koo, H., & Hajishengallis, G. (2018). The oral microbiota: dynamic communities and
1292 host interactions. *Nature reviews microbiology*, 16(12), 745–759.
- 1293 Leitich, H., & Kaider, A. (2003). Fetal fibronectin—how useful is it in the prediction of preterm birth?
1294 *BJOG: An International Journal of Obstetrics & Gynaecology*, 110, 66–70.
- 1295 Le Leu, R. K., Hu, Y., Brown, I. L., Woodman, R. J., & Young, G. P. (2010). Synbiotic intervention of
1296 bifidobacterium lactis and resistant starch protects against colorectal cancer development in rats.
1297 *Carcinogenesis*, 31(2), 246–251.
- 1298 León, R., Silva, N., Ovalle, A., Chaparro, A., Ahumada, A., Gajardo, M., ... Gamonal, J. (2007).
1299 Detection of porphyromonas gingivalis in the amniotic fluid in pregnant women with a diagnosis
1300 of threatened premature labor. *Journal of periodontology*, 78(7), 1249–1255.
- 1301 Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with burrows–wheeler transform.
1302 *bioinformatics*, 25(14), 1754–1760.
- 1303 Li, N., Lu, B., Luo, C., Cai, J., Lu, M., Zhang, Y., ... Dai, M. (2021). Incidence, mortality, survival,

- 1304 risk factor and screening of colorectal cancer: A comparison among china, europe, and northern
1305 america. *Cancer letters*, 522, 255–268.
- 1306 Li, R., Miao, Z., Liu, Y., Chen, X., Wang, H., Su, J., & Chen, J. (2024). The brain–gut–bone axis in
1307 neurodegenerative diseases: insights, challenges, and future prospects. *Advanced Science*, 11(38),
1308 2307971.
- 1309 Li, X., Yu, D., Wang, Y., Yuan, H., Ning, X., Rui, B., ... Li, M. (2021). The intestinal dysbiosis of
1310 mothers with gestational diabetes mellitus (gdm) and its impact on the gut microbiota of their
1311 newborns. *Canadian Journal of Infectious Diseases and Medical Microbiology*, 2021(1), 3044534.
- 1312 Li, Y., Qian, F., Cheng, X., Wang, D., Wang, Y., Pan, Y., ... Tian, Y. (2023). Dysbiosis of oral microbiota
1313 and metabolite profiles associated with type 2 diabetes mellitus. *Microbiology spectrum*, 11(1),
1314 e03796–22.
- 1315 Lim, J. W., Park, T., Tong, Y. W., & Yu, Z. (2020). The microbiome driving anaerobic digestion and
1316 microbial analysis. In *Advances in bioenergy* (Vol. 5, pp. 1–61). Elsevier.
- 1317 Lin, H., Eggesbø, M., & Peddada, S. D. (2022). Linear and nonlinear correlation estimators unveil
1318 undescribed taxa interactions in microbiome data. *Nature communications*, 13(1), 4946.
- 1319 Lin, H., & Peddada, S. D. (2020). Analysis of compositions of microbiomes with bias correction. *Nature
1320 communications*, 11(1), 3514.
- 1321 Lin, H., & Peddada, S. D. (2024). Multigroup analysis of compositions of microbiomes with covariate
1322 adjustments and repeated measures. *Nature Methods*, 21(1), 83–91.
- 1323 Listgarten, M. A. (1986). Pathogenesis of periodontitis. *Journal of clinical periodontology*, 13(5),
1324 418–425.
- 1325 Lloyd-Price, J., Abu-Ali, G., & Huttenhower, C. (2016). The healthy human microbiome. *Genome
1326 medicine*, 8, 1–11.
- 1327 López-Aladid, R., Fernández-Barat, L., Alcaraz-Serrano, V., Bueno-Freire, L., Vázquez, N., Pastor-
1328 Ibáñez, R., ... Torres, A. (2023). Determining the most accurate 16s rrna hypervariable region for
1329 taxonomic identification from respiratory samples. *Scientific reports*, 13(1), 3974.
- 1330 Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for
1331 rna-seq data with deseq2. *Genome biology*, 15, 1–21.
- 1332 Magnúsdóttir, S., & Thiele, I. (2018). Modeling metabolism of the human gut microbiome. *Current
1333 opinion in biotechnology*, 51, 90–96.
- 1334 Magurran, A. E. (2021). Measuring biological diversity. *Current Biology*, 31(19), R1174–R1177.
- 1335 Mandic, M., Safizadeh, F., Niedermaier, T., Hoffmeister, M., & Brenner, H. (2023). Association of
1336 overweight, obesity, and recent weight loss with colorectal cancer risk. *JAMA network Open*, 6(4),
1337 e239556–e239556.
- 1338 Mann, H. B., & Whitney, D. R. (1947). On a test of whether one of two random variables is stochastically
1339 larger than the other. *The annals of mathematical statistics*, 50–60.
- 1340 Manolis, A. A., Manolis, T. A., Melita, H., & Manolis, A. S. (2022). Gut microbiota and cardiovascular
1341 disease: symbiosis versus dysbiosis. *Current Medicinal Chemistry*, 29(23), 4050–4077.
- 1342 Martin, C. R., Osadchiy, V., Kalani, A., & Mayer, E. A. (2018). The brain-gut-microbiome axis. *Cellular*

