Non-significant influence between aerobic and anaerobic sample transport materials on gut (fecal) microbiota in healthy and fat-metabolic disorder Thai adults

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Background. The appropriate sample handling for human fecal microbiota studies is essential to prevent changes in bacterial composition and quantities that could lead to misinterpretation of the data. **Methods.** This study thereby firstly identified potential effect of aerobic and anaerobic fecal sample collection and transport materials on microbiota and quantitative microbiota in healthy and fat-metabolic disorder Thai adults aged 23-43 years, utilizing metagenomics followed 16S rRNA gene sequencing and 16S rRNA gene qPCR. We analyzed taxonomic composition, alpha diversity, beta diversity, bacterial quantification, Pearson's correlation with clinical factors for fat-metabolic disorder, and the microbial community and species potential metabolic functions. **Results.** Our study successfully obtained microbiota results in percent and quantitative compositions. The quality sequences in each sample had > 99% Good's coverage index, relatively plateau rarefaction curve, and alpha diversity indices that showed no statistical difference in percent and quantitative microbiota OTU richness and evenness, between aerobic and anaerobic sample transport materials. Obligate and facultative anaerobic species were analyzed and also showed no significantly different. Supportively, the beta diversity analysis by non-metric multidimensional scale (NMDS) could not distinguish microbiota community structures between aerobic and anaerobic sample transport groups (P = 0.864); yet could distinguish microbiota community structures between healthy and fat-metabolic disorder groups (P = 0.049), along the Pearson's correlated clinical parameters (i.e. age, liver stiffness, GGT, BMI, and TC), the significant associated bacterial species, and their microbial metabolic functions. For examples, genera such as *Ruminococcus* and *Biofidobacterium* were exhibited in healthy human gut and provided

functions in metabolisms of cofactors and vitamins, biosynthesis of secondary metabolites against gut bacterial pathogens, energy metabolisms, digestive system, and carbohydrate metabolism; and these characteristics were also predicted as healthy subject markers with the significant LEfSe scores. In conclusion, this study demonstrated that short term aerobic sample collection and transport (< 48 hours) did not statistically affected the microbiota and quantitative microbiota analyses in alpha and beta diversity measurements. The study also demonstrated that the short term aerobic sample collection and transport still allowed fecal microbiota differentiations between healthy and fat-metabolic disorder subjects, similar with anaerobic sample collection and transport. Moreover, the microbiota related metabolic function potentials and bacterial species biomarkers in healthy and fat-metabolic disorder were suggested with statistic bioinformatics, albeit limitation of inter-individual human differences such as sex and diet patterns.

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Running Title: Aerobic & anaerobic transport microbiota

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Abstract

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Methods. This study thereby firstly identified potential effect of aerobic and anaerobic fecal sample collection and transport materials on microbiota and quantitative microbiota in healthy and fat-metabolic disorder Thai adults aged 23-43 years, utilizing metagenomics followed 16S rRNA gene sequencing and 16S rRNA gene qPCR. We analyzed taxonomic composition, alpha diversity, beta diversity, bacterial quantification, Pearson's correlation with clinical factors for fat-metabolic disorder, and the microbial community and species potential metabolic functions.

Results. Our study successfully obtained microbiota results in percent and quantitative compositions. The quality sequences in each sample had > 99% Good's coverage index, relatively plateau rarefaction curve, and alpha diversity indices that showed no statistical difference in percent and quantitative microbiota OTU richness and evenness, between aerobic and anaerobic sample transport materials. Obligate and facultative anaerobic species were

analyzed and also showed no significantly different. Supportively, the beta diversity analysis by non-metric multidimensional scale (NMDS) could not distinguish microbiota community structures between aerobic and anaerobic sample transport groups ($P = 0.864$); yet could distinguish microbiota community structures between healthy and fat-metabolic disorder groups ($P = 0.049$), along the Pearson's correlated clinical parameters (i.e. age, liver stiffness, GGT, BMI, and TC), the significant associated bacterial species, and their microbial metabolic functions. For examples, genera such as *Ruminococcus* and *Biofidobacterium* were exhibited in healthy human gut and provided functions in metabolisms of cofactors and vitamins, biosynthesis of secondary metabolites against gut bacterial pathogens, energy metabolisms, digestive system, and carbohydrate metabolism; and these characteristics were also predicted as healthy subject markers with the significant LEfSe scores. In conclusion, this study demonstrated that short term aerobic sample collection and transport (< 48 hours) did not statistically affected the microbiota and quantitative microbiota analyses in alpha and beta diversity measurements. The study also demonstrated that the short term aerobic sample collection and transport still allowed fecal microbiota differentiations between healthy and fat-metabolic disorder subjects, similar with anaerobic sample collection and transport. Moreover, the microbiota related metabolic function potentials and bacterial species biomarkers in healthy and fat-metabolic disorder were suggested with statistic bioinformatics, albeit limitation of inter-individual human differences such as sex and diet patterns.

Keywords

Human gut microbiome, Microbiota, 16S rRNA gene sequencing, Bacteria diversity, Aerobic and anaerobic sample transport, Fecal, Fat-metabolic disease

Introduction

Human gastrointestinal microbiota is the most complex and dynamic microbial diversity of estimated trillion bacterial cells, which include culture-independent and anaerobic bacteria. These bacterial communities were also found diverse among different ethnics, ages, and health status (*Reynoso-García et al., 2022; HMP Consortium, 2012a; HMP Consortium, 2012b*). The gut bacterial diversity plays a crucial role in a health (symbiosis) status, for instances, supporting proper fat metabolisms and gut leakage immune responses, antimicrobials, metabolites, and nutrients e.g. vitamins B and K (*Reynoso-García et al., 2022; Valdes et al., 2018*). Perturbation of the gut microbiota (dysbiosis) can cause risks to multiple diseases, such as fat metabolic disorders, inflammatory bowel disease and other autoimmune diseases, lupus erythrematosous, and cancer (*Hrncir, 2022*). Fat (or lipid) metabolism disorders are defined an improper process human's body uses to make energy from food, and can cause a harmful amount of lipids deposited in your body that may damage important tissues and organs, such as peripheral blood, liver, and brain (*Handzlik et al., 2023; Yan et al., 2023*). Subsequently, the studies of gut bacterial communities via fecal samples using 16S rRNA gene high-throughput sequencing have become widely performed owning to its powerful identification in a culture-independent manner (*Caporaso et al., 2011; Dailey et al., 2019; Kousgaard et al., 2020; Human Microbiome Project Consortium, 2012a*), and our study thereby compared influences of aerobic and anaerobic sample transport materials on gut (fecal) microbiota and also analyzed if the differences might affect when interpretation in healthy and gut disease, in which the fat-metabolic disease presents as example.

92

93 Many studies had investigated effects of sample collection preservatives and length of
94 sample storage to guideline fecal sample handling and storage for gut microbiome analyses. For
95 instances, -80°C was respected a standard choice for long-term (≥ 6 months) sample storage
96 temperature, and a common chemical such as 70% ethanol was reported sufficient for sample
97 preservative while other researches presented FTA cards and the OMNIgene Gut kit *(Hsu et al.,*
98 *2019; Ma et al., 2020; Song et al., 2016; Watson et al., 2019*). Fecal microbiota in these different
99 preservatives showed variable generally after 1 week of sample storage. For sample processing,
100 general protocols recommended cold sample transport (≤ 4°C) within 24-48 h after sample
101 collection (*Gorzelak et al., 2015; Liang et al., 2020; Moossavi et al., 2019; Song et al., 2016*).
102 Subsequently, our study processed metagenomic extraction immediately after each sample
103 collection and cold transport (within 24 h) to prevent any bias. Our analysis of aerobic vs.
104 anaerobic sample transport materials present an interesting factor in local clinical sample
105 collection settings. In local clinical settings, an anaerobic sample transport material is sometimes
106 unaccessible and the samples are collected-transported aerobically in typical sterile
107 polypropylene containers in 24-48 h without DNA preservatives (*Dore et al., 2015; Wesolowska-*
108 *Andersen et al., 2014*). This aerobic condition may cause an oxygen toxicity to extremely
109 oxygen-sensitive bacteria, affecting fecal microbiota and quantitative microbiota analyses
110 (*Ndongo et al., 2020; Taur et al., 2018*). For examples, some bacteria such as *Faecalibacterium*
111 spp. are unable to retain cell viability for > 2 min of oxygen exposure (*Duncan et al., 2002*). To
112 date, a limited studies have investigated an impact of aerobic and anaerobic sample transport
113 materials without DNA preservative on quantitative microbiota whether this affects an ability to
114 differentiate between healthy and metabolic-disease gut microbial diversity (*Fofanov et al.,*

115 *2018; Jenkins et al., 2018; Martínez et al., 2019*). Our analyses included taxonomic composition,
116 alpha diversity, beta diversity, bacterial quantification, correlation with clinical factors for fat-
117 metabolic disorder, and the microbial community and species potential metabolic functions.

