Performance evaluation of a new custom, multi-component DNA isolation method optimized for use in shotgun metagenomic sequencing-based aerosol microbiome research

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ABSTRACT

Background

Aerosol microbiome research advances our understanding of bioaerosols, including how airborne microorganisms affect our health and surrounding environment. Traditional microbiological/molecular methods are commonly used to study bioaerosols, but do not allow for generic, unbiased microbiome profiling. Recent studies have adopted shotgun metagenomic sequencing (SMS) to address this issue. However, SMS requires relatively large DNA inputs, which are challenging when studying low biomass air environments, and puts high requirements on air sampling, sample processing and DNA isolation protocols. Previous SMS studies have consequently adopted various mitigation strategies, including long-duration sampling, sample pooling, and whole genome amplification, each associated with some inherent drawbacks/limitations.

Results

Here, we demonstrate a new custom, multi-component DNA isolation method optimized for SMS-based aerosol microbiome research. The method achieves improved DNA yields from filter-collected air samples by isolating DNA from the entire filter extract, and ensures unbiased microbiome representation by combining chemical, enzymatic and mechanical lysis. Benchmarking against two state-of-the-art DNA isolation methods was performed with a mock microbial community and real-world subway air samples. All methods demonstrated similar performance regarding DNA yield and community representation with the mock community. However, with subway air samples, the new method obtained drastically improved DNA yields, while SMS revealed that the new method reported higher diversity and gave better taxonomic

coverage. The new method involves intermediate filter extract separation into a pellet and supernatant fraction. Using subway air samples, we demonstrate that supernatant inclusion results in improved DNA yields. Furthermore, SMS of pellet and supernatant fractions revealed overall similar taxonomic composition but also identified differences that could bias the microbiome profile, emphasizing the importance of processing the entire filter extract.

Conclusions

By demonstrating and benchmarking a new DNA isolation method optimized for SMS-based aerosol microbiome research with both a mock microbial community and real-world air samples, this study contributes to improved selection, harmonization, and standardization of DNA isolation methods. Our findings highlight the importance of ensuring end-to-end sample integrity and using methods with well-defined performance characteristics. Taken together, the demonstrated performance characteristics suggest the new method could be used to improve the quality of SMS-based aerosol microbiome research in low biomass air environments.

KEYWORDS

Aerosol Microbiome; Air Sampling; DNA Isolation; Shotgun Metagenomic Sequencing

BACKGROUND

The study of bioaerosols is an emerging and expanding research discipline [1], with several important study applications, including surveillance of clinically relevant microbes [2-5], air quality monitoring [6-8] and biodefense [9]. Bioaerosol research has traditionally relied on culture methods; however, few microorganisms grow under standard laboratory conditions, resulting in underrepresentation of the true microbial diversity [10-13]. Although culture methods are still in use, culture-independent methods are now widespread. Due to the low amount of DNA that is typically obtained from air samples, most culture-independent bioaerosol studies to date have used PCR to target either the bacterial 16S rRNA gene [14, 15] or the fungal 18S rRNA gene/internal transcribed spacer (ITS) region, followed by amplicon sequencing [16, 17]. In contrast to the amplicon sequencing approach, shotgun metagenomic sequencing (SMS) allows for generic, unbiased interrogation of microbial diversity in a sample. However, SMS will typically require a higher quality and quantity of DNA for analysis than other molecular methods. SMS has been used to characterize the human microbiome [18] and environmental microbiomes [19, 20], and has recently been implemented in a few aerosol microbiome studies [2, 21, 22].

Although bioaerosols originate from many different sources and are ubiquitous in almost any indoor and outdoor environment, air is still a very low biomass environment compared to e.g. soil, feces and water [23]. The low biomass makes it challenging to obtain sufficient DNA amounts for downstream analyses, especially in the context of SMS [21]. An important first step in recovering sufficient biomass and a representative sample from air involves the use of well-characterized air samplers that are capable of rapid and efficient biomass collection [24]. Filter-based aerosol collection is a commonly used method, and the use of hand-portable, high-volume filter-based air sampling equipment may improve the spatiotemporal resolution in aerosol

97 microbiome research [24, 25]. The post-sampling processing steps are also important since the
98 filter-collected biomass must be transformed into a representative high quality DNA sample with
99 minimal loss. It is therefore essential to use a well-characterized DNA isolation method that is
100 capable of thorough unbiased biomass lysis, sufficient inhibitor removal and sample clean-up,
101 and high efficiency recovery of DNA [25]. In short, the main challenges are typically obtaining
102 sufficient DNA amounts and capturing representative samples that reflect the true diversity of the
103 sampled air environment [2, 22, 25, 26].

104 With recent advancements in sequencing technology, along with the development of
105 improved strategies for air sampling and sample processing, it should be possible to mitigate the
106 low biomass challenge. Mitigation strategies that have been attempted in the past include long-
107 duration sampling (days to weeks), pooling of multiple air samples, whole genome amplification
108 (WGA) techniques, and modification of commercial DNA isolation kits originally developed for
109 other environmental matrices such as water and soil [2, 21, 27-29]. Increasing the air sampling
110 time is a common strategy to improve the DNA yield, but this approach may not always be
111 practical. For example, in studies where the aim is to address spatiotemporal variability, the need
112 for long-duration air sampling (e.g. days to weeks) exclude the possibility of aerosol microbiome
113 investigations on shorter timescales. Another challenge with increased air sampling time is that
114 long-duration filter collection may compromise the integrity of stress-sensitive microorganisms,
115 e.g. due to desiccation and osmotic shock [27], and thereby cause a potential loss of DNA from
116 organisms that become membrane-compromised, ruptured or lysed during filter extraction and
117 subsequent processing steps prior to DNA isolation. Liquid extraction of aerosol filters often
118 results in sample volumes that are too large to process with most commercial DNA isolation kits.
119 This introduces a need for adopting additional post-extraction filtration or centrifugation steps to
120 reduce the sample volume before DNA isolation, which may result in loss of both intact

121 microorganisms and DNA, and thereby compromise the sample integrity regarding both yield and
122 composition (diversity). Furthermore, long-duration, high-volume air sampling alone does not
123 always translate into successful recovery of sufficient DNA amounts for SMS [2, 21, 28, 29].
124 This may be due to the use of different downstream sample processing and DNA isolation
125 methods that have not been sufficiently evaluated regarding their specific performance on air
126 samples, and which therefore may deliver suboptimal performance regarding biomass lysis
127 and/or DNA recovery efficiency. Various modifications of existing sample processing and DNA
128 isolation methods have been proposed to improve the DNA yield from filter-collected air
129 samples. Jiang et al. modified the DNeasy (former MO-BIO) PowerSoil Kit by replacing the
130 silica spin column with AMPure XP beads, and introduced sample pre-treatment steps and a
131 secondary filtration step [28]. Yooseph et al. introduced a WGA step to generate sufficient DNA
132 amounts from air samples for SMS [21]. King et al. performed liquid extraction of aerosol filters
133 followed by a secondary filtration step and DNA isolation with the DNeasy PowerWater Kit, and
134 precipitated DNA from the original filtrate before combining the two DNA fractions [2].
135 Dommergue et al., who also used the DNeasy PowerWater Kit, placed the aerosol filters directly
136 in PowerBead tubes, introduced sample pre-treatment steps, and a centrifugation step to
137 maximize lysate recovery from PowerBead tubes [29]. Recovery of sufficient DNA amounts and
138 preservation of unbiased microbial diversity from air samples is essential to ensure reliable
139 results in SMS-based aerosol microbiome research. Several studies on other sample matrices
140 have looked into how DNA yields can be improved and microbial diversity preserved. Tighe et
141 al. found that using a multi-enzyme cocktail (MetaPolyzyme) that targets bacterial and fungal cell
142 wall components resulted in improved DNA yields [30]. Yuan et al. evaluated different DNA
143 isolation methods for human microbiome samples, and found bead beating and enzymatic lysis to
144 be essential for obtaining an accurate representation of microorganisms in a complex mock

145 community [31]. Abusleme et al. found that bead beating may limit the DNA yield, but also that
146 bead beating was necessary to detect all organisms in a complex mock bacterial community [32].
147 These observations suggest that biomass lysis based on a combination of chemical, enzymatic
148 and mechanical principles may be useful to minimize microbiome composition (diversity) bias
149 resulting from insufficient biomass lysis during isolation of DNA from complex environmental
150 assemblages.

151 It is well established that the choice of DNA isolation method should be based on careful
152 consideration of the specific study aims, including type of targeted organisms and environmental
153 matrices [33]. However, substantial uncertainty exists regarding the extent of microbiome
154 composition (diversity) bias that may be introduced by the use of different sample processing and
155 DNA isolation methods, which makes it difficult to reliably compare microbiome results between
156 different studies and environments. Consequently, several attempts have in recent years been
157 made to improve the harmonization and standardization of DNA isolation methods, especially for
158 common sample matrices such as human [31, 34], soil [35], and water [36] samples. Lear et al.
159 recommended DNA isolation kits for different environmental matrices such as soil, plant and
160 animal tissue, and water [37]. The Earth Microbiome Project demonstrated how procedural
161 standardization allows for comparison of microbial diversity in samples from across the globe
162 [35]. Dommergue et al. proposed an air sampling, filter extraction and DNA isolation method
163 where microbial diversity and chemical composition in air can be investigated using existing
164 high-volume particulate matter samplers used for atmospheric pollution monitoring [29].
165 Nevertheless, despite substantial effort several unresolved issues remain, e.g., the current reliance
166 on long-duration air sampling raises some questions regarding sample integrity and only offers
167 support for low temporal resolution studies since the necessary sampling time may be days or
168 even weeks. Hence, performance benchmarking, harmonization, and standardization of air

169 sampling, sample processing and DNA isolation methods is a topic that warrants further study,
170 and especially in the context of SMS-based aerosol microbiome research, which is a research
171 field still largely in its infancy.

172 The aim of this study was to demonstrate a new custom, multi-component DNA isolation
173 method optimized for SMS-based aerosol microbiome research and perform a comprehensive
174 performance benchmarking of the new method. The custom, multi-component DNA isolation
175 method was specifically developed to maximize the DNA yield and ensure unbiased biomass
176 lysis from low biomass environmental air samples. The DNA isolation method, hereafter referred
177 to as the "MetaSUB method", was developed for the MetaSUB Consortium (www.metasub.org)
178 to complement an ongoing global effort to characterize subway and urban environment
179 microbiomes using surface swab samples, by extending the effort to also include air samples. The
180 MetaSUB method was benchmarked against two other state-of-the-art DNA isolation methods: a
181 custom multi-component DNA isolation method developed for use in aerosol microbiome
182 research published by Jiang et al. [28], and the commercial ZymoBIOMICS DNA Microprep Kit
183 commonly used in environmental microbiome studies [38-41]. The performance of the three
184 DNA isolation methods was evaluated using both a mock microbial community and real-world
185 low biomass subway air samples. As part of this study, we also describe an end-to-end high-
186 volume filter-based air sampling, filter processing and DNA isolation method, hereafter referred
187 to as the "end-to-end MetaSUB method". Since the MetaSUB method, when used as an
188 integrated element of the end-to-end MetaSUB method, involves intermediate separation of the
189 filter extract into a pellet (subjected to additional lysis) and supernatant fraction that is combined
190 before final DNA purification, the relative contribution of the two fractions to the total DNA
191 yield and observed aerosol microbiome profile was also evaluated using subway air samples.
192

METHODS

MetaSUB method

The end-to-end MetaSUB method consists of an integrated air sampling, filter processing and DNA isolation scheme (Figure 1). The method relies on the use of high-volume, battery-operated, hand-portable, electret filter-based air samplers that allow for flexible, user-adjustable sampling time and rapid change of sampling locations, which in turn provides support for high spatiotemporal resolution air (aerosol biomass) sampling campaigns. Following air sampling, the electret microfibrous filter is subjected to a liquid filter extraction procedure, after which the entire filter extract is processed to avoid the need for downstream filtration or centrifugation steps to reduce the sample volume prior to DNA isolation, which may compromise the sample integrity regarding both biomass and DNA yield and composition (diversity).

Bioaerosol collection

Air (aerosol biomass) samples were collected with SASS3100 (Research International, Monroe, WA, USA), a high-volume electret microfibrous filter-based air sampler. The air sampler was powered by UBI-2590 lithium-ion rechargeable batteries (Ultralife batteries, NY, USA), operated at a flowrate of 265 liters of air per minute (LPM), and mounted on a tripod (~1.5 meters above ground) with the inlet facing downward (45°) to avoid direct deposition of large particles. After sampling, the electret filters were stored in 50 ml polypropylene tubes at -80 °C until further processing.

Filter extraction

Liquid extraction of filter-collected aerosol biomass from the electret filters was performed by removing the filters from their housing and transferring them into 50 ml polypropylene tubes pre-

loaded with 10 ml NucliSENS Lysis Buffer (BioMérieux, Marcy-l'Étoile, France). The sample tube was vortexed at maximum speed for 20 seconds before the filter was transferred into a 10 ml syringe with sterile forceps to extract residual liquid back into the sample tube before discarding the filter. The sample tube was centrifuged (7000 x g, 30 minutes) and the supernatant transferred to a new 50 ml polypropylene tube (referred to as filter extract supernatant).