- 1343 and molecular gastroenterology and hepatology, 6(2), 133–148.
- 1344 Mayer, E. A., Tillisch, K., Gupta, A., et al. (2015). Gut/brain axis and the microbiota. *The Journal of*
1345 *clinical investigation*, 125(3), 926–938.
- 1346 Melguizo-Rodríguez, L., Costela-Ruiz, V. J., Manzano-Moreno, F. J., Ruiz, C., & Illescas-Montes, R.
1347 (2020). Salivary biomarkers and their application in the diagnosis and monitoring of the most
1348 common oral pathologies. *International journal of molecular sciences*, 21(14), 5173.
- 1349 Merrill, L. C., & Mangano, K. M. (2023). Racial and ethnic differences in studies of the gut microbiome
1350 and osteoporosis. *Current Osteoporosis Reports*, 21(5), 578–591.
- 1351 Miller, C. S., Ding, X., Dawson III, D. R., & Ebersole, J. L. (2021). Salivary biomarkers for discriminating
1352 periodontitis in the presence of diabetes. *Journal of clinical periodontology*, 48(2), 216–225.
- 1353 Morita, T., Yamazaki, Y., Mita, A., Takada, K., Seto, M., Nishinoue, N., ... Maeno, M. (2010). A cohort
1354 study on the association between periodontal disease and the development of metabolic syndrome.
1355 *Journal of periodontology*, 81(4), 512–519.
- 1356 Na, H. S., Kim, S. Y., Han, H., Kim, H.-J., Lee, J.-Y., Lee, J.-H., & Chung, J. (2020). Identification of
1357 potential oral microbial biomarkers for the diagnosis of periodontitis. *Journal of clinical medicine*,
1358 9(5), 1549.
- 1359 Nemoto, T., Shiba, T., Komatsu, K., Watanabe, T., Shimogishi, M., Shibasaki, M., ... others (2021).
1360 Discrimination of bacterial community structures among healthy, gingivitis, and periodontitis
1361 statuses through integrated metatranscriptomic and network analyses. *Msystems*, 6(6), e00886–21.
- 1362 Nesbitt, M. J., Reynolds, M. A., Shiau, H., Choe, K., Simonsick, E. M., & Ferrucci, L. (2010). Association
1363 of periodontitis and metabolic syndrome in the baltimore longitudinal study of aging. *Aging clinical*
1364 *and experimental research*, 22, 238–242.
- 1365 Network, C. G. A., et al. (2012). Comprehensive molecular characterization of human colon and rectal
1366 cancer. *Nature*, 487(7407), 330.
- 1367 Nibali, L., Sousa, V., Davrandi, M., Spratt, D., Alyahya, Q., Dopico, J., & Donos, N. (2020). Differences
1368 in the periodontal microbiome of successfully treated and persistent aggressive periodontitis.
1369 *Journal of Clinical Periodontology*, 47(8), 980–990.
- 1370 Novaković, J. D., Veljović, A., Ilić, S. S., Papić, Ž., & Tomović, M. (2017). Evaluation of classification
1371 models in machine learning. *Theory and Applications of Mathematics & Computer Science*, 7(1),
1372 39.
- 1373 Obuchowski, N. A., & Bullen, J. A. (2018). Receiver operating characteristic (roc) curves: review of
1374 methods with applications in diagnostic medicine. *Physics in Medicine & Biology*, 63(7), 07TR01.
- 1375 Offenbacher, S., Katz, V., Fertik, G., Collins, J., Boyd, D., Maynor, G., ... Beck, J. (1996). Periodontal
1376 infection as a possible risk factor for preterm low birth weight. *Journal of periodontology*, 67,
1377 1103–1113.
- 1378 Ojesina, A. I., Pedamallu, C. S., Kostic, A., Jung, J., Auclair, D., Lohr, J., ... Meyerson, M. (2013). High
1379 throughput sequencing-based pathogen discovery in multiple myeloma. *Blood*, 122(21), 5322.
- 1380 Omundiagbe, D. A., Veeramani, S., & Sidhu, A. S. (2019). Machine learning classification techniques
1381 for breast cancer diagnosis. In *Iop conference series: materials science and engineering* (Vol. 495,