118

119 ## Materials and Methods

120 **Participant's recruitment, fecal sample collections and metagenomic extraction**

121 Nine healthy and eleven fat-metabolic disorder Thai participants, males and females of
122 age range 24-43 years, were recruited and all methods used in this study were in accordance with
123 the guidelines by the ethic approval. The ethical approval for the study was granted by
124 Institutional Review Board, Faculty of Medicine, Chulalongkorn University (no. 735/61).
125 Written informed consent was obtained from all participants in this study. Fecal samples were
126 collected in fecal containers with one aerobic and one anaerobic transport materials. For aerobic
127 transport material, the fecal container was sealed and placed in a plastic bag. For anaerobic
128 transport material, the fecal container was sealed and placed in the AnaeroPack-Anaero
129 (Mitsubishi Gas Chemical, Tokyo, Japan) (less than 0.1% O_2 and more than 15% CO_2) (*van*
130 *Horn et al., 1997; Wen et al., 2021*). The samples were transported on the same day of fecal
131 collection in cold temperature (≤ 4°C) and processed immediately for metagenomic extraction
132 using DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) following manufacturer's
133 instruction (*Wongsaroj et al., 2021; Ondee et al., 2022*). The metagenomic DNA was qualified
134 and quantified by agarose gel electrophoresis and nanodrop spectrophotometry (A260 and
135 A260/A280, respectively). Schematic experimental design was illustrated in Fig. 1.

136

16S rRNA gene V3-V5 library preparation and MiSeq sequencing

PCR amplification of the 16S rRNA gene at the V3-V5 region was performed using the universal prokaryotic primers 342F (5′-GGRGGCAGCAGTNGGGAA-3′) and 895R (5′-TGCGDCCGTACTCCCCA-3′) with appended barcode and adaptor sequences (*Castelino et al., 2017; Wongsaroj et al., 2021; Dityen et al., 2022*). Briefly, each PCR reaction comprised 1× EmeraldAmp GT PCR Master Mix (TaKaRa, Shiga, Japan), 0.2 μM of each primer, and 50-100 ng of the genomic DNA in a total volume of 75 μL. The PCR conditions were 94°C 3 min, and 25 cycles of 94°C 45 s, 50°C 1 min and 72°C 1 min 30 s, followed by 72°C 10 min. A minimum of two independent PCR reactions were performed and pooled to prevent PCR stochastic bias. Then, the ~640-base pair (bp) amplicon was excised from agarose gel resolution and purified using PureDireX PCR Clean-Up & Gel Extraction Kit (Bio-Helix, Keelung, Taiwan), and quantified using a Qubit 3.0 Fluorometer and Qubit dsDNA HS Assay kit (Invitrogen, Waltham, USA). Finally, 180 ng of each barcoded amplicon product was pooled for sequencing using the Miseq600 platform (Illumina, San Diego, CA, USA), along with the sequencing primers and index sequence (*Caporaso et al., 2012; Wongsaroj et al., 2021; Dityen et al., 2022; Ondee et al., 2022*), at the Omics Sciences and Bioinformatics Center, Chulalongkorn University (Bangkok, Thailand).

Quantification of total bacteria copy number

The 16S rRNA gene qPCR was performed to quantify total bacteria in copy unit, using universal primers 1392F (5'-CGGTGAATACGTTCYCGG-3) and 1492R (5′-GGTTACCTTGTTAC GACTT-3′), and Quantinova SYBR green PCR Master Mix (Qiagen, Hilden, Germany) in a 20 μL total volume and 1 ng metagenomic DNA (or reference DNA), as

previously established (*Suzuki et al., 2000; Oldham & Duncan 2012; Wongsaroj et al., 2021*). The qPCR thermocycling conditions were 95°C 5 min, followed by 40 cycles of 95°C 5 s and 60°C 10 s, and ended with a 50-99°C melting curve analysis to validate a single proper amplicon peak (i.e. neither primer-dimer nor non-specific amplification). The reference for copy number computation was *Escherichia coli*, in which the ~120-bp 1392F-1492R amplicon fragments were cloned into pGEM-T-Easy Vector (Promega, Wisconsin, USA) and the recombinant plasmids were transformed into competent *E. coli* DH5α for expression (*Hanahan et al., 1991*). The inserted fragments were verified by colony PCR using the primers M13F (on vector) and 1492R (inserted fragment). Ten-fold serial dilutions of the extracted plasmids (10^5-10^{10} copies/μL) were used as the reference standard curves in the bacterial copy number computation as following equation (*Smith et al., 2006*).

$$\text{Copy number per } \mu\text{L} = \frac{\text{concentration (ng/}\mu\text{L)} \times 6.023 \times 10^{23}(\text{copies/mol})}{\text{length (bp)} \times 6.6 \times 10^{11}(\text{ng/mol})}$$

The qPCR experiments were performed using Rotor-GeneQ (Qiagen, Hilden, Germany). Three replicates were conducted per reaction. The bacteria copy number of each sample was quantified against the reference standard curve by Rotor-Gene Q Series Software (Qiagen).

Bioinformatic and statistical analyses for bacterial microbiota diversity and potential metabolisms

Raw sequences (reads) were processed following Mothur 1.39.5's standard operation procedures for MiSeq (*Schloss et al., 2009*) (https://github.com/mothur/mothur/releases/), including removal of (a) reads shorter than 100 nucleotides (nt) excluding primer and barcode sequences, (b) ambiguous bases ≥ 4, (c) chimera sequences, and (d) homopolymer of > 7

182 homopolymers. The quality sequences were aligned with the 16S rRNA gene SILVA 13.2
183 (*McDonald et al., 2012*) and Greengenes 13.8 *(Quast et al., 2013*) to remove lineages of
184 mitochondria, chloroplasts and eukaryotes, and were clustered into operational taxonomic units
185 (OTU) with 97% nt similarity (78% for phylum, 88% order, 91% class, 93% family, 95% genus,
186 and 97% species) based on naïve Bayesian taxonomic method with default parameters (*Wang et*
187 *al., 2007*). Samples were normalized for an equal sequencing depth (7,137 quality sequences per
188 sample), and the total bacteria copy number from the 16S rRNA gene qPCR data were analyzed
189 together with the percent microbiota composition to yield the quantitative microbiota
190 (*Vandeputte et al., 2017a; Vandeputte et al., 2017b; Jian et al., 2018;Wongsaroj et al., 2021*).
191 Alpha diversity including Good's coverage index (percent sequence coverage to true estimate),
192 rarefaction curve, Chao1 richness, inverse Simpson and Shannon diversity; and beta diversity
193 including Smith theta (Thetan), Sorenson (Sorabund), Morisita–Horn, Yue and Clayton theta
194 (Thetayc), Bray-Curtis (BC), Jaccard (jclass), and Lennon (Lennon) coefficients, and two-
195 dimension non-metric multidimensional scaling (NMDS), were computed using Mothur 1.39.5
196 (*Schloss et al., 2009; Schloss, 2020*). Estimate of the microbial metabolic profiles was
197 determined by PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of
198 Unobserved States) based on the reference genome annotations in KEGG (Kyoto Encyclopedia
199 of genes and genomes pathways) *(Langille et al., 2013*), and statistically compared by STAMP
200 (Statistical Analysis of Metagenomic Profiles) (*Parks et al., 2014*). The differences in microbial
201 metabolic profiles were further analyzed by linear discriminant analysis effect size (LEfSe)
202 method with pairwise Kruskal–Wallis and Wilcoxon tests to identify the microbial metabolic
203 biomarker representing healthy and disease groups. For general statistics, the non-parametric
204 multiple *t*-tests were used and a P-value < 0.05 was considered significant.