DNA isolation

The pellet from the sample tube (referred to as filter extract pellet) was transferred to a polypropylene microcentrifuge tube with 1 ml PBS (pH 7.5, Sigma-Aldrich, St. Louis, MO, USA) and centrifuged (17 000 x g, 5 minutes). The resulting supernatant was carefully removed and combined with the filter extract supernatant. The pellet was dissolved in 150 µl PBS (pH 7.5). MetaPolyzyme (Sigma-Aldrich), a multi-enzyme cocktail, was prepared by dissolving the enzyme powder in 1 ml PBS (pH 7.5), and 10 µl MetaPolyzyme (5 mg/ml) and 5 µl sodium azide (0.1 M, Sigma-Aldrich) was added to the dissolved pellet sample. Enzymatic digestion was performed at 35°C for 1 hour in a Thermomixer (Eppendorf, Hamburg, Germany) at 1400 rpm. Subsequently, the sample was transferred to ZR BashingBead Lysis Tubes (0.1/0.5 mm beads, Zymo Research, Irvine, CA, USA) prefilled with 550 µl PowerSoil Bead Solution (Qiagen, Hilden, Germany) and 60 µl PowerSoil Solution C1 (Qiagen). Bead tubes were subjected to bead beating (17 000 x g, 3 minutes) in a Mini Bead Beater-8 (BioSpec Products, Bartlesville, OK, USA). Bead tubes were centrifuged (13 000 x g, 2 minutes) and the supernatant treated with Solution C2 and C3 according to the DNeasy PowerSoil protocol (Qiagen). The resulting supernatant was combined with the original filter extract supernatant before DNA purification. DNA was purified according to the manual protocol of the NucliSENS Magnetic Extraction Reagents kit (BioMérieux) with two modifications; magnetic silica suspension volume was

239 increased to 90 µl and incubation time was increased to 20 minutes. DNA samples were stored at
240 -80°C until further processing.
241
242 **DNA isolation method described by Jiang et al. (Jiang method)**
243 The custom, multi-component DNA isolation method (protocol steps 13-24) for air samples
244 published by Jiang et al. [28] is based on the DNeasy PowerSoil Kit and AMPure XP magnetic
245 bead separation. Jiang et al. introduced an incubation step in water bath (65°C) before bead
246 vortexing, and found that magnetic bead capture recovered more DNA than standard PowerSoil
247 spin columns. The DNA isolation method (protocol steps 13-24) published by Jiang et al.
248 (hereafter referred to as "Jiang") was used in this study with some minor modifications. Briefly,
249 all samples were pretreated with MetaPolyzyme (as described for the MetaSUB method), before
250 transfer to PowerBead tubes and continuation of DNA isolation according to the Jiang protocol.
251
252 **ZymoBIOMICS DNA Microprep Kit (Zymobiomics method)**
253 DNA isolation was performed according to the ZymoBIOMICS DNA Microprep Kit (Zymo
254 Research) protocol (hereafter referred to as "Zymobiomics") with some minor modifications.
255 Briefly, all samples were pretreated with MetaPolyzyme (as described for the MetaSUB method)
256 and bead beating was performed in a Mini Bead Beater-8 (BioSpec Products) for 3 minutes.
257
258 **Performance evaluation using mock microbial community**
259 The MetaSUB method was compared to the Jiang and Zymobiomics methods using a mock
260 microbial community with a defined quantity and composition. The ZymoBIOMICS Microbial
261 Community Standard (Zymo Research) contains ten microorganisms, eight bacteria (five Gram-

positives and three Gram-negatives) and two yeasts. For each sample, the mock community (10 µl), corresponding to a theoretical total DNA content of approximately 267 ng, was added to 140 µl PBS (pH 7.5) and treated with MetaPolyzyme (as described for the MetaSUB method) before DNA isolation according to the three DNA isolation methods. Total DNA and 16S rRNA gene copy yields were measured for four sample pairs processed with MetaSUB (N=4) and Jiang (N=4) and six sample pairs processed with MetaSUB (N=6) and Zymobiomics (N=6). The within-sample differences in total DNA and 16S rRNA gene copy yields were evaluated with one-sample t-tests (H_0: difference=0). All statistical analyses were performed in R (version3.4.3, www.R-project.org). A subset of the mock community samples were subjected to SMS (N=12): MetaSUB (N=4), Jiang (N=4), and Zymobiomics (N=4).

Performance evaluation using subway air samples

The MetaSUB method was compared to the Jiang and Zymobiomics methods using subway air samples. Only the DNA isolation part of the end-to-end MetaSUB method was evaluated since the air sampling and filter-processing steps were used to collect and process subway air samples to generate equal aliquots of aerosol biomass for paired difference comparisons. An overview of the common sample processing steps and the three evaluated DNA isolation methods is given in Table 1. Air samples were collected for 1 hour, corresponding to ~16 m^3 of air sampled (60 minutes sampling at 265 LPM), during daytime hours at subway stations (Tøyen, Grønland, Stortinget, Nationaltheateret and Majorstuen) in Oslo, Norway, in the period between October 2017 and May 2018. The filter-collected samples were extracted in 10 ml NucliSENS lysis buffer and split into two equal filter extract aliquots. The aliquots were centrifuged (7000 x g, 30 minutes) and only the pellet fractions were used for the comparison of DNA isolation methods. The supernatant fractions were subjected to DNA isolation separately (as described below) and

used to investigate the distribution of DNA in the intermediate pellet and supernatant fractions of the MetaSUB method. For the DNA isolation method comparison, 24 air samples were split and the pellets processed with either MetaSUB (N=10) and Jiang (N=10) or MetaSUB (N=14) and Zymobiomics (N=14), to enable within-sample comparisons between the MetaSUB method and the two other methods. Since the supernatant fraction was not included in the MetaSUB method for the DNA isolation method comparison, 10 ml of fresh NucliSENS lysis buffer was used. Negative controls (reagents) were included for each DNA isolation method. Total DNA and 16S rRNA gene copy yields were examined and within-sample differences were evaluated with one-sample t-tests (H_0: difference=0). All statistical analyses were performed in R (version3.4.3, www.R-project.org). A subset of the subway air samples (N=6) that had been split into two equal aliquots and processed with the three DNA isolation methods were subjected to SMS (N=12): MetaSUB (N=3) v. Jiang (N=3) and MetaSUB (N=3) v. Zymobiomics (N=3). A negative control (reagents) for each DNA isolation method was also subjected to SMS (N=3).

DNA distribution in intermediate pellet/supernatant fractions of the MetaSUB method

The filter extraction procedure of the MetaSUB method generates two intermediate fractions (pellet and supernatant) that are usually recombined before the final DNA purification (Figure 1). Differences in total DNA and 16S rRNA gene copy yields between pellet (N=24) and supernatant (N=24) fractions were therefore investigated. DNA was isolated from the supernatant fractions from subway air samples (described above) with the NucliSENS Magnetic Extraction Reagents kit as described for the MetaSUB method. Furthermore, to identify potential differences in DNA composition (diversity) between the pellet and supernatant fractions, DNA isolated with the MetaSUB method from six paired pellet and supernatant fractions (N=12) and one negative control (reagents; N=1) was subjected to SMS.

310

311 **Quantification of total DNA and 16S rRNA gene copies**

312 Total DNA was quantified with Qubit dsDNA HS assays (Life Technologies, Carlsbad, CA,
313 USA) on a Qubit 3.0 Fluorimeter (Life Technologies). Bacterial 16S rRNA gene copies were
314 determined with a 16S rRNA gene qPCR assay performed according to Liu et al. [42] on a
315 LightCycler 480 instrument (Roche Diagnostics, Oslo, Norway). Serial dilutions of *Escherichia*
316 *coli* DNA (seven 16S rRNA gene copies per genome) were used to generate a standard curve.

317

318 **Shotgun metagenomic sequencing (SMS)**

319 DNA isolated from mock community samples were subjected to SMS (150 bp paired-end)
320 multiplexed on a MiSeq (~24-30 M paired-end reads, Illumina, San Diego, CA, USA). Library
321 preparation was done with the Nextera DNA Flex kit (Illumina) according to the recommended
322 protocol. DNA isolated from subway air samples were subjected to SMS (150 bp paired-end)
323 multiplexed on one lane (~80-130M paired-end reads) on a HiSeq 3000 (Illumina). Library
324 preparation was done with the ThruPLEX DNA-Seq kit (Takara Bio, Mountain View, CA, USA)
325 according to the recommended protocol and 18 amplification cycles. Raw sequence reads were
326 demultiplexed, quality trimmed (Trim Galore, v0.4.3; ≥Q20, ≥50 bp) and underwent adapter
327 removal (Cutadapt, v1.16), before analysis on the One Codex platform with default settings [43].
328 One Codex taxonomic feature tables were imported into R and analyzed in the phyloseq package
329 [44].

330 All sequence reads not taxonomically assigned to the species level were removed from the
331 12 mock community samples. Since the aim was to gauge the relative contribution of the ten
332 bacterial and fungal species in the mock community across the three DNA isolation methods,

non-target features were binned as “other”. The comparison was made by plotting normalized abundances across all 12 samples.

For the six subway air samples that were split into equal aliquots and processed with the three DNA isolation methods, MetaSUB (N=3) v. Jiang (N=3) and MetaSUB (N=3) v. Zymobiomics (N=3), all taxonomic features not assigned to the genus or species level, along with human reads, were removed. Prevalent features reported in the negative control samples (>1% of within-sample reads, four in total, accounting for 94.5% of all reads in the negative controls) were stripped from the entire dataset before removing the negative controls. The cleaned samples varied in the number of assigned reads, ranging from 1 160 976 to 5 530 138. After examining the effect of rarefication on the α-diversity measures "Observed", "Shannon", and "Simpson" (Figure S1), all samples were rarified to the lowest common depth (1 160 976).

The six paired pellet and supernatant fractions from subway air samples processed with the MetaSUB method underwent the same procedure: removing features not assigned to the genus or species level, along with human reads, and prevalent features in the negative control (12 features, accounting for 99.3% of all reads in the negative control). The effect of rarefication was evaluated (Figure S2), and all samples were rarified to the lowest common depth (453 218).

The cleaned SMS datasets were divided into six groups corresponding to the three comparisons (MetaSUB v. Jiang, MetaSUB v. Zymobiomics, and MetaSUB pellet v. supernatant) before summarizing the top phyla, families, genera and species within each group. Taxonomic features with species-level assignment were extracted for analyses of within-sample diversity (α-diversity: "Observed", "Shannon", "Simpson"), where relevant groups were compared by fitting linear models. All features (read counts) were conglomerated to the genus level for analyses of among sample differences (β diversity); Bray Curtis distances were ordinated with PCoA and analyzed with MetaSUB/Jiang, MetaSUB/Zymobiomics, and pellet/supernatant, as predictors in

separate PERMANOVA tests. Distance estimation and PERMANOVA was performed with vegan (v.2.6.0, https://github.com/vegandevs/vegan/). Sample clustering was visualized with PCoA ordination. MegaBLAST analysis of forward reads against the NCBI non-redundant nucleotide database, followed by taxonomic binning using the native lowest common ancestor (LCA) algorithm in MEGAN6 [45], was used to perform a cross-kingdom analysis on the pellet/supernatant samples. Lastly, random forest classification models were performed, using 10 001 trees, with MetaSUB/Jiang, MetaSUB/Zymobiomics, and pellet/supernatant, as response variable and One Codex (species-level) taxonomic features as predictor variables. Separate tests using 501 trees and 1000 permutations were performed to evaluate statistical significance. The random forest models were built using randomForest [46].

Accession numbers

The sequence data has been deposited in the NCBI Sequence Read Archive under Bioproject ID# PRJNA542423 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA542423).

RESULTS

Performance evaluation using mock microbial community

The total DNA and 16S rRNA gene copy yields from mock community samples showed no significant differences between the MetaSUB method and the other two methods (Figure 2; Table 2A). However, the MetaSUB method obtained a higher 16S rRNA gene copy yield than Jiang with borderline significance ($P = 0.055$; Figure 2; Table 2A). The 12 mock community samples that were subjected to SMS showed similar distributions of all ten microbial species in the mock

community across the three methods, with MetaSUB and Zymobiomics being nearly identical (Figure 3).

Performance evaluation using subway air samples

The total DNA and 16S rRNA gene copy yields from subway air samples showed that the MetaSUB method obtained significantly higher total DNA and 16S rRNA gene copy yields than both Jiang and Zymobiomics (all $P < 0.001$; Figure 4; Table 2B).

The subway air samples that had been isolated with the MetaSUB method resulted in higher numbers of assigned reads than both Jiang (5 017 442 v. 2 630 115) and Zymobiomics (5 085 947 v. 4 601 016). Note that these results are average numbers from six individual air samples that were split and processed with the different method pairs, MetaSUB (N=3) v. Jiang (N=3) and MetaSUB (N=3) v. Zymobiomics (N=3). All samples reached saturation with regard to α-diversity at the lowest common assigned read depth (1 160 976, Figure S1) , which was the depth at which all samples were rarified to. Taxonomic distributions at the family level were highly similar between the samples processed with MetaSUB and Zymobiomics (Figure 5). The samples processed with MetaSUB and Jiang were also highly similar, but a skew was observed in the relative abundances for two of the three Jiang samples (Figure 5). In the MetaSUB v. Zymobiomics comparison, the top ten most abundant phyla were identical between the method pairs, but not identical in their ordering by abundance (Table 3). Of the top ten families, one was uniquely found in the MetaSUB results (*Staphylococcaceae*; lowest abundance) and one in the Zymobiomics results (*Rhodobacteraceae*; second lowest abundance; Table 3). Among the ten top genera, only two where unique for MetaSUB (*Hymenobacter* and *Staphylococcus*) and two for Zymobiomics (*Dietzia* and *Paracoccus*; Table 3). Among the top ten species in each group, only one was unique to MetaSUB (*Chlorogloea sp. CCALA 695*) and one to Zymobiomics

(*Lecanicillium sp. LEC01*; Table 3). In the MetaSUB v. Jiang comparison, there were more pronounced differences. The top ten phyla were not identical; *Acidobacteria* was only found in the MetaSUB results and *Planctomycetes* only in the Jiang results (Table 4). The top ten families were identical (but not in ordering); however, Jiang reported a substantially higher relative abundance of the family that was most abundant for both methods (*Micrococcaceae*, MetaSUB: 14% and Jiang: 25.6%; Table 4). Among the ten top genera, two where unique for MetaSUB (*Corynebacterium* and *Hymenobacter*) and two for Jiang (*Dietzia* and *Marmoricola*; Table 4). Here, the most abundant genus in Jiang (*Micrococcus*: 11.7%) was not the most abundant in MetaSUB (second most abundant; 5.60%) Among the top ten species in each group, only five species were present in both MetaSUB and Jiang results (Table 4).