- 1382 p. 012033).
- 1383 O'Sullivan, D. E., Sutherland, R. L., Town, S., Chow, K., Fan, J., Forbes, N., ... Brenner, D. R. (2022).
1384 Risk factors for early-onset colorectal cancer: a systematic review and meta-analysis. *Clinical*
1385 *gastroenterology and hepatology*, 20(6), 1229–1240.
- 1386 Paganini, D., & Zimmermann, M. B. (2017). The effects of iron fortification and supplementation on the
1387 gut microbiome and diarrhea in infants and children: a review. *The American journal of clinical*
1388 *nutrition*, 106, 1688S–1693S.
- 1389 Pan, A. Y. (2021). Statistical analysis of microbiome data: the challenge of sparsity. *Current Opinion in*
1390 *Endocrine and Metabolic Research*, 19, 35–40.
- 1391 Papapanou, P. N., Sanz, M., Buduneli, N., Dietrich, T., Feres, M., Fine, D. H., ... others (2018).
1392 Periodontitis: Consensus report of workgroup 2 of the 2017 world workshop on the classification of
1393 periodontal and peri-implant diseases and conditions. *Journal of periodontology*, 89, S173–S182.
- 1394 Parizadeh, M., & Arrieta, M.-C. (2023). The global human gut microbiome: genes, lifestyles, and diet.
1395 *Trends in Molecular Medicine*.
- 1396 Park, J., Park, S. H., Lee, D., Lee, J. E., Lee, D., Na, K. J., ... Im, H.-J. (2024). Detecting cancer microbiota
1397 using unmapped rna reads on spatial transcriptomics. *Cancer Research*, 84(6_Supplement), 4881–
1398 4881.
- 1399 Payne, M. S., Newnham, J. P., Doherty, D. A., Furfarro, L. L., Pendal, N. L., Loh, D. E., & Keelan, J. A.
1400 (2021). A specific bacterial dna signature in the vagina of australian women in midpregnancy
1401 predicts high risk of spontaneous preterm birth (the predict1000 study). *American journal of*
1402 *obstetrics and gynecology*, 224(2), 206–e1.
- 1403 Peirce, J. M., & Alviña, K. (2019). The role of inflammation and the gut microbiome in depression and
1404 anxiety. *Journal of neuroscience research*, 97(10), 1223–1241.
- 1405 Peltomaki, P. (2003). Role of dna mismatch repair defects in the pathogenesis of human cancer. *Journal*
1406 *of clinical oncology*, 21(6), 1174–1179.
- 1407 Pezzino, S., Sofia, M., Greco, L. P., Litrico, G., Filippello, G., Sarvà, I., ... Latteri, S. (2023). Microbiome
1408 dysbiosis: a pathological mechanism at the intersection of obesity and glaucoma. *International*
1409 *Journal of Molecular Sciences*, 24(2), 1166.
- 1410 Premaraj, T. S., Vella, R., Chung, J., Lin, Q., Hunter, P., Underwood, K., ... Zhou, Y. (2020). Ethnic
1411 variation of oral microbiota in children. *Scientific reports*, 10(1), 14788.
- 1412 Raut, J. R., Schöttker, B., Holleczeck, B., Guo, F., Bhardwaj, M., Miah, K., ... Brenner, H. (2021).
1413 A microrna panel compared to environmental and polygenic scores for colorectal cancer risk
1414 prediction. *Nature Communications*, 12(1), 4811.
- 1415 Rebersek, M. (2021). Gut microbiome and its role in colorectal cancer. *BMC cancer*, 21(1), 1325.
- 1416 Redanz, U., Redanz, S., Treerat, P., Prakasam, S., Lin, L.-J., Merritt, J., & Kreth, J. (2021). Differential
1417 response of oral mucosal and gingival cells to corynebacterium durum, streptococcus sanguinis, and
1418 porphyromonas gingivalis multispecies biofilms. *Frontiers in cellular and infection microbiology*,
1419 11, 686479.
- 1420 Relvas, M., Regueira-Iglesias, A., Balsa-Castro, C., Salazar, F., Pacheco, J., Cabral, C., ... Tomás, I.