205

206 **Availability of supporting data**

207 Nucleic acid sequences in this study were deposited in an NCBI open access Sequence
208 Read Archive database, accession number PRJNA1020208.

209

210 ## Results

211 **16S rRNA gene sequencing results and percent microbiota compositions**

212 The 16S rRNA gene sequencing yielded 2,365,959 total raw sequences (Table S1:
213 aerobic sample transport 1,517,643 sequences, and anaerobic sample transport 848,316
214 sequences), and 1,623,517 total quality sequences (aerobic sample transport 1,062,335
215 sequences, and anaerobic sample transport 561,182 sequences). The average quality sequences
216 per sample was 40,587 ± 24,139 (avg. ± SD), and the numbers of OTUs ranged 5-10 at phylum
217 (Table 1: aerobic sample transport 6.80 ± 1.19), and anaerobic sample transport 848,316
218 sequences), 55-93 genus, and 77-133 species levels, respectively (Table S1 and Table 1). The
219 number of OTUs at phylum, genus and species levels were found approximately equal between
220 aerobic and anaerobic sample transports (Table 1: phylum OTUs 6.55 ± 1.19 aerobic, 7.05 ± 1.23
221 anaerobic; genus OTUs 71.40 ± 10.45 aerobic, 72.70 ± 11.29 anaerobic; and species OTUs
222 101.15 ± 16.83 aerobic, 101.60 ± 15.67 anaerobic). Following the successfully high number of
223 quality sequences, the Good's coverage (percent sequence coverage to true estimate) of all
224 samples were above 99.5% at phylum, genus and species level OTUs (Table S1), provided that
225 the Good's coverage index at phylum was averagely 100%, genus 99.82% and species 99.72%
226 (Table 1). Subsequently, once performed data normalization of all samples each to the same

sequencing depth received the relative plateau rarefaction curves with avg. > 99% Good's coverages (Fig. S1), and confirmed our further microbiota bioinformatic analyses without bias from various quality sequencing numbers per sample.

The distribution of percent bacterial compositions at phylum, genus, and species levels across participants (adults) albeit healthy or fat-metabolic disorder disorders were compared between aerobic vs. anaerobic sample transport material groups, and no statistical difference between groups were found (AMOVA, $P > 0.05$) (Fig. 2). In brief, five core phyla ranging from Firmicutes (averagely, 52.03 ± 17.30%), Bacteroidetes (24.32 ± 14.11%), Proteobacteria, Actinobacteria, to Fusobacteria, were dominated. The latter three phyla accounted averagely < 24% in total. Twenty-two bacterial genera and 24 bacterial species OTUs that exhibited more than 1% relative abundances, each were compared between aerobic vs. anaerobic sample transport material groups, and no statistical difference were found ($P > 0.05$) (Table S2). This indicated no statistical difference in percent microbiota compositions at phylum, genus and species levels, between aerobic and anaerobic sample transport groups.

Quantitative microbiota composition analyses between aerobic and anaerobic sample transport groups

Following the quantification of bacteria by the universal 16S rRNA gene qPCR, the quantity of bacterial counts and the quantitative microbiota compositions could be analyzed. The quantity of bacterial counts were found no significantly different between aerobic and anaerobic sample transport groups (Fig. 3A: $P = 0.057$). Noted that the relatively low in aerobic sample

transport group was due to ID3a and the relatively high in anaerobic sample transport group was due to ID1an; if except these two, the average bacterial counts of both groups will even become closer to each other and *P* value increases (Fig. S2).

Next, the prevalence of nine bacterial genera corresponding to obligate (or strictly) anaerobic and facultative anaerobic bacteria were quantitatively compared, and no significant difference in quantity between aerobic vs. anaerobic sample transport groups were pointed (Fig. 3B). The obligate anaerobic *Bacteroides* spp. were found most dominated than other obligate anaerobic bacterial genera in both groups and presented in approximately comparable counts, followed by *Prevotella*, *Faecalibacterium*, *Oscillospira*, *Bifidobacterium*, and the facultative anaerobic *Haemophilus*, *Streptococcus* and *Enterococcus*, respectively. Nonetheless, the slight but non-statistically significant higher counts of obligate anaerobic bacteria were showed, but this trend was minute and found inconsistent for facultative anaerobic bacteria genera (Fig. 3B), highlighting the differences in obligate vs. facultative oxygen requirement effect yet at the non-significant statistic. Overall, the percent microbiota composition and the quantitative microbiota did not demonstrate significant difference between aerobic and anaerobic sample transport materials. Subsequently, the alpha diversity by OTU species richness (OTUs and Chao1) and OTU species diversity (inverse Simpson and Shannon) showed very high *P* values between 0.3827 and 0.9497 (Fig. 4), and the beta diversity among individual samples belonging aerobic and anaerobic sample transport groups showed no separate clustering pattern (Fig. 5A). Noted that the detail analyses of alpha diversity at OTU phylum and genus levels were also analyzed and no statistic differences were found ($P > 0.05$) (Fig. S3). Additionally, other beta diversity coefficients, such as Sorabund, Morisita-Horn, Thetayc and Bray-Curtis, were computed and all

dissimilarity coefficient indices did not separate the microbiota community differences between aerobic and anaerobic sample transport groups (Table S3: $P > 0.05$).

Quantitative microbiota analyses between healthy and fat-metabolic disorder groups

When we analyzed the quantitative microbiota structure differences based on beta diversity coefficients and statistics between healthy vs. fat-metabolic disorder (from now on referred as "unhealthy") groups, both community structures demonstrated relatively separate clusters with significant statistic (Fig. 5B: enclosed green circle for healthy subjects and red circle for mostly unhealthy subjects, $P = 0.049$). Together, many clinical parameters corresponding to fat-metabolic disorders demonstrated statistically ($P < 0.05$: age, liver stiffness, GGT, BMI, and TC) and non-statistically (i.e., AST, ALT, TG, LDL, and CAP) associated the same direction with the unhealthy microbiota community structure (Fig. 5C). Fig. 5D exhibited bacterial species that significantly associated with healthy community structure pattern such as *Prevotella*, *Haemophilus* and *Bacteroides plebeius*; and unhealthy community structure such as *Clostridium*, *Ruminococcus* and Bacteroides.

Metabolic function prediction levels via quantitative profiles of prevalent health-associated bacteria, and microbial metabolic function species biomarkers for healthy and fat-metabolic disorder groups

Potentially important microbial related metabolic functions were analyzed via quantitative profiles of reported and prevalent health-associated bacterial OTUs, including *Bacteroides, Prevotella, Megamonas, Bifidobacterium, Hemophilus, Clostridium, Ruminococcus*

and Pasteurellaceae (*Wu, Bushmanc & Lewis, 2013; Schirmer et al., 2019; Sun et al., 2020; Sabo & Dumitrascu, 2021*). The general most active microbial related functions were in metabolism pathway (49.92%: primarily amino acid and carbohydrate metabolisms followed by energy, cofactors and vitamins, lipid and xenobiotics biodegradation metabolisms), 19.94% in genetic information processing, 16.22% environmental information processing, 3.11% cellular process, 0.91% human diseases, 0.65% organismal systems, and 5.09% poorly characterized. The OTUs representing *Bacteroides* and *Prevotella copri* represented the general topmost bacterial metabolisms (Fig. 6A). Nonetheless, the relative abundances of these two species and other prevalent health-associated bacteria showed the dynamic functions in categories of metabolisms, cellular process, and genetic information processing between healthy and fat-metabolic disorder groups, in orderly. For instances, the relatively more abundances of amino acid, carbohydrate and energy metabolism functions, cellular process, genetic information processing and human diseases were reported in the fat-metabolic disorder group. *Prevotella copri, Prevotella stercorea* and *Bacteroides plebeius* were estimated more diverse and abundant functions in the fat-metabolic disorder group while *Bacteroides* and *Bifidobacterium longum* were estimated the more diverse and abundant in healthy group (Fig. S4). These microbial metabolism differences between groups allowed LEfSe to identify the specific microbial metabolic functions along the bacterial species as the biomarkers to differentiate between heathy vs. fat-metabolic disorder groups, with statistic *P* values. *Prevotella copri* and *Bacteroides plebeius* were the biomarkers for the fat-metabolic disorder and their microbial metabolic functions included many functions involved in diseases (immune system diseases, metabolic diseases, and neurodegenerative diseases). On the other hand, more numbers of bacterial species and their microbial metabolic functions were the biomarkers for the healthy group, supporting the diverse microbial related