Linear regression of within-sample α-diversity indices showed that MetaSUB reported significantly higher diversity estimates compared to Zymobiomics (Observed: *est*=734.3, *P*=0.01; Shannon: *est*=0.22, *P*=0.002; Simpson: *est*=0.00079, *P*=0.001; Figure 6), but no differences were shown between MetaSUB and Jiang α-diversity estimates (Observed: *est*=6531; Shannon: *est*=2.75; Simpson: *est*=0.028; all *P*>0.12; Figure 6). PERMANOVA tests of PCoA ordinated Bray Curtis distances found no significant differences among MetaSUB and Jiang (*P*=0.1) or MetaSUB and Zymobiomics (*P*=0.1; Figure 7).

The random forest classification analysis, where species-level features were scored by their ability to correctly classify the DNA isolation method used, had a perfect out-of-bag error of 0%, and a significant permutation test (*P*>0.02) for MetaSUB v. Zymobiomics. For MetaSUB v. Jiang, the classification model had an out-of-bag error of 16%, but also here the permutation test was significant (*P*=0.01). For MetaSUB v. Zymobiomics, the proportions of archaea, bacteria and fungi across the dataset and in the 100 species most important for correctly classifying samples as either MetaSUB or Zymobiomics were highly similar. However, for MetaSUB v. Jiang, 6.0% of

all assigned species were fungi, while among the 100 species most important for classification, 20 were fungi. These 20 fungal species all had higher abundances in the MetaSUB results (Figure S4). The top 30 most important features for both classification models are shown in Figure 8.

DNA distribution in intermediate pellet/supernatant fractions of the MetaSUB method

The distribution of DNA in terms of both amount and composition (diversity) in the intermediate pellet and supernatant fractions of the MetaSUB method was investigated by separately isolating DNA from the two fractions from subway air samples. The results revealed that the supernatant fraction contained 42%±6 of the total DNA yield and 32%±12 of the total 16S rRNA gene copy yield (Figure S2).

The SMS results showed that the pellet samples had a higher number of assigned reads than supernatant samples (2 584 159 v. 1 609 457). Rarefication plots of pellet and supernatant samples indicated that α-diversity indices (particularly Shannon and Simpson) reached saturation before the lowest common assigned read depth (453 218, Figure S2), which was the depth at which all samples were rarified to. The taxonomic distributions in pellet and supernatant samples were largely similar (Table 5; Figure 9). The top ten phyla were identical in the pellet and supernatant group, but not identical in their ordering by abundance (Table 5). Of the top ten families, one was uniquely found in the pellet group (*Rhodobacteraceae*; second lowest abundance) and one only in the supernatant group (*Deinococcaceae*; lowest abundance; Table 5). Among the ten top genera, only one was unique for the pellet group (*Marmoricola*) and one for the supernatant group (*Deinococcus*; Table 5). Among the top ten species in each group, seven species were present in both (Table 5). Linear regression of within-sample α-diversity indices revealed no significant differences between pellet and supernatant samples (Figure 10; all $P>0.38$). A PERMANOVA test of PCoA ordinated Bray Curtis distances found that whether

452 samples were pellet or supernatant explained 51.7% of the among-sample variance in diversity
453 (Figure 11; P=0.004).

454 The cross-kingdom analysis revealed substantial differences in the relative representation
455 of almost all examined groups (archaea, bacteria, fungi, plants, human, and other animals)
456 between the pellet and supernatant samples (Figure 12). While very few reads were assigned to
457 archaea, only pellet samples had any coverage within this group. Pellet samples also had a higher
458 relative number of assigned reads across all sample pairs within bacteria and fungi. The
459 supernatant had a higher relative number of reads assigned as human and other animals, while
460 plants saw similar representation in pellet and supernatant samples.

461 The random forest classification analysis, where species-level features were scored by
462 their ability to correctly classify the pellet and supernatant groups, had a perfect out-of-bag error
463 of 0%, and the permutation test was statistically significant (P>0.001). In the entire dataset, 6.0%
464 of the features were assigned as fungi and 0.3% were assigned as archaea, while among the 100
465 species with the highest variable importance in our classification model, 56 were fungi and two
466 where archaea. Among the top 50 species, 30 were fungi and one archaea. The top 30 most
467 important features are shown in Figure 13.

468

469 **DISCUSSION**

470

471 Here, we have demonstrated a new custom, multi-component DNA isolation method ("the
472 MetaSUB method") optimized for SMS-based aerosol microbiome research. By processing the
473 entire filter extract, in combination with thorough chemical, enzymatic and mechanical lysis and
474 DNA purification using magentic beads, the MetaSUB method drastically improves the DNA
475 yield from low biomass air samples and reduces the risk of introducing microbiome profile bias.

476 Comprehensive performance benchmarking of the MetaSUB method against two other state-of-
477 the-art DNA isolation methods was done with both a mock microbial community and real-world
478 subway air samples. The benchmarking revealed that the MetaSUB method obtains significantly
479 higher DNA yields from subway air samples than the other two methods, which is an important
480 performance parameter for successful implementation of SMS on low biomass air samples. SMS
481 of subway air samples revealed that the MetaSUB method resulted in higher numbers of assigned
482 reads than the other two methods, reported higher diversity than Zymobiomics, and gave better
483 representation of certain fungal species than Jiang. All three DNA isolation methods performed
484 similarly well on mock microbial community samples, both in terms of DNA yield and
485 community representation. As part of this study, we have also described an end-to-end air
486 sampling, filter processing and DNA isolation method ("the end-to-end MetaSUB method")
487 optimized for SMS-based aerosol microbiome research. The end-to-end MetaSUB method relies
488 on the use of SASS 3100 high-volume electret microfibrous filter-based air samplers and was
489 shown to be capable of recovering sufficient DNA yields from short-duration subway air
490 samples, which corresponded to ~8 m^3 of air sampled (30 minutes sampling at 265 LPM) in this
491 study, to facilitate high temporal resolution SMS-based aerosol microbiome investigations.
492 The performence evaluation of the three DNA isolation methods (MetaSUB, Jiang and
493 Zymobiomics) revealed no significant differences regarding total DNA and 16S rRNA gene copy
494 yields when isolating DNA from mock microbial community samples (Figure 2). Furthermore,
495 SMS of mock community samples showed that the three methods gave highly similar
496 representation of the ten microbial species present in the mock community (Figure 3). However,
497 on subway air samples, the MetaSUB method outperformed both Jiang and Zymobiomics
498 regarding total DNA and 16S rRNA gene copy yields (Figure 4). SMS analyses of subway air
499 samples that had been split and isolated with either MetaSUB and Jiang or MetaSUB and

Zymobiomics revealed significant differences among the three methods. The numbers of assigned reads were higher for MetaSUB in both comparisons, which is congruent with the higher DNA yields seen for the MetaSUB method (Figure 4). We also observed significantly higher α-diversity estimates for MetaSUB compared to Zymobiomics (Figure 6). One of the three samples processed with Jiang showed higher α-diversity than all three MetaSUB samples, while the other two Jiang samples showed substantially lower diversity estimates (Figure 6), which rendered the comparison against MetaSUB non-significant for all α-diversity indices. We have no conclusive explanation for this pattern; however, we observed that the two low-scoring Jiang samples had high duplicate sequence read proportions (62.4% and 71.8%) compared to all other samples (average: 18.6%), and postulate that the variable performance may be related to the recovery of insufficient DNA yields from two of the Jiang samples to allow for reliable SMS. Furthermore, the random forest classification analysis indicates that the Jiang method does not produce the same representation for certain fungal species as the MetaSUB method, since out of the 100 most important species for distinguishing between MetaSUB and Jiang processed samples, 20 where fungal, while across the entire dataset, only 6% of the species were fungal. All of these 20 fungal species had higher representation in MetaSUB samples (Figure S4).

Our findings highlight the importance of benchmarking DNA isolation methods with both mock communities and real-world samples since the complexity found in the real-world environment is not easily recreated. The observed DNA yield differences among the three methods can probably be attributed to a combination of sub-process efficiency differences, since the methods rely on different combinations of lysis (chemical, enzymatic, and/or mechanical), inhibitor removal and sample clean-up, and DNA purification (magnetic beads and silica spin filters) principles (Table 1). During customization of DNA isolation methods it is therefore important to keep in mind that even subtle procedural differences, including choice of bead

524 solution, intensity and time settings for the bead beating process [47, 48], and different enzyme
525 combinations, may have a large effect on the ultimate biomass lysis efficiency [31]. By replacing
526 the spin columns in the PowerSoil Kit with AMPure XP Beads (magnetic bead purification),
527 Jiang et al. [28] observed a three-fold increase in DNA yield. The multi-component MetaSUB
528 method was developed by adopting and customizing sub-processes from several different DNA
529 isolation methods in an effort to ensure maximized DNA recovery and thorough unbiased
530 biomass lysis. Note that for the performance benchmarking of DNA isolation methods in this
531 study, only the intermediate pellet fraction of the MetaSUB method was used to facilitate an
532 equal comparison between the three different DNA isolation methods (Figure 1). The
533 intermediate supernatant fraction would normally also be included in the MetaSUB method and
534 would have constituted approximately 72% of additional DNA, thereby making the DNA yield
535 differences even more pronounced.

536 Since the filter extraction procedure in the MetaSUB method produces intermediate pellet
537 and supernatant fraction that are combined before DNA purification, we investigated differences
538 in DNA amount and composition (diversity) between the two fractions in an effort to better
539 understand the benefit of including supernatants (i.e., increased DNA yield) and the risk of not
540 including them (i.e., microbiome profile bias). The observed microbial diversity in paired pellet
541 and supernatant samples was highly similar at the phylum (Table 5), family (Table 5; Figure 9),
542 genus (Table 5) and species (Table 5) levels. Note, however, with direct examination of only the
543 most abundant taxonomic groups in Table 5 and Figure 9, the similarities do not necessarily
544 extend to groups with low abundance. While we did not find any differences among the pellet
545 and supernatant samples in α-diversity (Figure 10), which describes within-sample diversity,
546 there was significant diversity nested among samples, of which the pellet/supernatant grouping
547 explained 51.7% (Figure 11). The cross-kingdom analyses revealed differences in the taxonomic

548 composition of pellet and supernatant samples (Figure 12). While human DNA constituted a
549 relatively large proportion of eukaryotic reads, it did not account for all of the difference
550 observed among pellet and supernatant samples within this kingdom; on average, human reads
551 constituted 18% of assigned reads in pellets and 42% in supernatants based on the cross-kingdom
552 analysis (Figure 12). Human reads reported by One Codex also had a higher relative abundance
553 in supernatants (31% and 67% of assigned reads in pellets and supernatants, respectively).
554 Features assigned as archaea were exclusively observed in pellets; however, caution should be
555 used when interpreting these results, since only eleven features were assigned to this kingdom.
556 The random forest classification model revealed that fungi were particularly important in
557 separating pellet and supernatant samples, especially when accounting for the relatively low
558 representation of fungi across all samples. A recent study by Mbareche et al. has shown that the
559 use of traditional processing methods, e.g., filter extract processing where the supernatant
560 fraction is discarded after a centrifugation step, may lead to an underrepresentation of fungi [49].
561 In conclusion, concerning the most abundant microbial groups and within-sample diversity
562 estimates, there is little difference between the pellet and supernatant fractions. However, the
563 between-sample diversity analyses show that potentially important diversity may be lost if the
564 entire filter extract is not processed, and that an appreciable amount of this diversity is nested in
565 fungi. In addition, a more general but potentially important reason for processing the entire filter
566 extract in the context of high-volume filter-collected air samples is the variable resistance
567 different types of microorganisms have against sampling-associated stress factors. While stress-
568 resistant microorganisms may be relatively unaffected by sampling-associated stress, stress-
569 sensitive organisms, e.g. Gram-negative bacteria, may become membrane-impaired, ruptured or
570 even completely lysed due to sampling-associated desiccation during high-volume dry filter
571 collection and subsequent osmotic shock during liquid filter extraction. DNA that becomes

572 liberated from membrane-impaired, ruptured or lysed microorganisms will generally not be
573 recovered by standard centrifugation or filtration processes intended for intact organism capture,
574 and may therefore remain in the supernatant or filtrate fraction.