- 1421 (2021). Relationship between dental and periodontal health status and the salivary microbiome:
1422 bacterial diversity, co-occurrence networks and predictive models. *Scientific reports*, 11(1), 929.
- 1423 Renson, A., Jones, H. E., Beghini, F., Segata, N., Zolnik, C. P., Usyk, M., ... others (2019). Sociodemographic variation in the oral microbiome. *Annals of epidemiology*, 35, 73–80.
- 1425 Renvert, S., & Persson, G. (2002). A systematic review on the use of residual probing depth, bleeding on
1426 probing and furcation status following initial periodontal therapy to predict further attachment and
1427 tooth loss. *Journal of clinical periodontology*, 29, 82–89.
- 1428 Rideout, J. R., Caporaso, G., Bolyen, E., McDonald, D., Baeza, Y. V., Alastuey, J. C., ... Sharma, K.
1429 (2018, December). *biocore/scikit-bio: scikit-bio 0.5.5: More compositional methods added*. Zenodo.
1430 Retrieved from <https://doi.org/10.5281/zenodo.2254379> doi: 10.5281/zenodo.2254379
- 1431 Rôças, I. N., Siqueira Jr, J. F., Santos, K. R., Coelho, A. M., & de Janeiro, R. (2001). “red complex”(*bacteroides forsythus*, *porphyromonas gingivalis*, and *treponema denticola*) in endodontic
1432 infections: a molecular approach. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology,*
1433 *and Endodontology*, 91(4), 468–471.
- 1435 Romero, R., Dey, S. K., & Fisher, S. J. (2014). Preterm labor: one syndrome, many causes. *Science*,
1436 345(6198), 760–765.
- 1437 Romero, R., Hassan, S. S., Gajer, P., Tarca, A. L., Fadrosh, D. W., Nikita, L., ... others (2014). The
1438 composition and stability of the vaginal microbiota of normal pregnant women is different from
1439 that of non-pregnant women. *Microbiome*, 2, 1–19.
- 1440 Rosan, B., & Lamont, R. J. (2000). Dental plaque formation. *Microbes and infection*, 2(13), 1599–1607.
- 1441 Schwabe, R. F., & Jobin, C. (2013). The microbiome and cancer. *Nature Reviews Cancer*, 13(11),
1442 800–812.
- 1443 Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011).
1444 Metagenomic biomarker discovery and explanation. *Genome biology*, 12, 1–18.
- 1445 Sen, P. C., Hajra, M., & Ghosh, M. (2020). Supervised classification algorithms in machine learning: A
1446 survey and review. In *Emerging technology in modelling and graphics: Proceedings of iem graph*
1447 2018 (pp. 99–111).
- 1448 Sepich-Poore, G. D., Zitvogel, L., Straussman, R., Hasty, J., Wargo, J. A., & Knight, R. (2021). The
1449 microbiome and human cancer. *Science*, 371(6536), eabc4552.
- 1450 Sharma, S., & Tripathi, P. (2019). Gut microbiome and type 2 diabetes: where we are and where to go?
1451 *The Journal of nutritional biochemistry*, 63, 101–108.
- 1452 Shi, N., Li, N., Duan, X., & Niu, H. (2017). Interaction between the gut microbiome and mucosal
1453 immune system. *Military Medical Research*, 4, 1–7.
- 1454 Simpson, E. (1949). Measurement of diversity. *Nature*, 163.
- 1455 Song, M., Chan, A. T., & Sun, J. (2020). Influence of the gut microbiome, diet, and environment on risk
1456 of colorectal cancer. *Gastroenterology*, 158(2), 322–340.
- 1457 Söreide, K., Janssen, E., Söiland, H., Körner, H., & Baak, J. (2006). Microsatellite instability in colorectal
1458 cancer. *Journal of British Surgery*, 93(4), 395–406.
- 1459 Sotiriadis, A., Papatheodorou, S., Kavvadias, A., & Makrydimas, G. (2010). Transvaginal cervical