317 metabolic functions in healthy human guts; and noted that the commonly reported functions were
318 in metabolism and organismal systems pathways meanwhile the human disease pathway was rare
319 for the healthy group (Fig. 6B).
320

321 ## Discussion

322 As intestine occupies the most number and diversity of bacteria in human body, fecal
323 microbiome has shown the important field to study many bacterial interactions with human
324 heathy (or diseases), and the fat-metabolic disorder represent one common related disorder with
325 fecal microbiota dysbiosis. Due to variation in sample transport materials, especially in local and
326 limited research resource settings, the anaerobic sample transport materials might be utilized.
327 Hence, this study analyzed influences of aerobic and anaerobic sample transport materials on
328 percent composition and quantitative composition of gut (fecal) microbiota, and also identified
329 whether these influences could affect the interpretation in microbiomes of healthy compared with
330 the fat-metabolic disorder. Further, we could describe the percent and quantitative microbiota
331 differences in heathy and fat-metabolic disorder subjects disrespect of aerobic or anaerobic
332 sample transport materials.
333

334 Our study successfully obtained microbiota results in percent and quantitative
335 compositions. The number of quality sequences in each sample allowed reliable Good's
336 coverage index score for OTU diversity and rarefaction curve. Comparing between aerobic and
337 anaerobic sample transport materials, the core bacterial compositions, in both percentages and
338 quantitative counts, showed no statistical difference. This supported the no statistic difference in
339 alpha diversity that included numbers of OTUs, Chao1 richness, inverse Simpson, and Shannon

diversity indices. Further, analyses of obligate anaerobic and facultative anaerobic bacteria were compared and still no statistical difference in these bacterial species between the aerobic and anaerobic sample transport groups. Supportively, the beta diversity analysis by NMDS could not separate bacterial communities of aerobic from anaerobic sample transport groups ($P = 0.864$). Overall, our study indicated no influence between aerobic and anaerobic sample transport materials during sample collection and sample transport (provided that the metagenomic extraction was performed within 2 days) on fecal microbiota and fecal quantitative microbiota. Our results were in consistent with Taguer et al. (*2021*) that short period of oxygen exposure did not affect the nucleic acid content and changes of bacterial microbiota. Moreover, studies reported that the fecal samples for microbiome studies might be kept without any DNA stabilizer reagent at 4^{o}C for up to 8 weeks and at -20^{o}C for the longer period (*Choo et al., 2015; Song et al., 2016*). Moreover, some obligate anaerobes could partially reduce pressure of aerobic (oxygen) environment by consuming oxygen via their bacterial oxidase enzymes *(Baughn & Malamy, 2004*): for examples, a conserved cytochrome *bd* family enzymes in many bacterial species in phyla Firmicutes, Bacteroidetes, Actinomycetes and Proteobacteria, allowing the bacterial tolerance in the presence of oxygen for several hours (*Borisov et al., 2021*). Yet, when possible, the minimizing oxygen exposure remains the gold standard fecal collection and transport (*Burz et al., 2019*).

Next, we analyzed if these microbiota communities remained associated and able to be distinguished by a fat-metabolic disorder, an example of well-known disease that could be affected by the gut microbiota dysbiosis (*Rothschild et al., 2018; HMP Consortium, 2012b; Zheng et al., 2020*). The beta diversity analyses by NMDS could distinguish the different

363 microbiota community structures between healthy and this disease state, and many clinical
364 factors representing the fat-metabolic disorders (*Dominianni et al., 2015; Loo et al., 2017; Liu et*
365 *al., 2019a; Xu et al., 2019; Zheng et al., 2020*) were statistically correlated with the fat-metabolic
366 disorder microbiota subjects (from both aerobic and anaerobic sample transport groups) (e.g.
367 age, liver stiffness, GGT, BMI, and TC). In addition, we could identify the bacterial OTUs that
368 statistically associated with the healthy vs. fat-metabolic disorder, their microbial metabolic
369 functions, and the potential biomarkers for bacterial species and correlated metabolisms in
370 healthy vs. fat-metabolic disorder. For instances, genera such as *Ruminococcus* and
371 *Biofidobacterium* were also reported previously in healthy human gut and provided functions in
372 short chain fatty acid producers, metabolisms of cofactors and vitamins, biosynthesis of
373 secondary metabolites against gut bacterial pathogens, energy metabolisms, digestive system,
374 and carbohydrate metabolism *(Ze et al., 2012; Christopherson et al., 2014; Matijašić et al.,*
375 *2014;*). Noted that the presence of *H. parainfluenzae* was reported no any negative effect in gut
376 health (*Kosikowska et al., 2016; Tanner et al., 2016*). Additionally, the microbial functions
377 involved human disease was rare found in the healthy than the fat-metabolic disorder groups
378 (Fig. 6B).
379
380 For fat-metabolic disorder group, *Prevotella copri* and *Bacteroides plebeius* had been
381 reported as potential gut pathogens for cardiac valve calcification and cardiovascular disease (*Liu*
382 *et al., 2019b*). However, the prevalence of genus *Prevotella* could be found in healthy gut, and
383 this genus was reported linked with high-fiber diet consumption (*Arumugam et al., 2011*).
384 Hence, the reason that we observed this genus correlated with the fat-metabolic disorder could be
385 biased by the subjects' diets, which we did not have information in study. Furthermore,

limitation in this study included a small number of samples, which could hinder the correlation and bacterial species identification of the microbiota and quantitative microbiota with the fat-metabolic disorder.

Conclusions

The study first analyzed fecal bacterial microbiota and quantitative microbiota, and revealed no influence of anaerobic sample transport material on the microbiota and quantitative microbiota. This supported studies that short term aerobic sample collection and transport does not statistically affected the microbiota analyses, with ≤ 4°C sample storage and sample processing within 48 h are required. Our study could also able to demonstrate the gut microbiota differences between healthy and fat-metabolic disorder in both aerobic and anaerobic sample transport collections, and analyze quantitative microbiota related microbial metabolic potentials and bacterial species biomarkers in Thai adult subjects. This disease gut microbiota dysbiosis factor exhibited albeit inter-individual human differences like sex and diet patterns.

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Authors' Contributions

NT performed molecular biology experiments and data analysis, and drafted the manuscript. LW helped molecular biology experiments and data analysis. KJ helped clinical sample collections. KP provided samples. KP and NS advised and conceived the study. NS designed the study, coordinated the experiments and data analysis, and wrote the manuscript. All authors read and approved the final manuscript

Conflict of Interest

The authors declare no conflict of interest.

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672

673 ## Figure Legends

674 **Fig. 1.** Schematic diagram of experimental design.

675

676 **Fig. 2.** Relative percent gut microbiota compositions of aerobic and anaerobic transport groups at
677 phylum, genus and species levels.
678 Color shades represent bacterial phyla: yellow (Firmicutes), blue (Bacteroidetes), red
679 (Proteobacteria), grey (Fusobacteria), purple (Actinobacteria), white (Other), and pink
680 (unclassified bacterial phyla). The OTUs where Mothur could not identify the genus (or species)
681 names were denoted by small letters (o_ abbreviates order; f_, family; g_, genus and s_, species)
682 to the deepest taxonomic names that could be identified; k_ abbreviated kingdom bacteria but
683 unclassified phylum; and "Other" represented < 1% phylum (or genus, or species) OTUs.

684

685 **Fig. 3.** Quantification of bacterial counts for (A) average total bacterial counts and (B) average
686 strictly anaerobic and facultative anaerobic bacterial genera, comparing between aerobic and
687 anaerobic sample transport groups.

Data were presented as average ± SD. Statistical differences between groups were tested using Student's *t*-test ($P < 0.05$), and no statistical difference was found.

Fig. 4. Scatter plots showing individual and mean range alpha diversity data at species OTUs of aerobic (filled circle) and anaerobic (empty square) sample transport groups, measured by (A) number of OTUs, (B) Chao1 richness, (C) inverse Simpson diversity, and (D) Shannon evenness. Statistical differences between groups were tested using Student's *t*-test ($P < 0.05$), and no statistical difference was found: $P > 0.05$.