575 Taken together, the demonstrated performance of the MetaSUB method, including
576 drastically improved DNA yield from subway air samples and reduced risk of microbiome profile
577 bias, highlights the benefit of isolating DNA from the entire filter extract. However, the need for
578 isolating DNA from a relatively large sample volume, a 10 ml filter extract in this work, limits
579 the available selection of out-of-the-box commercial DNA isolation kits and introduces a
580 customization need to ensure reliable performance regarding thorough unbiased biomass lysis,
581 sufficient inhibitor removal and sample clean-up, and efficient DNA recovery. The custom,
582 multi-component MetaSUB method is therefore a relatively hands-on (manual), labor-intensive
583 DNA isolation method compared to many out-of-the-box commercial DNA isolation kits.
584 However, an experienced operator can perform the MetaSUB method, including all processing
585 and incubation steps, in approximately three hours, while the estimated total processing time for
586 12 air samples is approximately four hours. Furthermore, even without considering the associated
587 benefits of isolating DNA from the entire filter extract, the use of a custom, multi-component
588 DNA isolation method, including extensively modified commercial DNA isolation kits, appears
589 to be necessary to overcome the unique and inherent challenges associated with SMS-based
590 aerosol microbiome research in complex low biomass air environments [2, 21, 28, 29].
591

592 **CONCLUSIONS**

593

594 By demonstrating and benchmarking a new custom, multi-component DNA isolation method (the
595 MetaSUB method) optimized for SMS-based aerosol microbiome research, this study contributes

596 to improved selection, harmonization, and standardization of DNA isolation methods. In the
597 context of SMS-based aerosol microbiome research in low biomass air environments, our
598 findings highlight the importance of ensuring end-to-end sample integrity and using DNA
599 isolation methods with well-defined performance characteristics regarding both DNA yield and
600 community representation. A comprehensive performance benchmarking of the MetaSUB
601 method against two other state-of-the-art DNA isolation methods (Jiang and Zymobiomics) was
602 done with both a mock microbial community and real-world subway air samples. All three DNA
603 isolation methods performed similarly well on mock community samples, both in terms of DNA
604 yield and community representation. However, the MetaSUB method obtained significantly
605 higher DNA yields than the other two methods from subway air samples, which is an important
606 performance parameter for successful implementation of SMS on low biomass air samples. We
607 also observed significant differences regarding SMS-based community representation across the
608 three methods when applying them to subway air samples. The MetaSUB method reported higher
609 α-diversity estimates than Zymobiomics, while Jiang appeared to underrepresent certain fungal
610 species. By processing the entire filter extract, in combination with thorough chemical, enzymatic
611 and mechanical biomass lysis, and efficient DNA recovery using magnetic beads, the MetaSUB
612 method may drastically improve the DNA yield from low biomass air samples and reduce the risk
613 of aerosol microbiome profile bias. Taken together, the demonstrated performance characteristics
614 suggest the MetaSUB method could be used to improve the quality of SMS-based aerosol
615 microbiome research in low biomass air environments. Furthermore, the MetaSUB method, when
616 used in combination with the described high-volume filter-based air sampling, filter processing
617 and DNA isolation scheme (the end-to-end MetaSUB method), could be used to improve the
618 temporal resolution in aerosol microbiome research by reducing the sampling time required to
619 obtain sufficient DNA yields for SMS analysis.

DECLARATIONS

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The sequence data has been deposited in the NCBI Sequence Read Archive under Bioproject ID# PRJNA542423 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA542423).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MD conceived, designed and led the study. JG performed the data analysis. LV-M contributed to the experimental work and the data analysis. KO-B performed the experimental work and contributed to the data analysis. All authors contributed to the manuscript writing and approved the final manuscript.

644

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TABLES AND FIGURES

Table 1 - Overview of the three DNA isolation methods evaluated in this work.

Method	**MetaSUB**	**Jiang**	**Zymobiomics**
Common processing steps (used to generate equal aerosol biomass aliquots for paired difference comparison)			
Filter extraction (filter-to-liquid)	NucliSENS lysis buffer		
Lysis (enzymatic)	MetaPolyzyme multi-enzyme cocktail		
Method-specific processing steps (used for paired difference comparison on equal aerosol biomass aliquots)			
Lysis (mechanical)	ZR BashingBead Tubes with PowerSoil Bead Solution and Solution C1. Bead beating for 3 min	PowerSoil Bead Tubes with PowerSoil Bead Solution and Solution C1 incubated at 65°C for 15 min. Bead vortexing for 15 min	ZR BashingBead Tubes with Zymobiomics lysis solution. Bead beating for 3 min
Inhibitor removal and sample clean-up	PowerSoil Solution C2 and C3	PowerSoil Solution C2 and C3	Zymo-Spin IV and Zymo-Spin IV-µHRC Columns
DNA purification	NucliSENS magnetic beads	AMPure XP magnetic beads	Zymo-Spin IC-Z Column

Table 2 – Benchmarking results for MetaSUB, Jiang, and Zymobiomics on mock microbial community and subway air samples.

A) Mock microbial community

Measure	Within-sample differences	Est	95% CI	T	df	P
Total DNA yield (ng)	MetaSUB – Jiang	-3	[-28.5 , 22.5]	-0.37	3	0.73
	MetaSUB – Zymobiomics	-7	[-46.5, 32.5]	-0.45	5	0.67
16S rRNA gene copy yield (copies)	MetaSUB – Jiang	17107	[-634, 34848]	3.07	3	0.055
	MetaSUB – Zymobiomics	-11452	[-83155, 60251]	-0.41	5	0.70

B) Subway air samples

Measure	Within-sample differences	Est	95% CI	T	df	P
Total DNA yield (ng)	MetaSUB – Jiang	1.07	[0.77, 1.37]	8.01	9	< 0.001
	MetaSUB – Zymobiomics	1.35	[0.86, 1.85]	5.94	13	< 0.001
16S rRNA gene copy yield (copies)	MetaSUB – Jiang	5046	[3882, 6211]	9.80	9	< 0.001
	MetaSUB – Zymobiomics	3451	[1741, 5162]	4.36	13	< 0.001

One-sample t-test on within-sample differences (H_0: difference in within-sample measurements = 0) for different method pairs with mock microbial community (A) and subway air samples (B). Measures from Jiang/Zymobiomics were subtracted from the MetaSUB measures: the estimate (*est*) gives the departure from zero of the resultant values (larger than zero values indicate that MetaSUB had a higher yield than Jiang/Zymobiomics).

Table 3 – Abundant microbial taxa in subway air samples (MetaSUB v. Zymobiomics method).

MetaSUB v. Zymobiomics							
MetaSub				**Zymobiomics**			
	prevalence		**abundance**		**prevalence**		**Abundance**
Phylum	**mean**	**total**		**Phylum**	**mean**	**total**	
Actinobacteria	2.7	9271	51.61 %	*Actinobacteria*	2.7	9301	57.72 %
Proteobacteria	2.3	14269	27.02 %	*Proteobacteria*	2.2	13421	23.67 %
Firmicutes	1.6	3250	4.76 %	*Ascomycota*	2.5	1899	4.91 %
Bacteroidetes	2.0	2999	4.74 %	*Basidiomycota*	2.4	622	4.75 %
Ascomycota	2.4	1843	4.42 %	*Firmicutes*	1.3	2705	3.30 %
Basidiomycota	2.3	606	3.84 %	*Bacteroidetes*	1.5	2257	2.77 %
Cyanobacteria	2.3	526	1.35 %	*Deinococcus-Thermus*	2.6	204	1.26 %
Deinococcus-Thermus	2.5	196	1.23 %	*Cyanobacteria*	1.9	432	0.66 %
Euryarchaeota	1.7	450	0.62 %	*Euryarchaeota*	1.5	393	0.58 %
Acidobacteria	2.5	112	0.08 %	*Acidobacteria*	2.6	114	0.08 %
	prevalence		**abundance**		**prevalence**		**Abundance**
Family	**mean**	**total**		**Family**	**mean**	**total**	
Micrococcaceae	2.8	801	15.72 %	*Micrococcaceae*	2.8	822	20.34 %
Nocardioidaceae	2.8	308	6.51 %	*Nocardioidaceae*	2.9	311	6.65 %
Microbacteriaceae	2.6	1222	4.89 %	*Microbacteriaceae*	2.6	1235	4.70 %
Sphingomonadaceae	2.8	1006	3.88 %	*Geodermatophilaceae*	3.0	150	4.24 %
Geodermatophilaceae	3.0	150	3.67 %	*Sphingomonadaceae*	2.8	1013	4.04 %
Moraxellaceae	1.9	608	3.54 %	*Intrasporangiaceae*	3.0	211	3.46 %
Intrasporangiaceae	3.0	211	3.15 %	*Corynebacteriaceae*	2.6	571	3.35 %
Comamonadaceae	2.6	800	2.94 %	*Comamonadaceae*	2.7	838	3.02 %
Corynebacteriaceae	2.6	568	2.93 %	*Rhodobacteraceae*	2.6	1484	2.28 %
Staphylococcaceae	2.3	401	2.17 %	*Moraxellaceae*	1.6	533	2.14 %
	prevalence		**abundance**		**prevalence**		**Abundance**
Genus	**mean**	**total**		**Genus**	**mean**	**total**	
Micrococcus	2.9	29	7.27 %	*Micrococcus*	2.9	29	9.34 %
Arthrobacter	2.8	399	5.32 %	*Arthrobacter*	2.9	413	6.44 %
Nocardioides	2.8	176	3.51 %	*Nocardioides*	2.9	177	3.57 %
Sphingomonas	2.9	461	3.09 %	*Kocuria*	2.7	79	3.44 %
Corynebacterium	2.6	568	2.93 %	*Corynebacterium*	2.6	571	3.35 %
Psychrobacter	2.0	141	2.85 %	*Sphingomonas*	2.9	464	3.23 %
Blastococcus	3.0	60	2.44 %	*Blastococcus*	3.0	60	2.92 %
Staphylococcus	2.2	308	2.02 %	*Psychrobacter*	2.7	184	1.82 %
Kocuria	2.7	79	2.02 %	*Dietzia*	3.0	51	1.70 %
Hymenobacter	2.9	98	1.92 %	*Paracoccus*	2.9	167	1.58 %
	prevalence		**abundance**		**prevalence**		**Abundance**
Species	**mean**	**total**		**Species**	**mean**	**total**	
Micrococcus luteus	3.0	3	1.13 %	*Micrococcus luteus*	3.0	3	1.44 %
Arthrobacter sp. H41	3.0	3	1.00 %	*Arthrobacter sp. H41*	3.0	3	1.38 %
Rubrobacter aplysinae	3.0	3	0.78 %	*Rubrobacter aplysinae*	3.0	3	0.97 %
Arthrobacter sp. Leaf234	3.0	3	0.67 %	*Arthrobacter sp. Leaf234*	3.0	3	0.89 %
Marmoricola sp. Leaf446	3.0	3	0.65 %	*Stereum hirsutum*	3.0	3	0.76 %
Chlorogloea sp. CCALA 695	3.0	3	0.63 %	*Marmoricola sp. Leaf446*	3.0	3	0.76 %
Deinococcus marmoris	3.0	3	0.60 %	*Fomitopsis pinicola*	3.0	3	0.68 %
Stereum hirsutum	3.0	3	0.50 %	*Blastococcus sp. DSM 44268*	3.0	3	0.64 %
Blastococcus sp. DSM 44268	3.0	3	0.48 %	*Deinococcus marmoris*	3.0	3	0.57 %
Fomitopsis pinicola	3.0	3	0.45 %	*Lecanicillium sp. LEC01*	3.0	3	0.57 %

Top ten microbial phyla, families, genera and species in subway air samples (N=3) that were split and processed with the MetaSUB (N=3) and Zymobiomics (N=3) methods.

869 **Table 4** – Abundant microbial taxa in subway air samples (MetaSUB v. Jiang method).