- length measurement for prediction of preterm birth in women with threatened preterm labor: a meta-analysis. *Ultrasound in Obstetrics and Gynecology: The Official Journal of the International Society of Ultrasound in Obstetrics and Gynecology*, 35(1), 54–64.
- Spss, I., et al. (2011). Ibm spss statistics for windows, version 20.0. *New York: IBM Corp*, 440, 394.
- Stafford, G., Roy, S., Honma, K., & Sharma, A. (2012). Sialic acid, periodontal pathogens and tannerella forsythia: stick around and enjoy the feast! *Molecular Oral Microbiology*, 27(1), 11–22.
- Stout, M. J., Conlon, B., Landeau, M., Lee, I., Bower, C., Zhao, Q., ... Mysorekar, I. U. (2013). Identification of intracellular bacteria in the basal plate of the human placenta in term and preterm gestations. *American journal of obstetrics and gynecology*, 208(3), 226–e1.
- Sultan, S., El-Mowafy, M., Elgaml, A., Ahmed, T. A., Hassan, H., & Mottawea, W. (2021). Metabolic influences of gut microbiota dysbiosis on inflammatory bowel disease. *Frontiers in physiology*, 12, 715506.
- Suzuki, N., Nakano, Y., Yoneda, M., Hirofumi, T., & Hanioka, T. (2022). The effects of cigarette smoking on the salivary and tongue microbiome. *Clinical and Experimental Dental Research*, 8(1), 449–456.
- Swidsinski, A., Khilkin, M., Kerjaschki, D., Schreiber, S., Ortner, M., Weber, J., & Lochs, H. (1998). Association between intraepithelial escherichia coli and colorectal cancer. *Gastroenterology*, 115(2), 281–286.
- Swift, D., Cresswell, K., Johnson, R., Stilianoudakis, S., & Wei, X. (2023). A review of normalization and differential abundance methods for microbiome counts data. *Wiley Interdisciplinary Reviews: Computational Statistics*, 15(1), e1586.
- Tanner, A. C., Kent Jr, R., Kanasi, E., Lu, S. C., Paster, B. J., Sonis, S. T., ... Van Dyke, T. E. (2007). Clinical characteristics and microbiota of progressing slight chronic periodontitis in adults. *Journal of clinical periodontology*, 34(11), 917–930.
- Tanner, A. C., Paster, B. J., Lu, S. C., Kanasi, E., Kent Jr, R., Van Dyke, T., & Sonis, S. T. (2006). Subgingival and tongue microbiota during early periodontitis. *Journal of dental research*, 85(4), 318–323.
- Tejeda, M., Farrell, J., Zhu, C., Haines, J. L., Wang, L.-S., Schellenberg, G. D., ... others (2021). Multiple viruses detected in human dna are associated with alzheimer disease risk. *Alzheimer's & Dementia*, 17, e054585.
- Teles, F., Wang, Y., Hajishengallis, G., Hasturk, H., & Marchesan, J. T. (2021). Impact of systemic factors in shaping the periodontal microbiome. *Periodontology 2000*, 85(1), 126–160.
- Thaiss, C. A., Zmora, N., Levy, M., & Elinav, E. (2016). The microbiome and innate immunity. *Nature*, 535(7610), 65–74.
- Tian, R., Liu, H., Feng, S., Wang, H., Wang, Y., Wang, Y., ... Zhang, S. (2021). Gut microbiota dysbiosis in stable coronary artery disease combined with type 2 diabetes mellitus influences cardiovascular prognosis. *Nutrition, Metabolism and Cardiovascular Diseases*, 31(5), 1454–1466.
- Tilg, H., Kaser, A., et al. (2011). Gut microbiome, obesity, and metabolic dysfunction. *The Journal of clinical investigation*, 121(6), 2126–2132.