Fig. 5. Non-metric multidimensional scaling (NMDS) constructed from Thetan coefficients displaying beta diversity among quantitative microbiota communities in aspects of (A) aerobic and anaerobic sample transport groups and (B-D) health and fat-metabolic disorder (denoted "unhealthy") groups.
In (A) and (B), AMOVA test was performed to determine statistical separation between designated groups ($P < 0.05$). In (C) and (D) showed the Pearson's correlations with health status parameters and the representing bacterial species OTUs, respectively. A vector direction and length represented the direction and strength of that parameter or OTU to the communities. A red arrow indicated a statistically significant correlation parameter ($P < 0.05$), and a black arrow indicated non-statistically significant correlation parameter ($P > 0.05$). In (C), GGT abbreviates gamma-glutamyl transferase; BMI, body mass index; stiffness, liver stiffness indicates the non-elasticity of the liver associated fat accumulation; TC, total cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TG, triglyceride; LDL, low-density lipoproteins; CAP, controlled attenuation parameter; and HDL, high-density lipoproteins.

711

712 **Fig. 6.** Metabolic functional prediction associated to quantitative profiles of (A) prevalent health-
713 associated bacteria OTUs and (B) linear discriminant analysis (LDA) combined effect size
714 (LEfSe) as bacterial species and associated microbial metabolic function biomarkers for healthy
715 or fat-metabolic disorder (denoted "unhealthy") groups.
716 Microbial metabolic functions were estimated according to KEGG pathways. In (A), a different
717 color from nude to tangerine represents the level of quantitative microbial metabolic function
718 abundance from absence to the highest presence level (scale in vertical bar chart). In (B), a
719 numeric in front of KEGG name represents the KEGG pathway category: 1, metabolism; 2,
720 organismal systems; 3, diseases; 4, environmental information processing; 5, genetic information
721 processing; and 6, cellular processes. The LDA score > 3.0 was referred microbial metabolic
722 function markers (ANOVA Welch's test, $P < 0.05$).

Figure 1

Schematic diagram of experimental design.

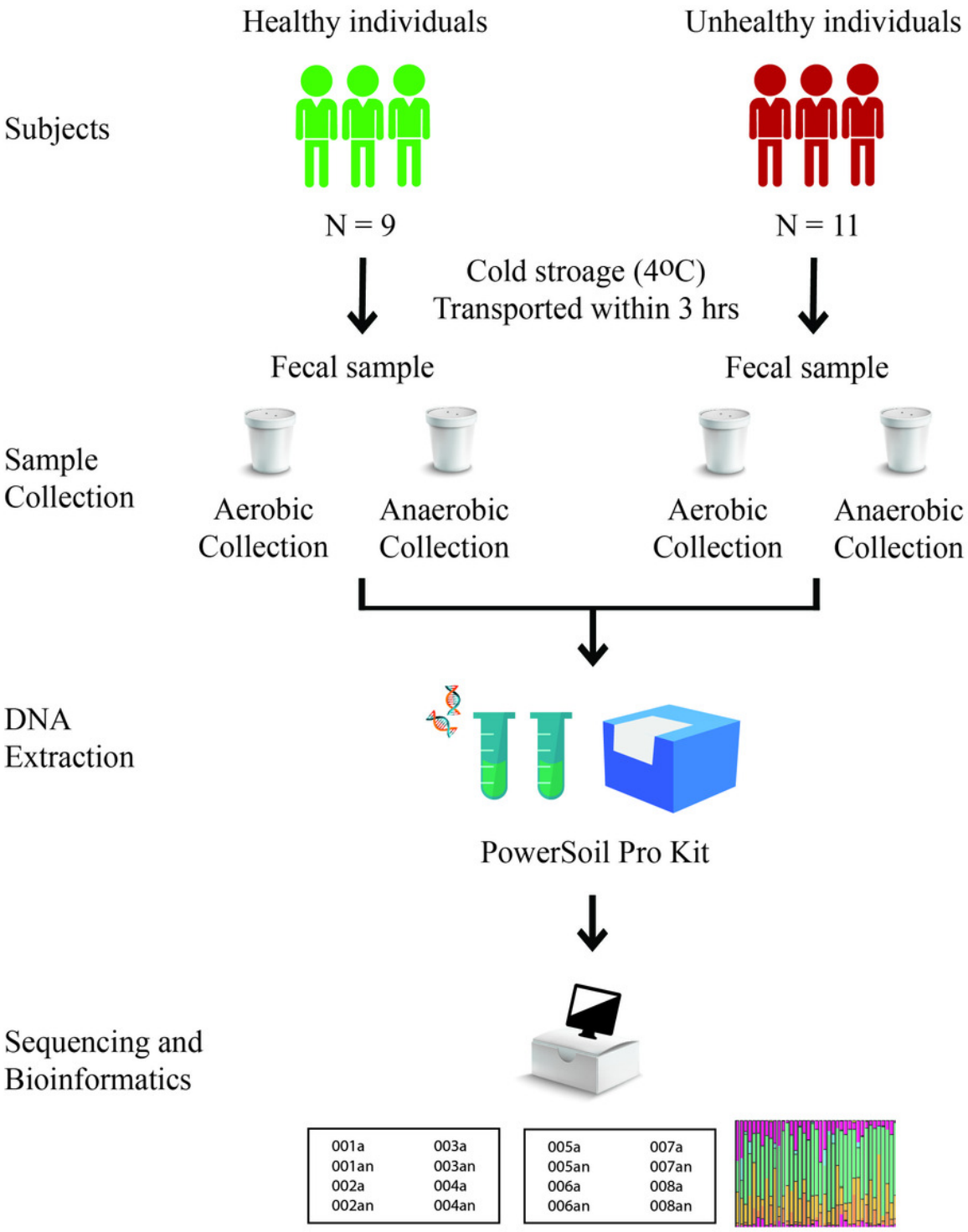
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Figure 2

Relative percent gut microbiota compositions of aerobic and anaerobic transport groups at phylum, genus and species levels.

Color shades represent bacterial phyla: yellow (Firmicutes), blue (Bacteroidetes), red (Proteobacteria), grey (Fusobacteria), purple (Actinobacteria), white (Other), and pink (unclassified bacterial phyla). The OTUs where Mothur could not identify the genus (or species) names were denoted by small letters (o_ abbreviates order; f_, family; g_, genus and s_, species) to the deepest taxonomic names that could be identified; k_ abbreviated kingdom bacteria but unclassified phylum; and “Other” represented < 1% phylum (or genus, or species) OTUs.

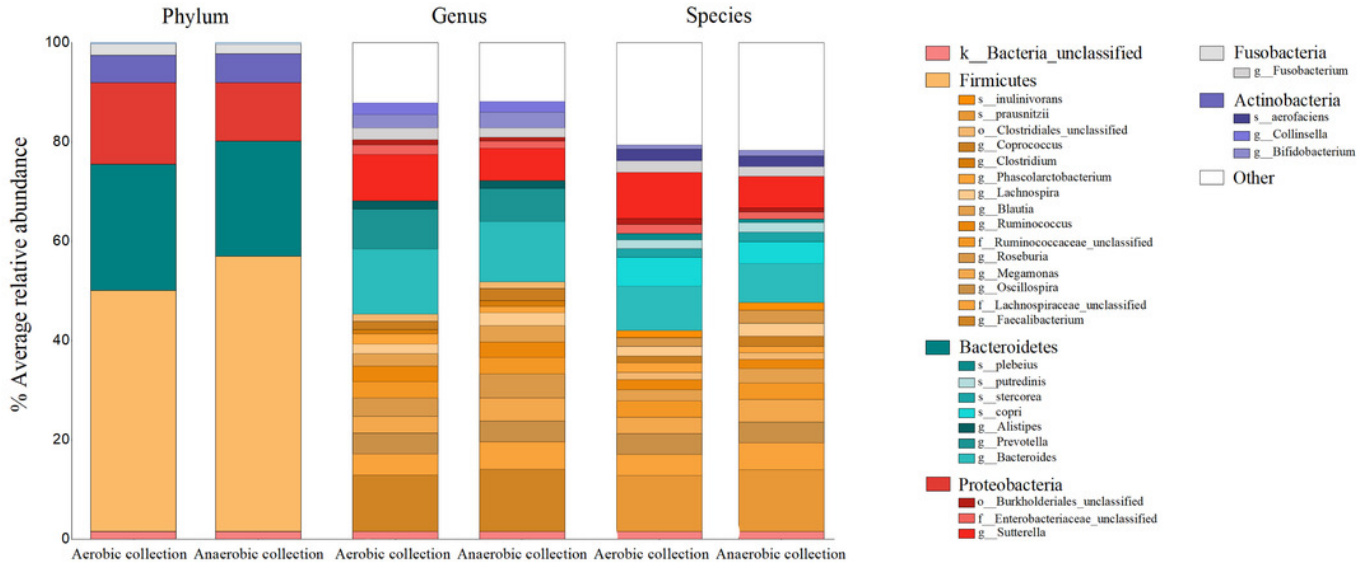

Figure 3

Quantification of bacterial counts for (A) average total bacterial counts and (B) average strictly anaerobic and facultative anaerobic bacterial genera, comparing between aerobic and anaerobic sample transport groups.