MetaSUB v. Jiang							
MetaSub				**Jiang**			
	prevalence		**abundance**		**prevalence**		**Abundance**
Phylum	**mean**	**total**		**Phylum**	**mean**	**total**	
Actinobacteria	2.7	9248	48.69 %	*Actinobacteria*	1.1	3625	60.08 %
Proteobacteria	2.3	14379	28.58 %	*Proteobacteria*	1.0	6090	23.40 %
Ascomycota	2.4	1843	5.28 %	*Firmicutes*	0.7	1510	4.58 %
Bacteroidetes	2.1	3148	5.10 %	*Bacteroidetes*	0.9	1436	3.40 %
Firmicutes	1.6	3250	4.52 %	*Ascomycota*	1.1	822	2.78 %
Basidiomycota	2.3	598	3.95 %	*Deinococcus-Thermus*	1.0	77	1.94 %
Cyanobacteria	2.3	518	1.44 %	*Basidiomycota*	1.1	286	1.91 %
Deinococcus-Thermus	2.4	190	1.35 %	*Cyanobacteria*	1.1	243	1.50 %
Euryarchaeota	1.9	495	0.63 %	*Euryarchaeota*	0.9	231	0.15 %
Acidobacteria	2.6	113	0.08 %	*Planctomycetes*	1.2	52	0.04 %
	prevalence		**abundance**		**prevalence**		**abundance**
Family	**mean**	**total**		**Family**	**mean**	**total**	
Micrococcaceae	2.7	785	13.96 %	*Micrococcaceae*	1.2	336	25.63 %
Nocardioidaceae	2.8	301	6.58 %	*Nocardioidaceae*	1.1	123	7.02 %
Microbacteriaceae	2.6	1213	5.05 %	*Geodermatophilaceae*	1.3	65	4.77 %
Sphingomonadaceae	2.8	1010	4.20 %	*Microbacteriaceae*	1.0	486	4.45 %
Moraxellaceae	2.0	655	4.12 %	*Intrasporangiaceae*	1.4	97	4.15 %
Comamonadaceae	2.4	750	3.68 %	*Moraxellaceae*	0.9	289	4.05 %
Geodermatophilaceae	3.0	149	3.59 %	*Sphingomonadaceae*	1.0	357	3.92 %
Intrasporangiaceae	3.0	210	3.23 %	*Comamonadaceae*	1.1	337	2.60 %
Hymenobacteraceae	2.9	182	2.17 %	*Staphylococcaceae*	0.9	169	2.39 %
Flavobacteriaceae	2.2	1594	2.04 %	*Dietziaceae*	1.1	19	1.99 %
	prevalence		**abundance**		**prevalence**		**abundance**
Genus	**mean**	**total**		**Genus**	**mean**	**total**	
Arthrobacter	2.7	391	5.63 %	*Micrococcus*	2.1	21	11.69 %
Micrococcus	2.8	28	5.60 %	*Arthrobacter*	1.1	153	9.34 %
Nocardioides	2.7	167	3.50 %	*Kocuria*	1.2	34	3.53 %
Psychrobacter	1.9	131	3.40 %	*Psychrobacter*	0.9	64	3.46 %
Sphingomonas	2.9	465	3.38 %	*Nocardioides*	1.0	61	3.38 %
Blastococcus	3.0	59	2.30 %	*Sphingomonas*	1.0	155	3.20 %
Corynebacterium	2.6	559	2.04 %	*Blastococcus*	1.3	26	3.16 %
Hymenobacter	2.9	98	2.02 %	*Marmoricola*	2.0	12	2.78 %
Staphylococcus	2.0	289	1.77 %	*Staphylococcus*	0.9	128	2.33 %
Kocuria	2.7	79	1.76 %	*Dietzia*	1.1	19	1.99 %
	prevalence		**abundance**		**prevalence**		**abundance**
Species	**mean**	**total**		**Species**	**mean**	**total**	
Arthrobacter sp. H41	3.0	3	1.14 %	*Arthrobacter sp. H41*	3.0	3	2.36 %
Micrococcus luteus	3.0	3	0.88 %	*Arthrobacter sp. Leaf234*	3.0	3	2.15 %
Chlorogloea sp. CCALA 695	3.0	3	0.74 %	*Marmoricola sp. Leaf446*	3.0	3	1.71 %
Rubrobacter aplysinae	3.0	3	0.71 %	*Deinococcus marmoris*	3.0	3	1.56 %
Arthrobacter sp. Leaf234	3.0	3	0.71 %	*Blastococcus sp. DSM 44268*	3.0	3	1.24 %
Aspergillus sp. MA 6041	3.0	3	0.69 %	*Arthrobacter agilis*	3.0	3	1.23 %
Marmoricola sp. Leaf446	3.0	3	0.67 %	*Chlorogloea sp. CCALA 695*	3.0	3	1.14 %
Deinococcus marmoris	3.0	3	0.66 %	*Marmoricola scoriae*	3.0	3	0.84 %
Acidovorax temperans	3.0	3	0.61 %	*Janibacter sp. Soil728*	3.0	3	0.82 %
Stereum hirsutum	3.0	3	0.57 %	*Mrakia frigida*	3.0	3	0.77 %

870 Top ten microbial phyla, families, genera and species in subway air samples (N=3) that were split

871 and processed with the MetaSUB (N=3) and Jiang (N=3) methods.

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876 **Table 5** – Abundant microbial taxa in pellet and supernatant fractions from subway air samples

877 (MetaSUB method).

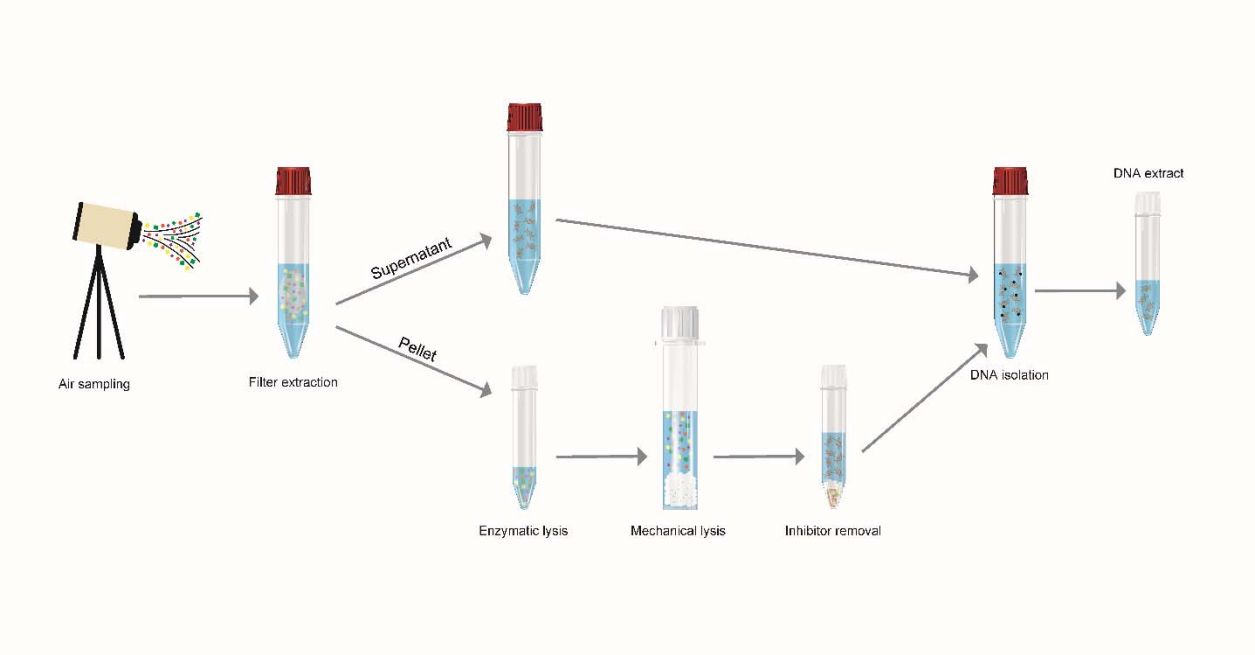
Supernatant				Pellet			
	Prevalence		abundance		Prevalence		abundance
Phylum	**mean**	**Total**		**Phylum**	**mean**	**Total**	
Actinobacteria	3.5	9479	51.21%	*Actinobacteria*	4.0	10653	53.53%
Proteobacteria	3.1	14453	28.87%	*Proteobacteria*	3.4	15894	24.50%
Bacteroidetes	2.8	3139	7.10%	*Basidiomycota*	3.7	717	5.42%
Firmicutes	2.5	3816	4.23%	*Ascomycota*	4.2	2109	5.23%
Deinococcus-Thermus	3.2	184	3.06%	*Bacteroidetes*	2.9	3202	4.44%
Basidiomycota	1.5	290	1.86%	*Firmicutes*	2.6	3874	2.68%
Ascomycota	2.4	1231	1.62%	*Deinococcus-Thermus*	3.7	214	2.05%
Cyanobacteria	2.8	431	1.47%	*Cyanobacteria*	3.6	557	1.03%
Euryarchaeota	1.5	260	0.17%	*Euryarchaeota*	2.1	367	0.66%
Acidobacteria	3.4	103	0.10%	*Acidobacteria*	4.4	132	0.11%
	Prevalence		abundance		Prevalence		abundance
Family	**mean**	**Total**		**Family**	**mean**	**Total**	
Micrococcaceae	3.6	735	11.27%	*Micrococcaceae*	3.9	795	14.70%
Nocardioidaceae	3.1	236	9.32%	*Nocardioidaceae*	3.0	222	8.26%
Microbacteriaceae	3.4	921	5.24%	*Geodermatophilaceae*	2.9	97	4.65%
Sphingomonadaceae	3.8	1119	5.20%	*Microbacteriaceae*	3.5	958	4.53%
Geodermatophilaceae	3.7	121	4.60%	*Sphingomonadaceae*	4.3	1260	4.22%
Moraxellaceae	2.9	794	4.14%	*Intrasporangiaceae*	3.8	199	4.20%
Hymenobacteraceae	3.5	150	3.93%	*Moraxellaceae*	2.6	717	2.77%
Intrasporangiaceae	3.7	192	3.81%	*Corynebacteriaceae*	4.2	881	2.61%
Corynebacteriaceae	3.7	789	3.77%	*Rhodobacteraceae*	4.3	1698	2.33%
Deinococcaceae	4.0	117	3.03%	*Hymenobacteraceae*	3.8	163	2.15%
	Prevalence		abundance		Prevalence		abundance
Genus	**mean**	**Total**		**Genus**	**mean**	**Total**	
Arthrobacter	3.3	320	6.31%	*Arthrobacter*	3.7	357	7.74%
Sphingomonas	3.7	512	4.40%	*Micrococcus*	3.9	39	4.19%
Nocardioides	3.0	127	3.87%	*Sphingomonas*	4.1	560	3.40%
Hymenobacter	3.4	89	3.85%	*Nocardioides*	2.9	120	3.14%
Corynebacterium	3.8	767	3.73%	*Blastococcus*	3.5	39	3.13%
Psychrobacter	2.5	143	3.24%	*Corynebacterium*	4.2	854	2.59%
Deinococcus	4.0	117	3.03%	*Marmoricola*	5.8	23	2.56%
Friedmanniella	6.0	18	2.85%	*Friedmanniella*	6.0	18	2.38%
Blastococcus	4.1	45	2.60%	*Psychrobacter*	2.3	133	2.37%
Micrococcus	4.6	46	2.31%	*Hymenobacter*	3.2	84	2.04%
	Prevalence		abundance		Prevalence		abundance
Species	**mean**	**Total**		**Species**	**mean**	**Total**	
Deinococcus marmoris	6.0	6	2.07%	*Arthrobacter sp. H41*	6.0	6	2.07%
Arthrobacter sp. Leaf234	6.0	6	1.91%	*Micrococcus luteus*	6.0	6	1.96%
Marmoricola sp. Leaf446	6.0	6	1.31%	*Rubrobacter aplysinae*	6.0	6	1.52%
Friedmanniella flava	6.0	6	1.05%	*Arthrobacter sp. Leaf234*	6.0	6	1.51%
Friedmanniella sagamiharensis	6.0	6	1.03%	*Marmoricola sp. Leaf446*	6.0	6	1.42%
Cutibacterium acnes	6.0	6	1.02%	*Blastococcus sp. DSM 44268*	6.0	6	1.32%
Mrakia frigida	6.0	6	1.02%	*Deinococcus marmoris*	6.0	6	1.27%
Blastococcus sp. DSM 44268	6.0	6	1.02%	*Arthrobacter agilis*	6.0	6	0.95%
Micrococcus luteus	6.0	6	1.01%	*Friedmanniella flava*	6.0	6	0.89%
Arthrobacter sp. H41	6.0	6	0.98%	*Stereum hirsutum*	6.0	6	0.86%

878 Top ten microbial phyla, families, genera and species in the intermediate pellet (N=6) and

879 supernatant (N=6) fractions from subway air samples (N=6) processed with the MetaSUB

880 method.

881 **Figure 1** – Overview of the end-to-end MetaSUB method.