- 1499 Tonetti, M. S., Greenwell, H., & Kornman, K. S. (2018). Staging and grading of periodontitis: Framework
1500 and proposal of a new classification and case definition. *Journal of periodontology*, 89, S159–S172.
- 1501 Tringe, S. G., & Hugenholtz, P. (2008). A renaissance for the pioneering 16s rRNA gene. *Current opinion*
1502 in *microbiology*, 11(5), 442–446.
- 1503 Tucker, C. M., Cadotte, M. W., Carvalho, S. B., Davies, T. J., Ferrier, S., Fritz, S. A., ... others (2017). A
1504 guide to phylogenetic metrics for conservation, community ecology and macroecology. *Biological*
1505 *Reviews*, 92(2), 698–715.
- 1506 Ulger Toprak, N., Yagci, A., Gulluoglu, B., Akin, M., Demirkalem, P., Celenk, T., & Soyletir, G. (2006).
1507 A possible role of *Bacteroides fragilis* enterotoxin in the aetiology of colorectal cancer. *Clinical*
1508 *microbiology and infection*, 12(8), 782–786.
- 1509 Ursell, L. K., Metcalf, J. L., Parfrey, L. W., & Knight, R. (2012). Defining the human microbiome.
1510 *Nutrition reviews*, 70(suppl_1), S38–S44.
- 1511 Utzschneider, K. M., Kratz, M., Damman, C. J., & Hullarg, M. (2016). Mechanisms linking the gut
1512 microbiome and glucose metabolism. *The Journal of Clinical Endocrinology & Metabolism*,
1513 101(4), 1445–1454.
- 1514 Vander Haar, E. L., So, J., Gyamfi-Bannerman, C., & Han, Y. W. (2018). *Fusobacterium nucleatum* and
1515 adverse pregnancy outcomes: epidemiological and mechanistic evidence. *Anaerobe*, 50, 55–59.
- 1516 Van der Maaten, L., & Hinton, G. (2008). Visualizing data using t-SNE. *Journal of machine learning*
1517 *research*, 9(11).
- 1518 Vasen, H. F., Mecklin, J.-P., Khan, P. M., & Lynch, H. T. (1991). The international collaborative group
1519 on hereditary non-polyposis colorectal cancer (icg-hnppcc). *Diseases of the Colon & Rectum*, 34(5),
1520 424–425.
- 1521 Vilar, E., & Gruber, S. B. (2010). Microsatellite instability in colorectal cancer—the stable evidence.
1522 *Nature reviews Clinical oncology*, 7(3), 153–162.
- 1523 Walker, M. A., Pedamallu, C. S., Ojesina, A. I., Bullman, S., Sharpe, T., Whelan, C. W., & Meyerson, M.
1524 (2018). Gatk pathseq: a customizable computational tool for the discovery and identification of
1525 microbial sequences in libraries from eukaryotic hosts. *Bioinformatics*, 34(24), 4287–4289.
- 1526 Weaver, W. (1963). *The mathematical theory of communication*. University of Illinois Press.
- 1527 Whiteside, S. A., Razvi, H., Dave, S., Reid, G., & Burton, J. P. (2015). The microbiome of the urinary
1528 tract—a role beyond infection. *Nature Reviews Urology*, 12(2), 81–90.
- 1529 Witkin, S. (2019). Vaginal microbiome studies in pregnancy must also analyse host factors. *BJOG: An*
1530 *International Journal of Obstetrics & Gynaecology*, 126(3), 359–359.
- 1531 Wong, T.-T., & Yeh, P.-Y. (2019). Reliable accuracy estimates from k-fold cross validation. *IEEE*
1532 *Transactions on Knowledge and Data Engineering*, 32(8), 1586–1594.
- 1533 Wyss, C., Moter, A., Choi, B.-K., Dewhirst, F., Xue, Y., Schüpbach, P., ... Guggenheim, B. (2004).
1534 *Treponema putidum* sp. nov., a medium-sized proteolytic spirochaete isolated from lesions of
1535 human periodontitis and acute necrotizing ulcerative gingivitis. *International journal of systematic*
1536 and *evolutionary microbiology*, 54(4), 1117–1122.
- 1537 Xia, Y. (2023). Statistical normalization methods in microbiome data with application to microbiome