Data were presented as average ± SD. Statistical differences between groups were tested using Student's *t*-test ($P < 0.05$), and no statistical difference was found.

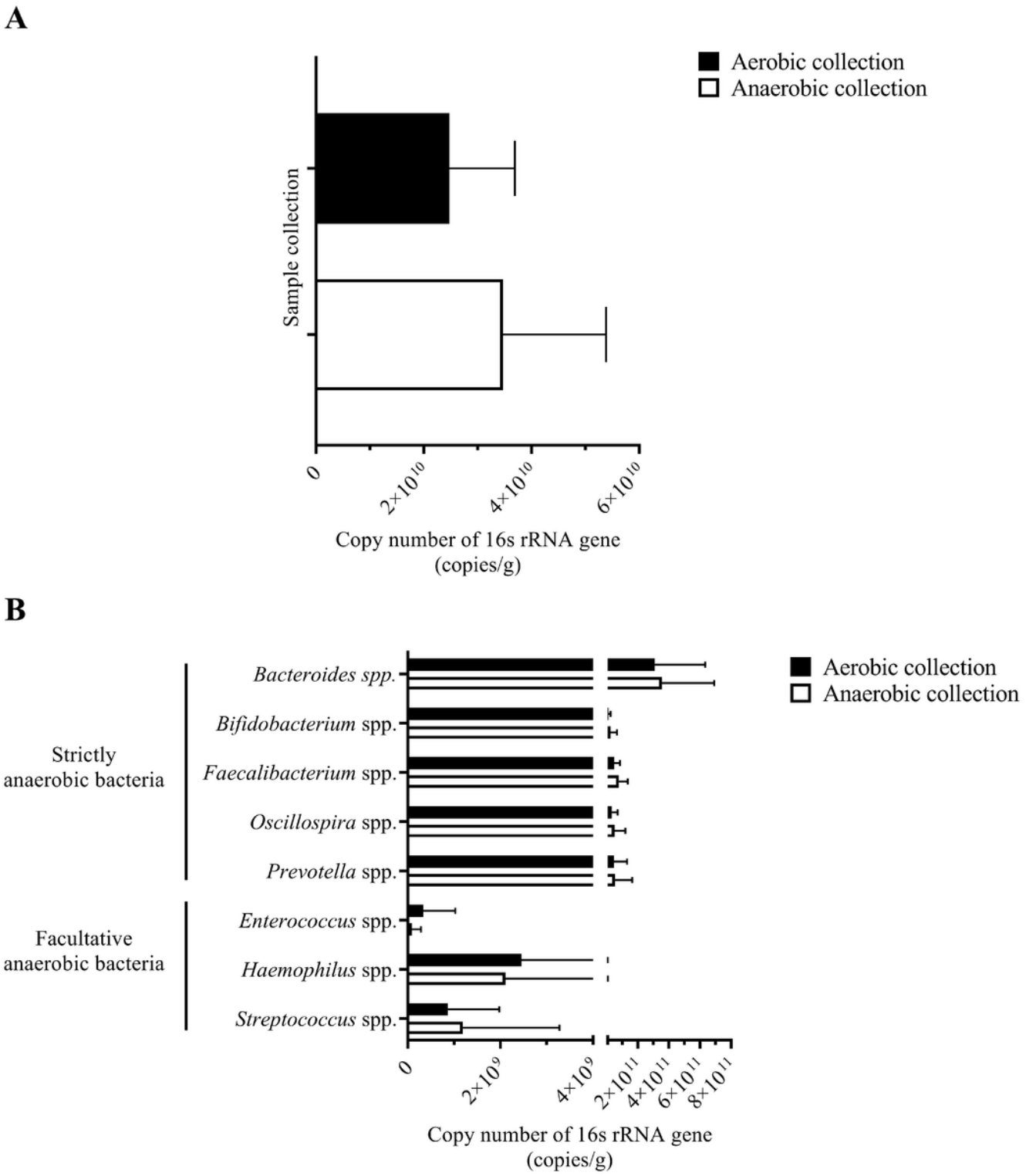
A
Aerobic collection
Anaerobic collection
Sample collection
0
2×10^10
4×10^10
6×10^10
Copy number of 16s rRNA gene (copies/g)
B
Aerobic collection
Anaerobic collection
Strictly anaerobic bacteria
Facultative anaerobic bacteria
Bacteroides spp.
Bifidobacterium spp.
Faecalibacterium spp.
Oscillospira spp.
Prevotella spp.
Enterococcus spp.
Haemophilus spp.
Streptococcus spp.
0
2×10^9
4×10^9
2×10^11
4×10^11
6×10^11
8×10^11
Copy number of 16s rRNA gene (copies/g)

Figure 4

Scatter plots showing individual and mean range alpha diversity data at species OTUs of aerobic (filled circle) and anaerobic (empty square) sample transport groups, measured by (A) number of OTUs, (B) Chao1 richness, (C) inverse Simpson diversity, and (D

Statistical differences between groups were tested using Student's *t*-test ($P < 0.05$), and no statistical difference was found: $P > 0.05$.