882 Air samples collected using SASS 3100 high-volume filter-based air samplers (Research
883 International) on SASS 3100 electret microfibrous filters (Research International) are extracted in
884 NucliSENS lysis buffer (BioMérieux) and centrifuged, resulting in intermediate separation of the
885 filter extract into a pellet and supernatant fraction. The pellet is subjected to additional lysis with
886 MetaPolyzyme (Sigma-Aldrich), a multi-enzyme cocktail, followed by bead beating with ZR
887 Bashing Tubes (Zymo Research) filled with PowerSoil Bead Solution (Qiagen) and Solution C1
888 (Qiagen). Inhibitor removal and sample clean-up is performed with Solution C2 and C3 (Qiagen).
889 The supernatant and pellet fractions are recombined and DNA purification performed according
890 to the manual protocol of the NucliSENS Magnetic Extraction Reagents kit (BioMérieux).
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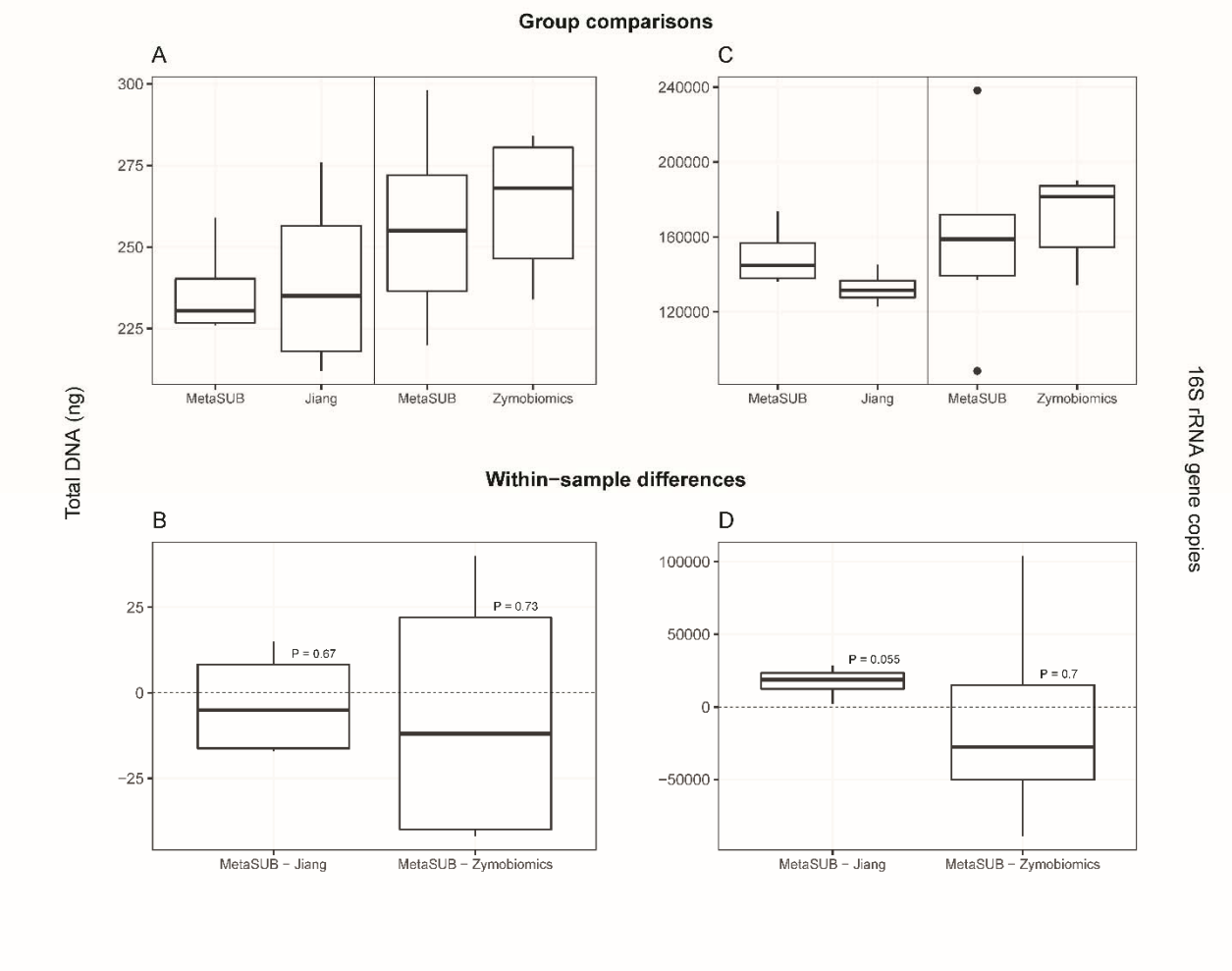
896 **Figure 2** – Benchmarking results for MetaSUB, Jiang, and Zymobiomics on mock microbial

897 community samples.



898 One sample t-tests were performed on within-sample differences (B, D) of total DNA yield (A),

899 and 16S rRNA gene copy yield (C) for MetaSUB (N=4) and Jiang (N=4), and MetaSUB (N=6)

900 and Zymobiomics (N=6).

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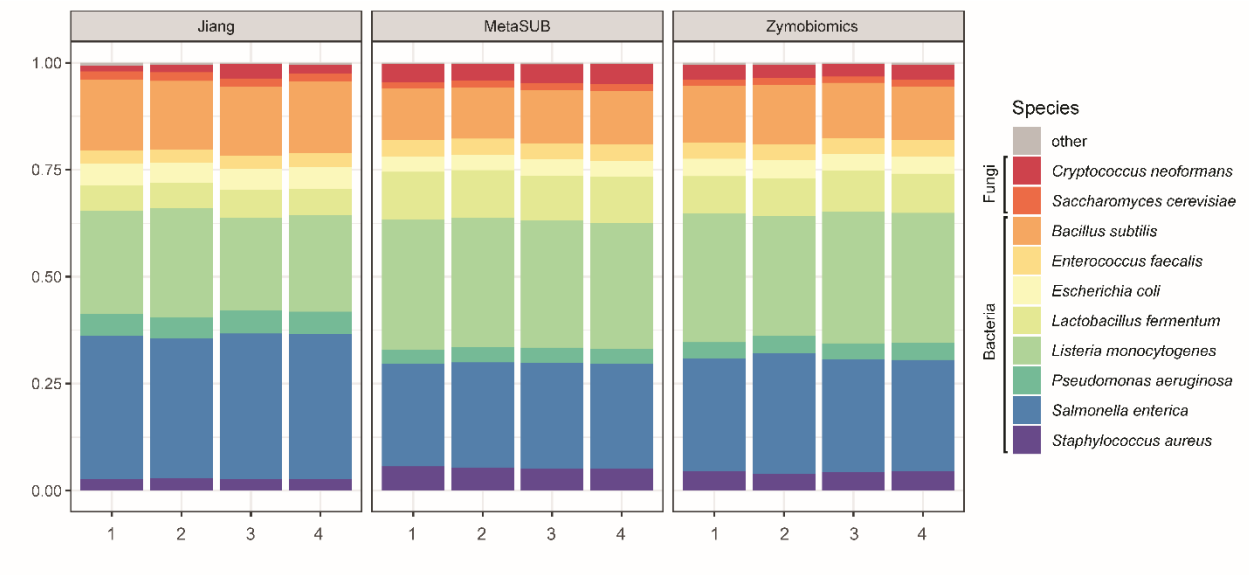
906 **Figure 3** – Relative distribution of the ten mock microbial community species for MetaSUB,

907 Jiang, and Zymobiomics.



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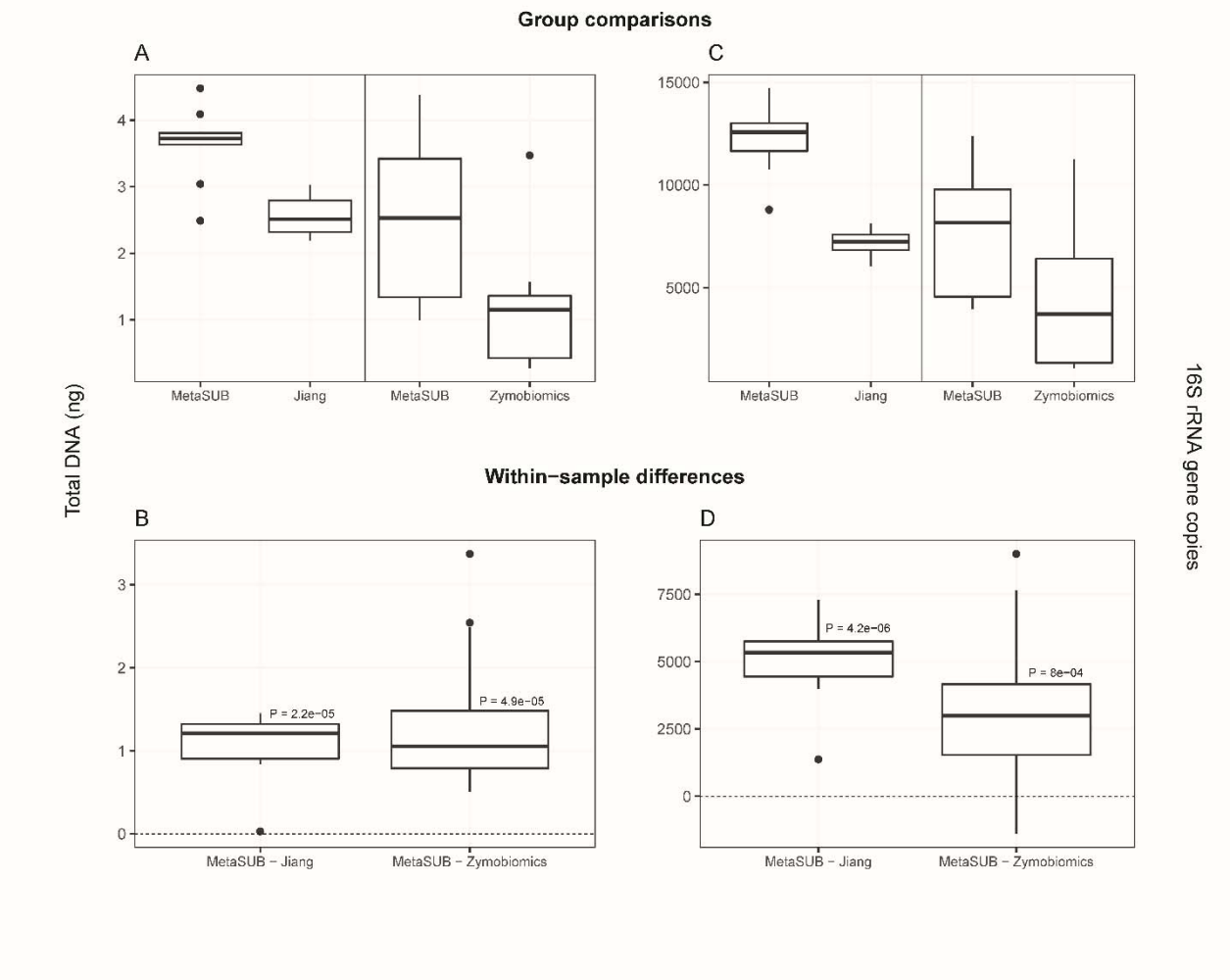
922 **Figure 4** – Benchmarking results for MetaSUB, Jiang, and Zymobiomics on split subway air
923 samples.



924 One sample t-tests were performed on within-sample differences (B, D) of total DNA yield (A),
925 and 16S rRNA gene copy yield (C) for MetaSUB (N=10) and Jiang (N=10), and MetaSUB
926 (N=14) and Zymobiomics (N=14).
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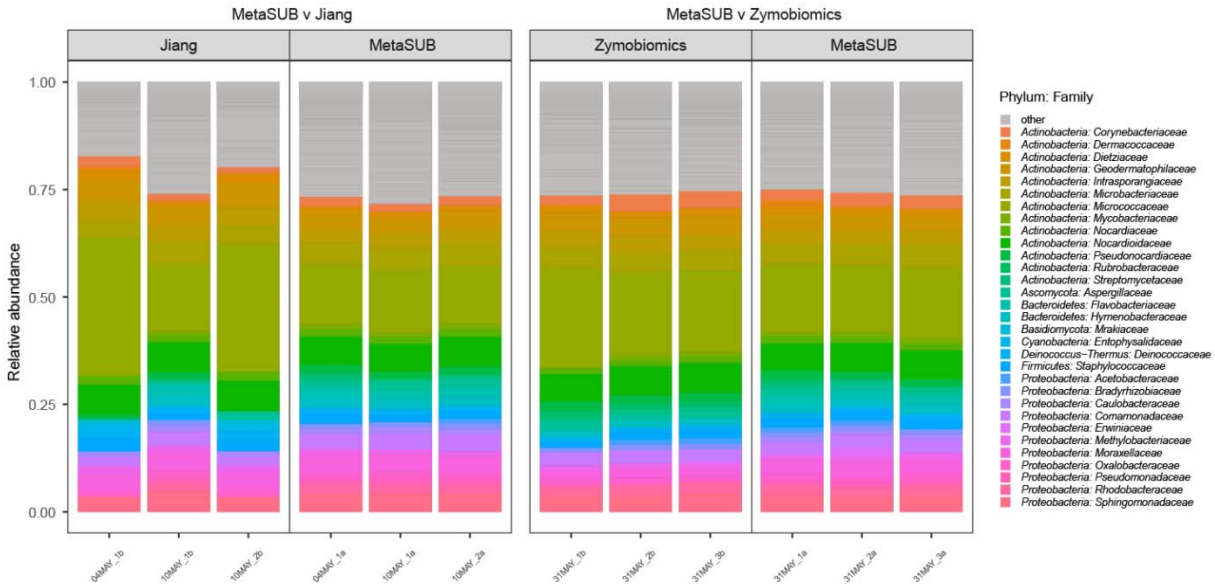
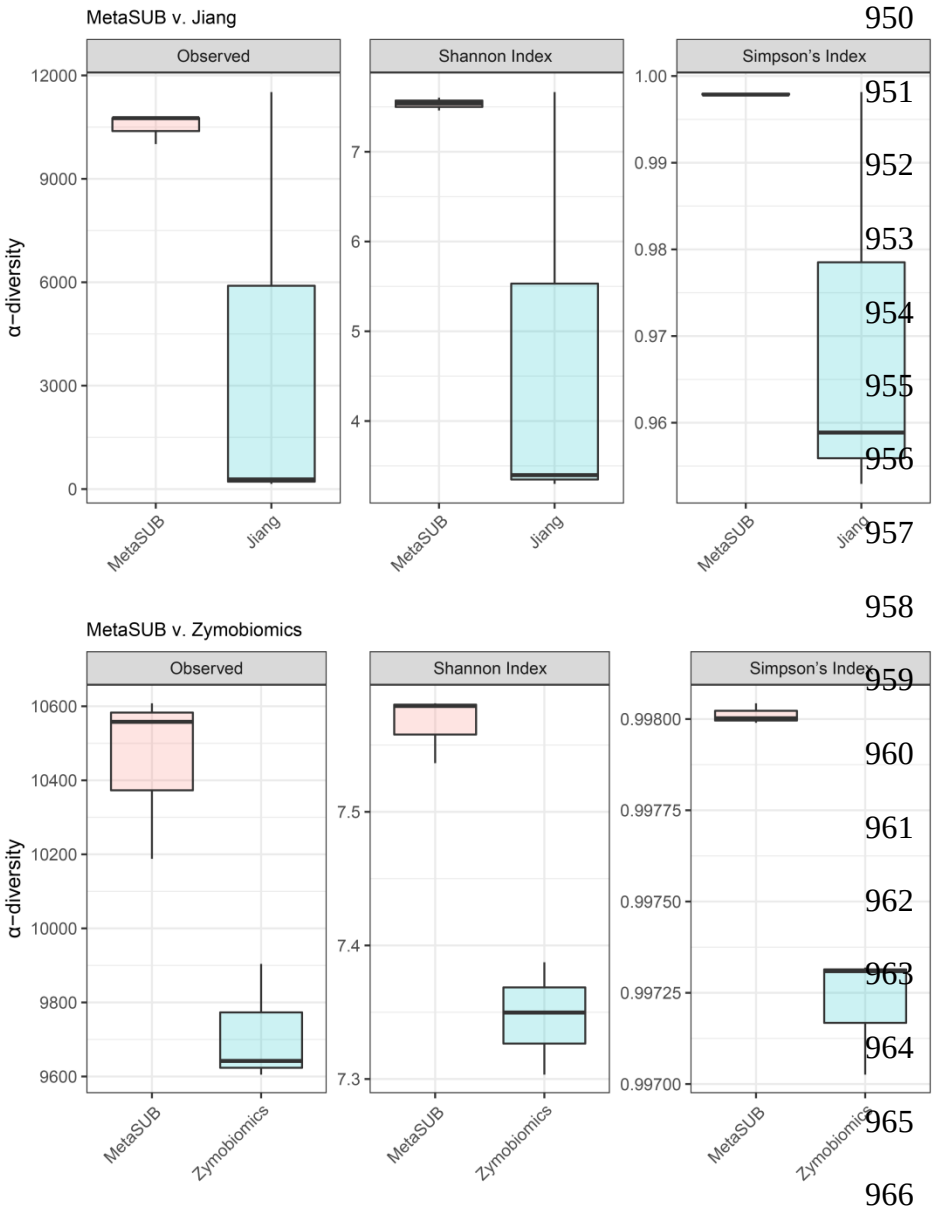
Figure 5 – Relative taxonomic (family-level) distribution in split subway air samples (MetaSUB v. Jiang, MetaSUB v. Zymobiomics).