- 1538 cancer research. *Gut Microbes*, 15(2), 2244139.
- 1539 Yaman, E., & Subasi, A. (2019). Comparison of bagging and boosting ensemble machine learning methods
1540 for automated emg signal classification. *BioMed research international*, 2019(1), 9152506.
- 1541 Yang, I., Claussen, H., Arthur, R. A., Hertzberg, V. S., Geurs, N., Corwin, E. J., & Dunlop, A. L. (2022).
1542 Subgingival microbiome in pregnancy and a potential relationship to early term birth. *Frontiers in*
1543 *cellular and infection microbiology*, 12, 873683.
- 1544 Yoshimura, F., Murakami, Y., Nishikawa, K., Hasegawa, Y., & Kawaminami, S. (2009). Surface
1545 components of porphyromonas gingivalis. *Journal of periodontal research*, 44(1), 1–12.
- 1546 Zhang, C.-Z., Cheng, X.-Q., Li, J.-Y., Zhang, P., Yi, P., Xu, X., & Zhou, X.-D. (2016). Saliva in the
1547 diagnosis of diseases. *International journal of oral science*, 8(3), 133–137.
- 1548 Zhou, X., Wang, L., Xiao, J., Sun, J., Yu, L., Zhang, H., ... others (2022). Alcohol consumption,
1549 dna methylation and colorectal cancer risk: Results from pooled cohort studies and mendelian
1550 randomization analysis. *International journal of cancer*, 151(1), 83–94.
- 1551 Zhu, W., & Lee, S.-W. (2016). Surface interactions between two of the main periodontal pathogens:
1552 *Porphyromonas gingivalis* and *tannerella forsythia*. *Journal of periodontal & implant science*,
1553 46(1), 2–9.
- 1554 Zhu, X., Han, Y., Du, J., Liu, R., Jin, K., & Yi, W. (2017). Microbiota-gut-brain axis and the central
1555 nervous system. *Oncotarget*, 8(32), 53829.
- 1556 Zhuang, Y., Wang, H., Jiang, D., Li, Y., Feng, L., Tian, C., ... others (2021). Multi gene mutation
1557 signatures in colorectal cancer patients: predict for the diagnosis, pathological classification, staging
and prognosis. *BMC cancer*, 21, 1–16.

Acknowledgments

1560 I would like to disclose my earnest appreciation for my advisor, Professor **Semin Lee**, who provided
 1561 solicitous supervision and cherished opportunities throughout the course of my research. His advice and
 1562 consultation encouraged me to become as a researcher and to receive all humility and gentleness. I am also
 1563 grateful to all of my committee members, Professor **Taejoon Kwon**, Professor **Eunhee Kim**, Professor
 1564 **Kyemyung Park**, and Professor **Min Hyuk Lim**, for their meaningful mentions and suggestions.

1565 I extend my deepest gratitude to my Lord, *the Flying Spaghetti Monster*, His Noodly Appendage
 1566 has guided me through the twist and turns of this academic journey. His presence, ever comforting and
 1567 mysterious, has been a source of strength and humor during both highs and lows. In moments of doubt, I
 1568 found solace in the belief that you were there, gently reminding me to keep faith in the process. His Holy
 1569 Noodle has nourished my mind, and for that, I am truly overwhelmed. May His Holy Noodle continue to
 1570 guide me in all my future endeavors. *R’Amen.*

1571 I would like to extend my heartfelt gratitude to Professor **You Mi Hong** for her invaluable guidance
 1572 and insightful advice on PTB study. Her expertise in maternal and fetal health, along with her deep under-
 1573 standing of statistical and clinical interpretations, greatly contributed to refining the analytical framework
 1574 of this study. Her constructive feedback and thoughtful discussions provided critical perspectives that
 1575 enhanced the robustness and relevance of the research findings. I sincerely appreciate her generosity
 1576 in sharing her knowledge and effort, as well as her encouragement throughout my Ph.D. journey. Her
 1577 support has been instrumental in strengthening this work, and I am truly grateful for her contributions.

1578 I also would like to express my sincere gratitude for Professor **Jun Hyeok Lim** for his invaluable
 1579 guidance and insightful advice on lung cancer study. His expertise in cancer genomics and data interpreta-
 1580 tion provided essential perspectives that greatly enriched the analytical approach of my Ph.D. journey. His
 1581 constructive feedback and thoughtful discussion helped refine methodologies and enhance the scientific
 1582 rigor of the research. I deeply appreciate his willingness to share his knowledge and expertise, which has
 1583 been instrumental in shaping key aspects of this work. His support and encouragement have been truly
 1584 inspiring, and I am grateful for the opportunity to have benefited from his mentorship.