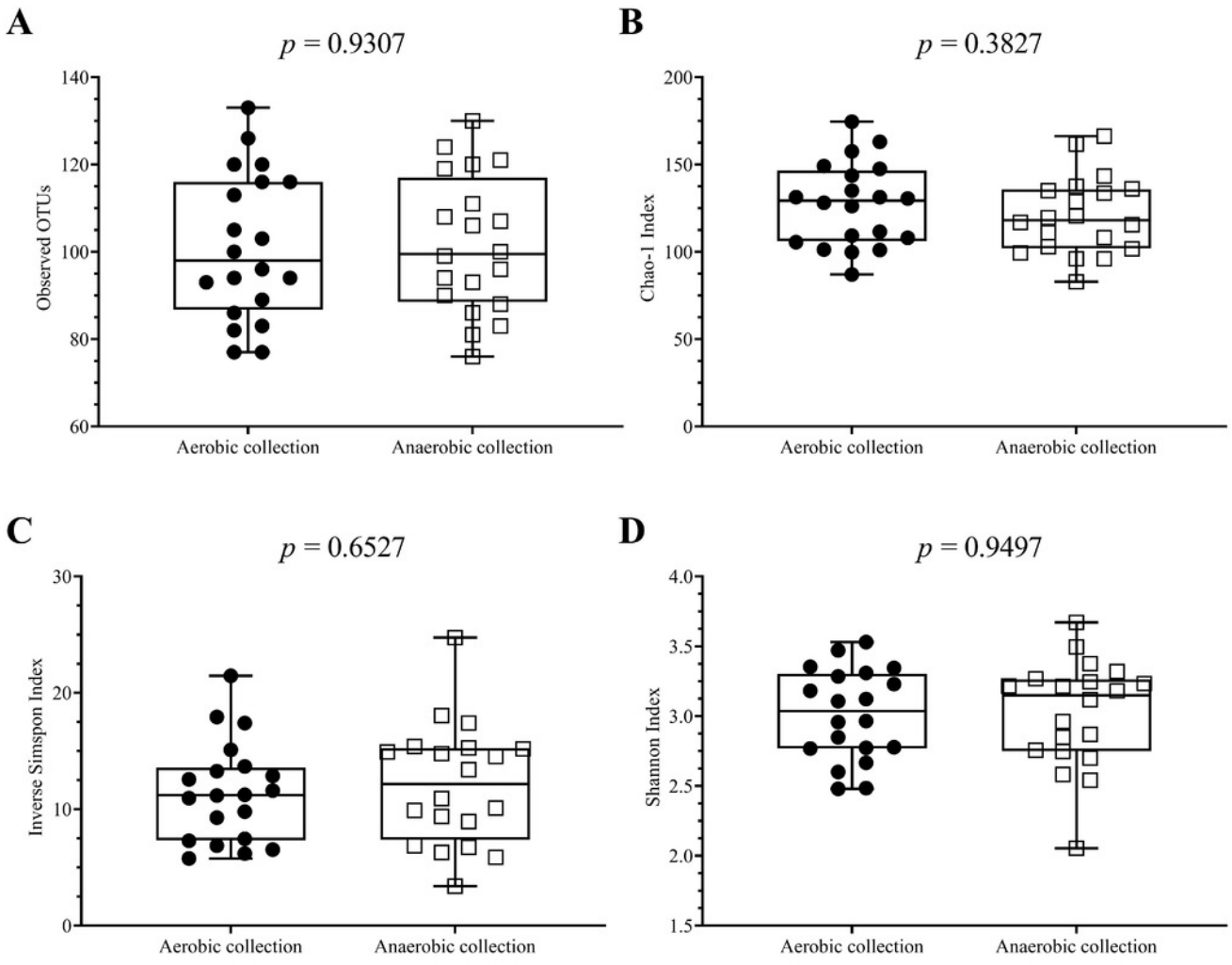

Figure 5

Non-metric multidimensional scaling (NMDS) constructed from Thetan coefficients displaying beta diversity among quantitative microbiota communities in aspects of (A) aerobic and anaerobic sample transport groups and (B-D) health and fat-metabolic disorder

In (A) and (B), AMOVA test was performed to determine statistical separation between designated groups ($P < 0.05$). In (C) and (D) showed the Pearson's correlations with health status parameters and the representing bacterial species OTUs, respectively. A vector direction and length represented the direction and strength of that parameter or OTU to the communities. A red arrow indicated a statistically significant correlation parameter ($P < 0.05$), and a black arrow indicated non-statistically significant correlation parameter ($P > 0.05$). In (C), GGT abbreviates gamma-glutamyl transferase; BMI, body mass index; stiffness, liver stiffness indicates the non-elasticity of the liver associated fat accumulation; TC, total cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TG, triglyceride; LDL, low-density lipoproteins; CAP, controlled attenuation parameter; and HDL, high-density lipoproteins.

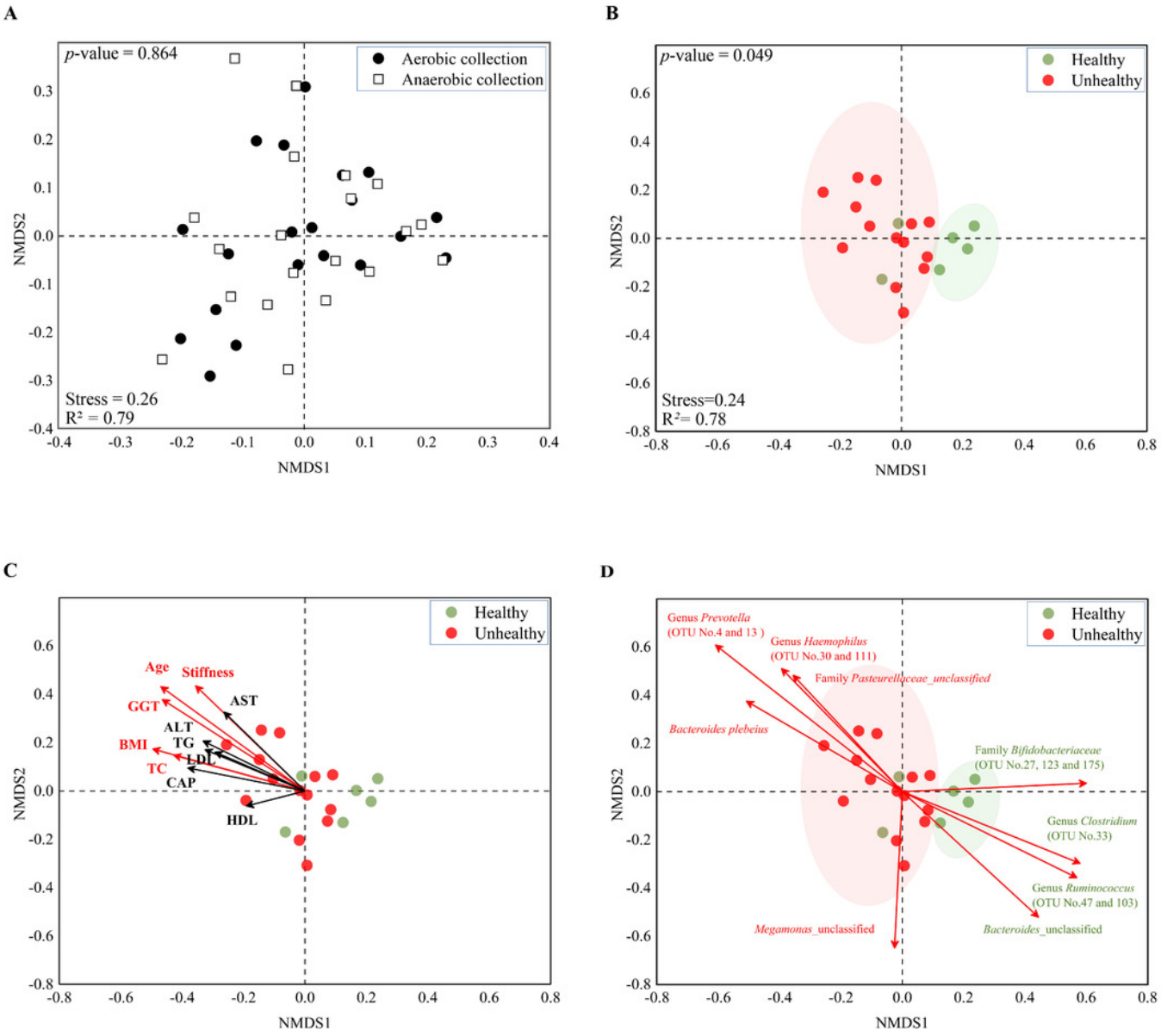
A
p-value = 0.864
Aerobic collection
Anaerobic collection
Stress = 0.26
R² = 0.79
NMDS1
NMDS2
B
p-value = 0.049
Healthy
Unhealthy
Stress=0.24
R²= 0.78
NMDS1
NMDS2
C
Healthy
Unhealthy
Age
Stiffness
GGT
AST
ALT
BMI
TG
LDL
TC
CAP
HDL
NMDS1
NMDS2
D
Healthy
Unhealthy
Genus Prevotella
(OTU No.4 and 13)
Genus Haemophilus
(OTU No.30 and 111)
Family Pasteurellaceae_unclassified
Bacteroides plebeius
Family Bifidobacteriaceae
(OTU No.27, 123 and 175)
Genus Clostridium
(OTU No.33)
Genus Ruminococcus
(OTU No.47 and 103)
Megamonas_unclassified
Bacteroides_unclassified
NMDS1
NMDS2

Figure 6

Metabolic functional prediction associated to quantitative profiles of (A) prevalent health-associated bacteria OTUs and (B) linear discriminant analysis (LDA) combined effect size (LEfSe) as bacterial species and associated microbial metabolic function b

Microbial metabolic functions were estimated according to KEGG pathways. In (A), a different color from nude to tangerine represents the level of quantitative microbial metabolic function abundance from absence to the highest presence level (scale in vertical bar chart). In (B), a numeric in front of KEGG name represents the KEGG pathway category: 1, metabolism; 2, organismal systems; 3, diseases; 4, environmental information processing; 5, genetic information processing; and 6, cellular processes. The LDA score > 3.0 was referred microbial metabolic function markers (ANOVA Welch's test, $P < 0.05$).