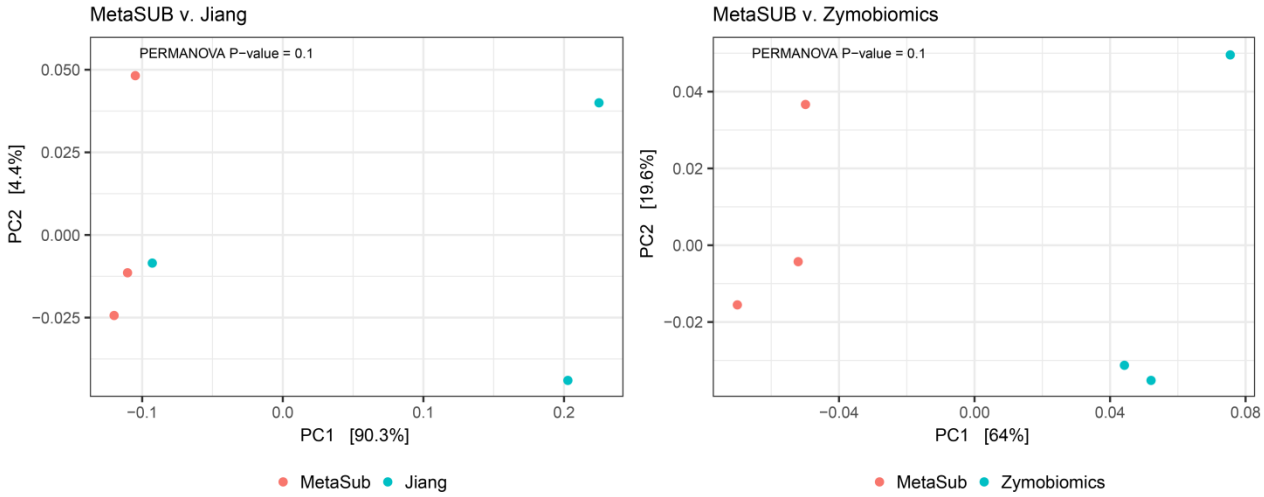
Relative taxonomic (family-level) distribution in subway air samples (N=6) that were split and processed with the MetaSUB (N=3) and Jiang (N=3) or MetaSUB (N=3) and Zymobiomics (N=3) methods. Families with <1% representation are listed as “other”.

Figure 6 – Diversity estimates (α-diversity) for split subway air samples (MetaSUB v. Jiang, MetaSUB v. Zymobiomics).



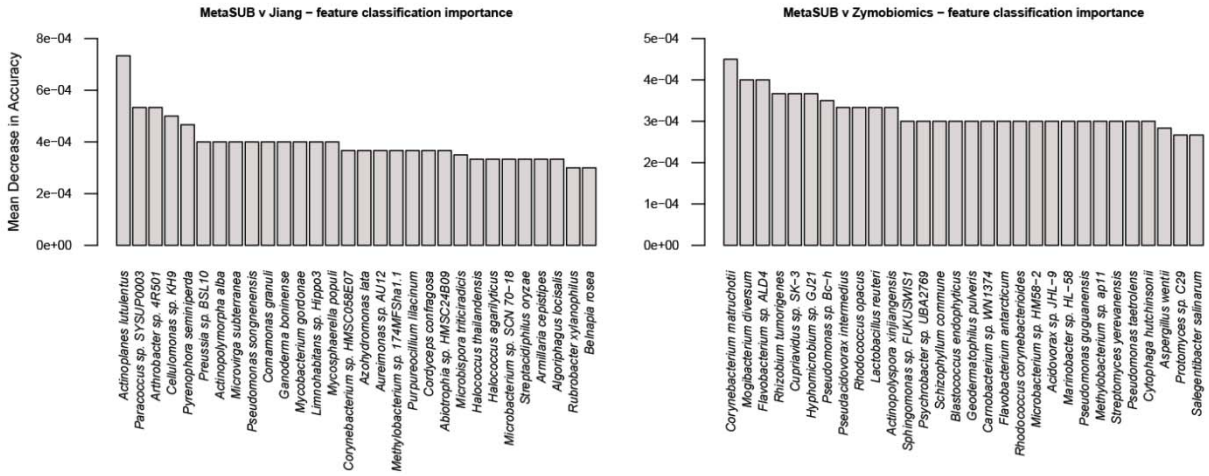
Comparison of diversity estimates (α-diversity) for subway air samples (N=6) that were split and processed with the MetaSUB (N=3) and Jiang (N=3) or MetaSUB (N=3) and Zymobiomics (N=3) methods.

Figure 7 – PCoA ordination plots (β-diversity) for split subway air samples (MetaSUB v. Jiang, MetaSUB v. Zymobiomics).



PCoA ordination plots using Bray Curtis distance estimation (β-diversity) for subway air samples (N=6) that were split and processed with the MetaSUB (N=3) and Jiang (N=3) or MetaSUB (N=3) and Zymobiomics (N=3) methods. PERMANOVA tests were performed on the MetaSUB/Jiang and MetaSUB/Zymobiomics groupings.

Figure 8 – Random forest classification analysis of split subway air samples (MetaSUB v. Jiang, MetaSUB v. Zymobiomics).



Random forest classification analysis of subway air samples (N=6) that were split and processed with the MetaSUB (N=3) and Jiang (N=3) or MetaSUB (N=3) and Zymobiomics (N=3) methods, showing taxonomic features with the highest classification variable importance for correctly identifying the DNA isolation method.

1006 **Figure 9** – Relative taxonomic (family-level) distribution in pellet and supernatant fractions from

1007 subway air samples (MetaSUB method).

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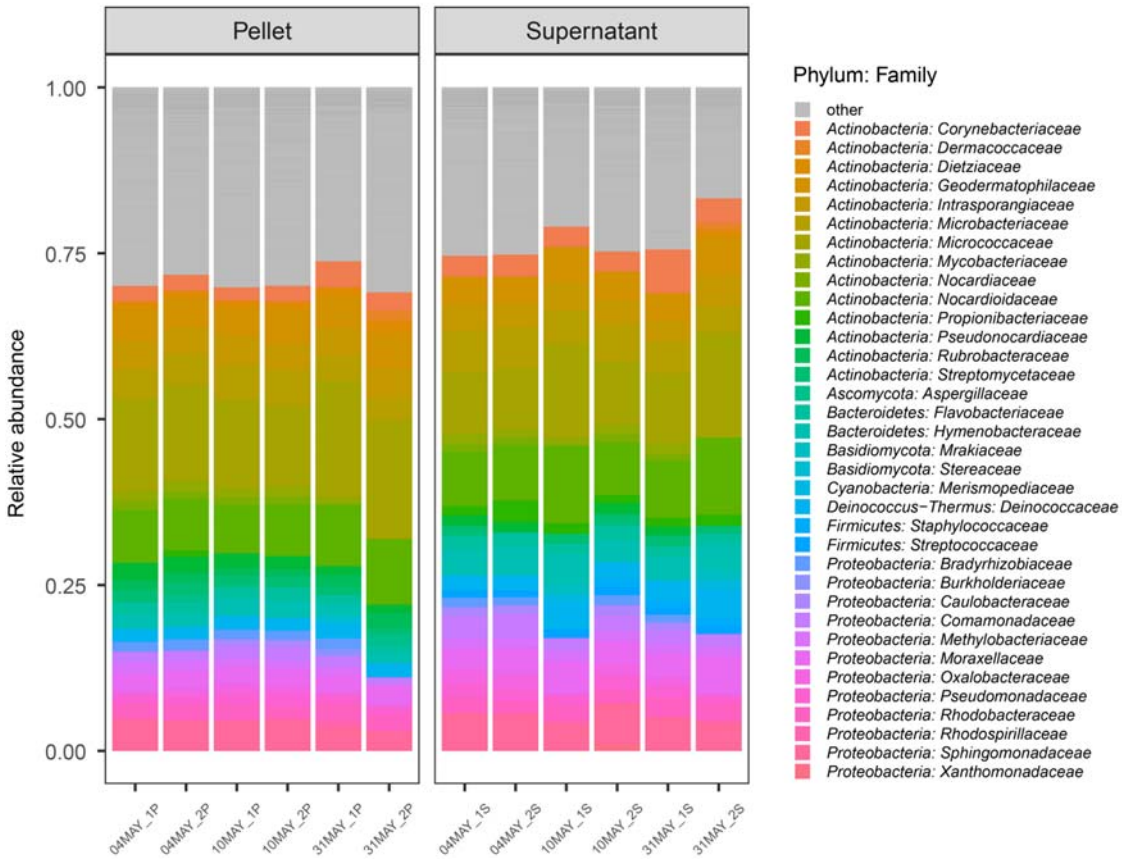

1009 Relative taxonomic (family-level) distribution for subway air samples (N=6) where the

1010 intermediate pellet (N=6) and supernatant (N=6) fractions were processed separately with the