1585 I would like to extend my heartfelt gratitude to my colleagues of the **Computational Biology Lab @**
 1586 **UNIST**, whose collaboration, friendship, brotherhood, and support have been an invaluable part of my
 1587 journey. Your willingness to share insights, engage in thoughtful discussions, and offer encouragement
 1588 during the challenging moments of research has significantly shaped my academic experience. The
 1589 camaraderie in Computational Biology Lab made even the most demanding days more enjoyable, and I
 1590 am deeply grateful for the collaborative environment we created together. I appreciate you for standing
 1591 by my side throughout this Ph.D. journey.

1592 I would like to express my heartfelt gratitude to **my family**, whose unwavering support has been the
 1593 foundation of everything I have achieved. Your love, encouragement, and belief in me have sustained me
 1594 through every challenge, and I could not have come this far without you. From your words of wisdom to
 1595 your patience and understanding, each of you has played a vital role in helping me navigate this journey.
 1596 The strength and comfort I have drawn from our family bond have been my greatest source of resilience.

1597 Your presence, both near and far, has filled my life with warmth and motivation. I am deeply grateful for
1598 your unconditional love and for always being there when I needed you the most. Thank you for being my
1599 constant source of strength and inspiration.

1600 I am incredibly pleased to my friends, especially my GSHS alumni (**이망특**), for their unwavering
1601 support and encouragement throughout this journey. The bonds we formed back in our school days have
1602 only grown stronger over the years, and I am fortunate to have had such loyal and understanding friends
1603 by my side. Your constant words of motivation, and even moments of levity during stressful times have
1604 helped keep me grounded. Whether it was a late-night conversations, a shared laugh, or a simple message
1605 of reassurance, you all have played a vital role in keeping me focused and motivated. I am relieved for the
1606 ways you celebrated each small achievement with me and how you patiently listened to my worries. The
1607 memories of our shared past provided me with comfort and a sense of stability when the road ahead felt
1608 uncertain. I could not have reached this point without the love and friendship that you all have generously
1609 given. Each of your, in your unique way, has contributed to this dissertation, even if indirectly, and for
1610 that, I am forever beholden. I look forward to continuing our friendship as we all grow in our individual
1611 paths, knowing that the support we share is something truly special.

1612 I would like to express my deepest recognition to **my girlfriend (expected)** for her unwavering
1613 support, patience, and companionship throughout my Ph.D. journey. Her presence has been a constant
1614 source of comfort and motivation, helping me navigate the challenges of research and writing with
1615 renewed energy. Through moments of frustration and accomplishment alike, her encouragement has
1616 reminded me of the importance of balance and perseverance. Her kindness, understanding, and belief
1617 in me have been invaluable, making even the most difficult days feel lighter. I am truly grateful for her
1618 support and for sharing this journey with me, and I look forward to all the moments we will continue to
1619 experience together.

1620 I would like to express my sincere gratitude to the amazing members of my animal protection groups,
1621 DRDR (**두루두루**) and UNIMALS (**유니멀스**), whose dedication and compassion have been a constant
1622 source of motivation. Your unwavering commitment to improving the lives of animals has inspired me
1623 throughout this journey. I am also thankful for the beautiful cats we have cared for, whose presence
1624 brought both joy and purpose to our allegiance. Their playful spirits and gentle companionship served as
1625 daily reminders of why we continue to fight for animal rights. The bond we share, both with each other
1626 and with the animals we protect, has enriched my life in countless ways. I appreciate you all again for
1627 your support, dedication, and for being part of this meaningful cause.

1628 I would like to express my deepest gratitude to **everyone** I have had the honor of meeting throughout
1629 this journey. Your kindness, encouragement, and support have carried me through both the challenging
1630 and rewarding moments of my life. Whether through a kind word, thoughtful advice, or simply being
1631 there when I needed it most, your presence has made all the difference. I am incredibly fortunate to have
1632 received such generosity and warmth from those around me, and I do not take it for granted. Every act
1633 of kindness, no matter how big or small, has been a source of strength and motivation for me. To all
1634 my friends, colleagues, mentors, and beloved ones, thank you for your unwavering support. I am truly
1635 grateful for each of you, and your kindness has left an indelible mark on my journey.

1636 My Lord, *the Flying Spaghetti Monster*,
1637 give us grace to accept with serenity the things that cannot be changed,
1638 courage to change the things that should be changed,
1639 and the wisdom to distinguish the one from the other.

1640
1641 Glory be to *the Meatball*, to *the Sauce*, and to *the Holy Noodle*.
1642 As it was in the beginning, is now, and ever shall be.

1643 *R'Amen.*



May your progress be evident to all