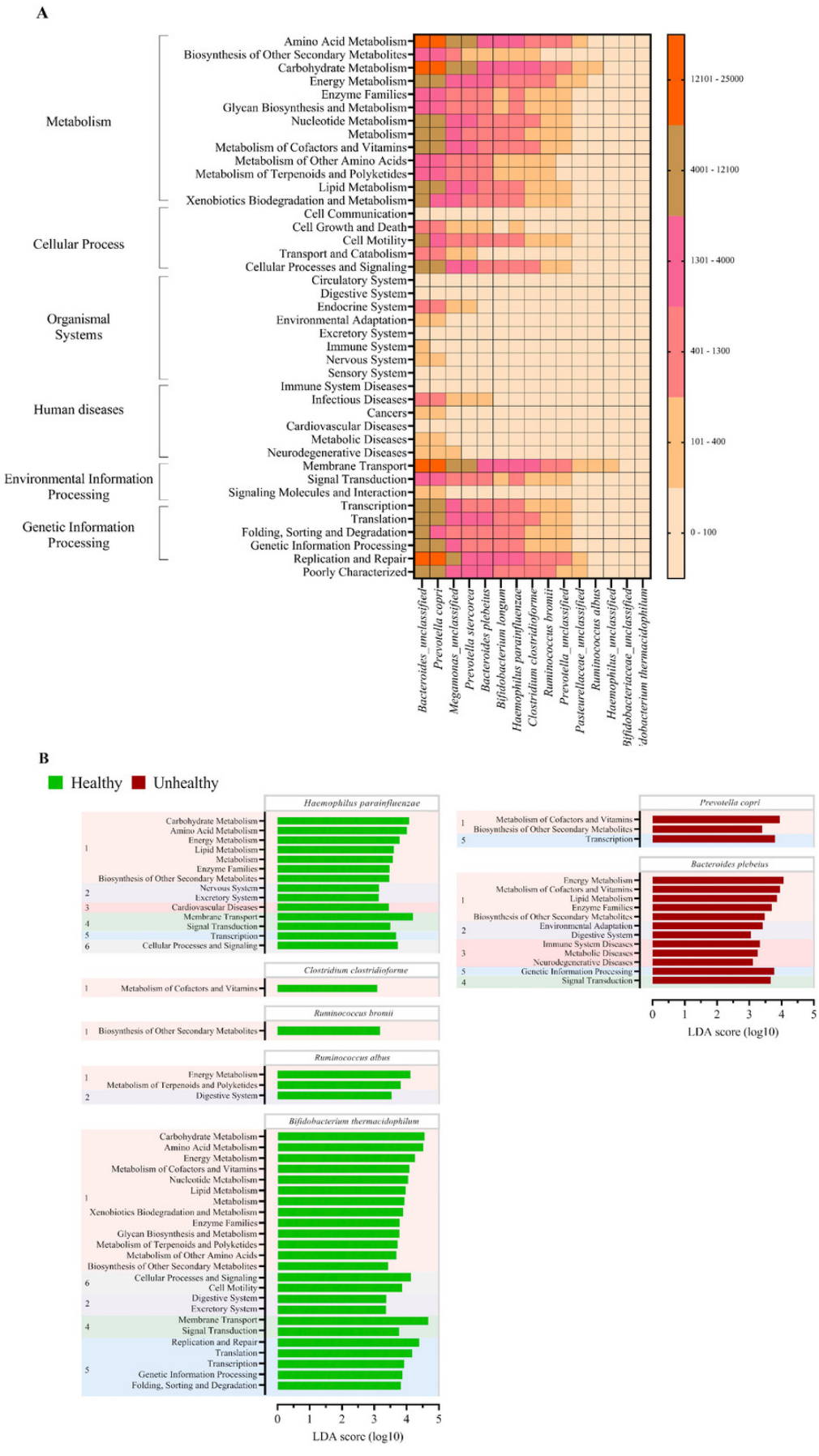
A
Metabolism
Cellular Process
Organismal Systems
Human diseases
Environmental Information Processing
Genetic Information Processing
Amino Acid Metabolism
Biosynthesis of Other Secondary Metabolites
Carbohydrate Metabolism
Energy Metabolism
Enzyme Families
Glycan Biosynthesis and Metabolism
Nucleotide Metabolism
Metabolism
Metabolism of Cofactors and Vitamins
Metabolism of Other Amino Acids
Metabolism of Terpenoids and Polyketides
Lipid Metabolism
Xenobiotics Biodegradation and Metabolism
Cell Communication
Cell Growth and Death
Cell Motility
Transport and Catabolism
Cellular Processes and Signaling
Circulatory System
Digestive System
Endocrine System
Environmental Adaptation
Excretory System
Immune System
Nervous System
Sensory System
Immune System Diseases
Infectious Diseases
Cancers
Cardiovascular Diseases
Metabolic Diseases
Neurodegenerative Diseases
Membrane Transport
Signal Transduction
Signaling Molecules and Interaction
Transcription
Translation
Folding, Sorting and Degradation
Genetic Information Processing
Replication and Repair
Poorly Characterized
12101 - 25000
4001 - 12100
1301 - 4000
401 - 1300
101 - 400
0 - 100
Bacteroides_unclassified
Prevotella copri
Megamonas_unclassified
Prevotella siercorea
Bacteroides plebeius
Bifidobacterium longum
Haemophilus parainfluenzae
Clostridium clostridioforme
Ruminococcus bromii
Prevotella_unclassified
Pasteurellaceae_unclassified
Ruminococcus albus
Haemophilus_unclassified
Bifidobacteriaceae_unclassified
dobacterium thermacidophilum
B
Healthy
Unhealthy
Haemophilus parainfluenzae
Carbohydrate Metabolism
Amino Acid Metabolism
Energy Metabolism
Lipid Metabolism
Metabolism
Enzyme Families
Biosynthesis of Other Secondary Metabolites
Nervous System
Excretory System
Cardiovascular Diseases
Membrane Transport
Signal Transduction
Transcription
Cellular Processes and Signaling
Clostridium clostridioforme
Metabolism of Cofactors and Vitamins
Ruminococcus bromii
Biosynthesis of Other Secondary Metabolites
Ruminococcus albus
Energy Metabolism
Metabolism of Terpenoids and Polyketides
Digestive System
Bifidobacterium thermacidophilum
Carbohydrate Metabolism
Amino Acid Metabolism
Energy Metabolism
Metabolism of Cofactors and Vitamins
Nucleotide Metabolism
Lipid Metabolism
Metabolism
Xenobiotics Biodegradation and Metabolism
Enzyme Families
Glycan Biosynthesis and Metabolism
Metabolism of Terpenoids and Polyketides
Metabolism of Other Amino Acids
Biosynthesis of Other Secondary Metabolites
Cellular Processes and Signaling
Cell Motility
Digestive System
Excretory System
Membrane Transport
Signal Transduction
Replication and Repair
Translation
Transcription
Genetic Information Processing
Folding, Sorting and Degradation
0 1 2 3 4 5
LDA score (log10)
Prevotella copri
Metabolism of Cofactors and Vitamins
Biosynthesis of Other Secondary Metabolites
Transcription
Bacteroides plebeius
Energy Metabolism
Metabolism of Cofactors and Vitamins
Lipid Metabolism
Enzyme Families
Biosynthesis of Other Secondary Metabolites
Environmental Adaptation
Digestive System
Immune System Diseases
Metabolic Diseases
Neurodegenerative Diseases
Genetic Information Processing
Signal Transduction
0 1 2 3 4 5
LDA score (log10)

Table 1(on next page)

Average quality reads, OTUs and Good's coverage (%) at phylum, genus and species level.

For number of quality reads and OTUs, data were displayed in average ± SD. Multiple t-tests were performed for OTUs and Good's coverages between aerobic and anaerobic sample transport groups and no statistical difference was determined ($P > 0.05$).

Table 1. Average quality reads, OTUs and Good's coverage (%) at phylum, genus and species level.

For number of quality reads and OTUs, data were displayed in average ± SD. Multiple *t*-tests were performed for OTUs and Good's coverages between aerobic and anaerobic sample transport groups and no statistical difference was determined ($P > 0.05$).

Groups	Quality sequences	Phylum		Genus		Species	
		OTUs	Good's coverage	OTUs	Good's coverage	OTUs	Good's coverage
All	40,587 ± 24,139	6.80 ± 1.22	100	72.05 ± 10.76	99.82	101.38 ± 16.05	99.72
Aerobic collection	53,116 ± 24211	6.55 ± 1.19	100	71.40 ± 10.45	99.83	101.15 ± 16.83	99.71
Anaerobic collection	28,059 ± 16,715	7.05 ± 1.23	100	72.70 ± 11.29	99.81	101.60 ± 15.67	99.72