1011 MetaSUB method. Families with <1% representation are listed as “other”.

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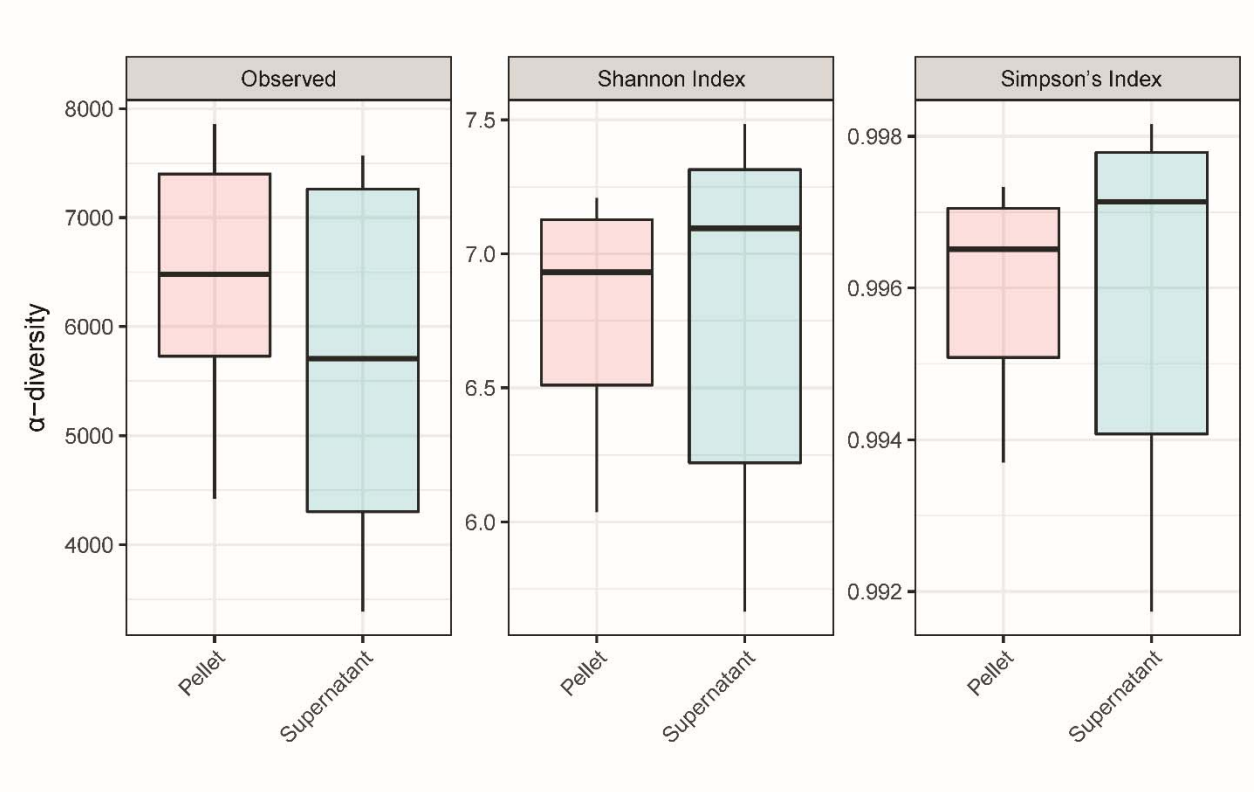
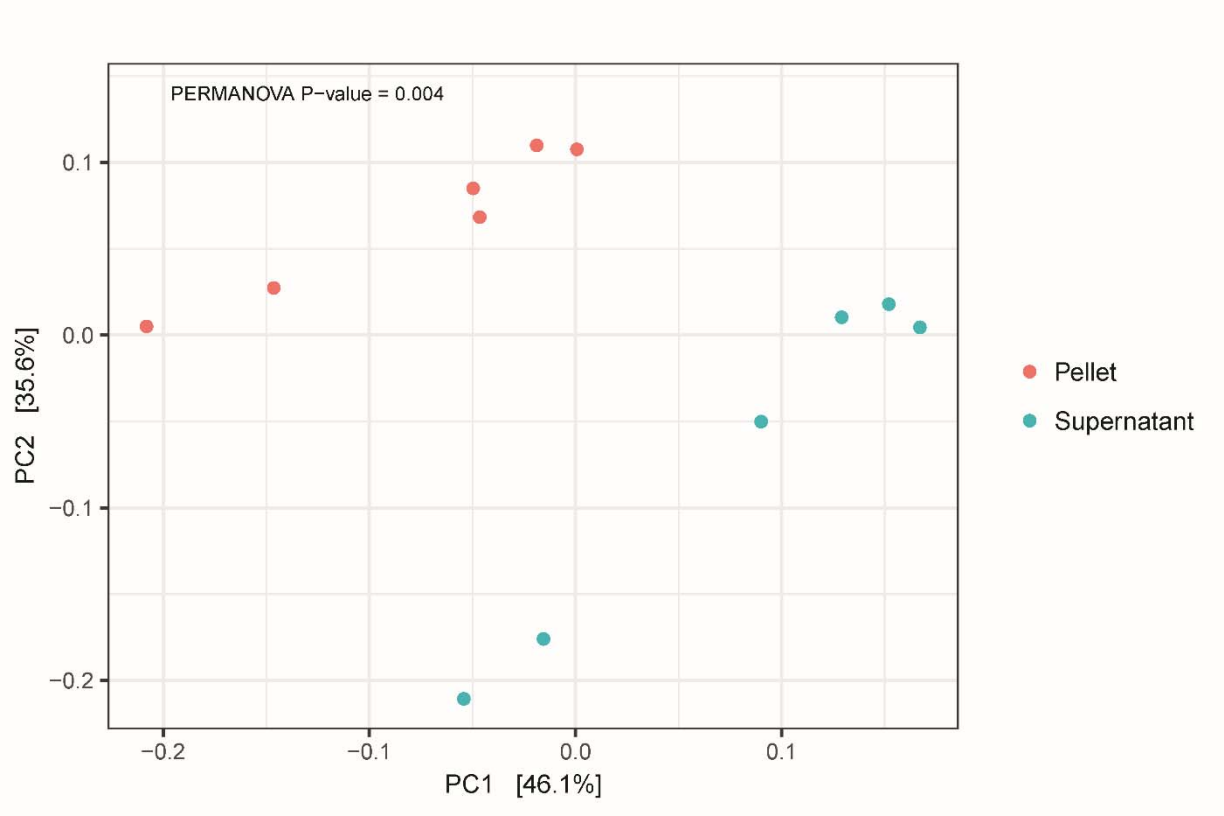
Figure 10 – Diversity estimates (α-diversity) for pellet and supernatant fractions from subway air samples (MetaSUB method).



Diversity estimates (α-diversity) for subway air samples (N=6) where the intermediate pellet (N=6) and supernatant (N=6) fractions were processed separately with the MetaSUB method.

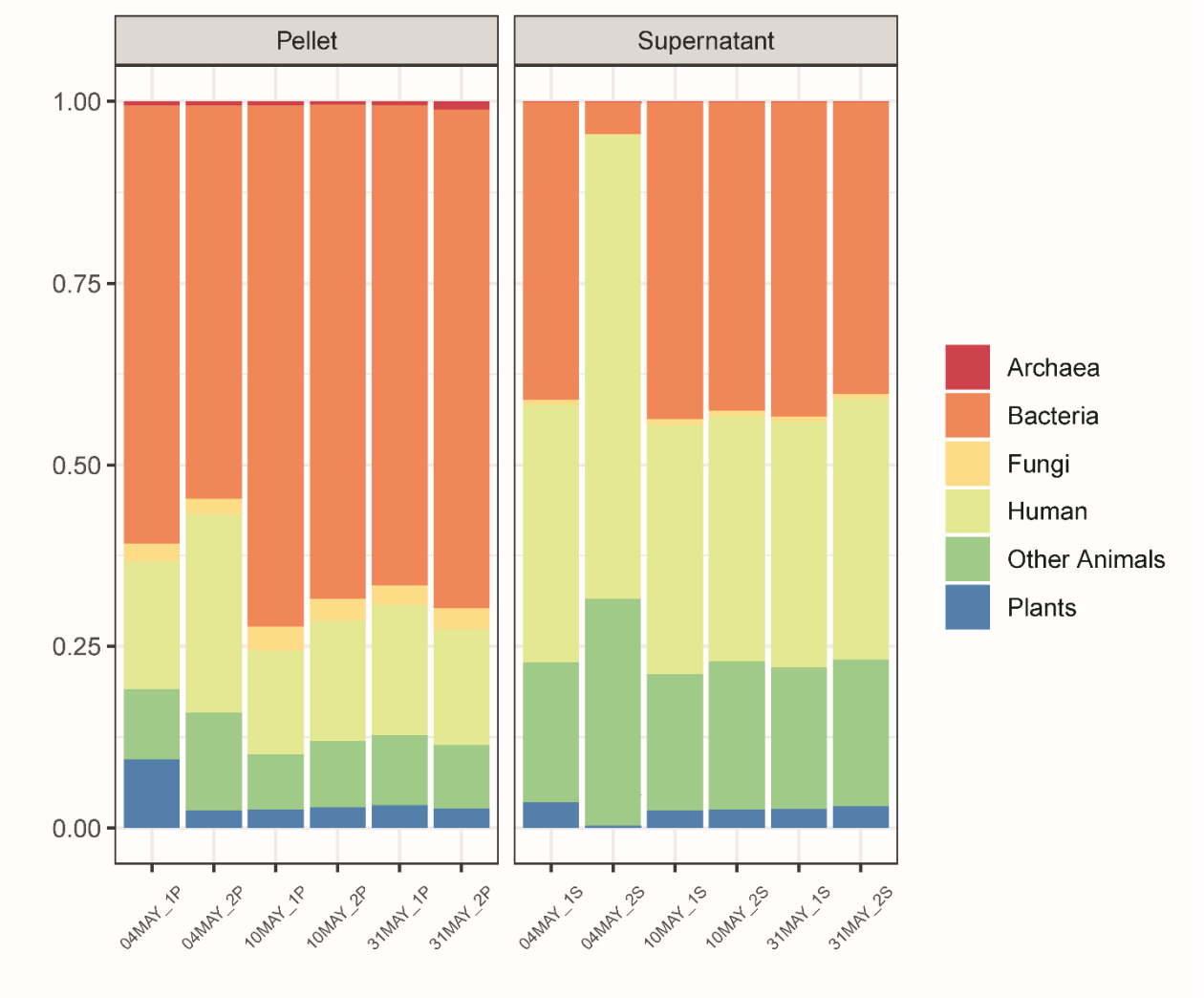
Figure 11 – PCoA ordination plot (β-diversity) for pellet and supernatant fractions from subway air samples (MetaSUB method).



PCoA ordination plot using Bray Curtis distance estimation (β-diversity) for subway air samples (N=6) where the intermediate pellet (N=6) and supernatant (N=6) fractions were processed separately with the MetaSUB method. PERMANOVA test was performed on pellet/supernatant grouping.

1044 **Figure 12** – Relative taxonomic (cross-kingdom) distribution in pellet and supernatant fractions

1045 from subway air samples (MetaSUB method).



1046 Relative taxonomic (cross-kingdom) distribution for subway air samples (N=6) where the

1047 intermediate pellet (N=6) and supernatant (N=6) fractions were processed separately with the

1048 MetaSUB method.

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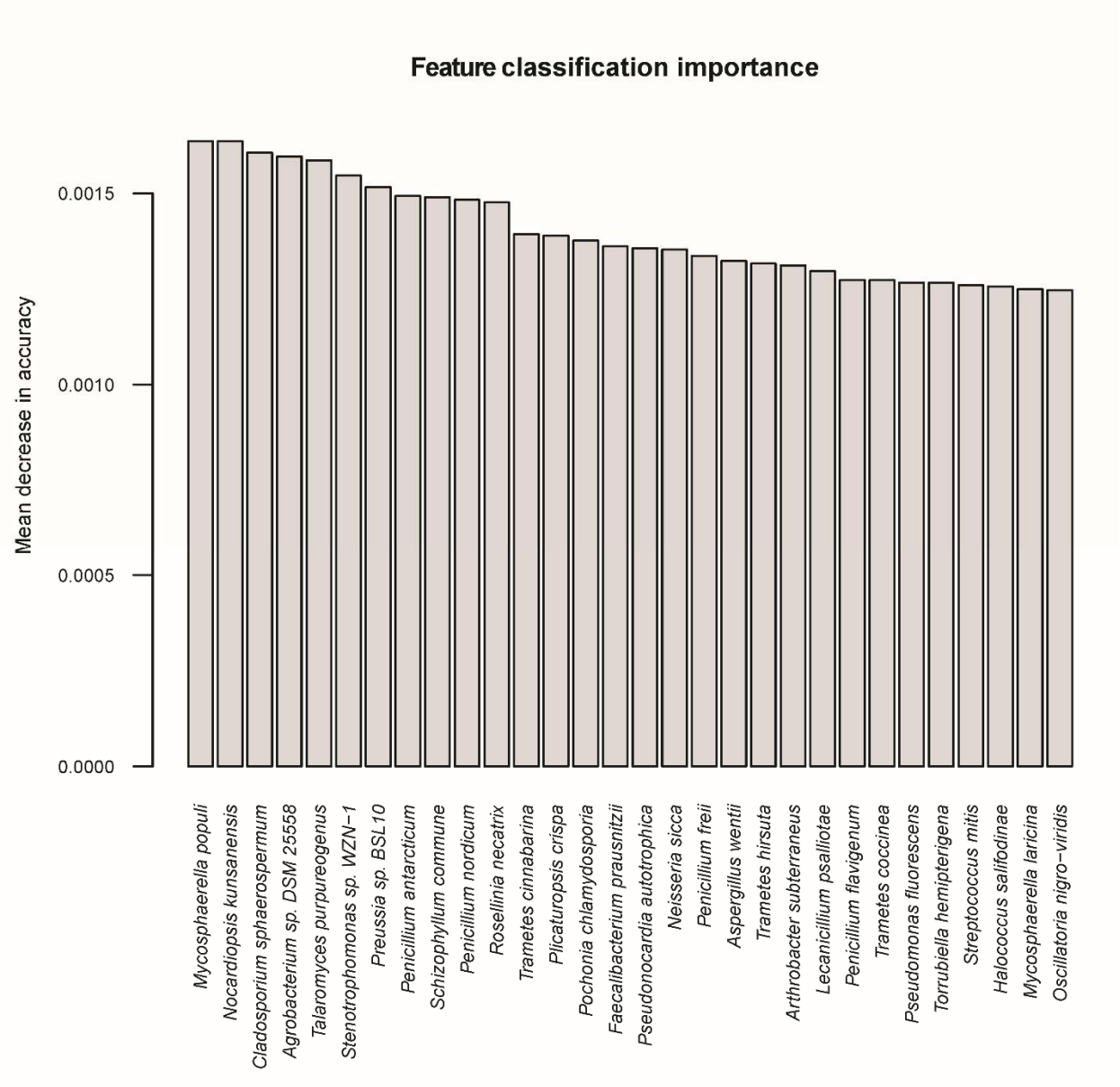
1053 **Figure 13** – Random forest classification analysis on pellet and supernatant fractions from

1054 subway air samples (MetaSUB method).



1055 Random forest classification analysis of subway air samples (N=6) where the intermediate pellet

1056 (N=6) and supernatant (N=6) fractions were processed separately with the MetaSUB method,

1057 showing taxonomic features with the highest classification variable importance for correctly

1058 identifying the pellet and supernatant fractions.

1059

SUPPLEMENTAL ONLINE MATERIAL

Figure S1 – Rarefaction curves with α-diversity measures: "Observed", "Shannon", and "Simpson" for subway air samples (N=6) that were split and processed with the MetaSUB (N=3) and Jiang (N=3) or MetaSUB (N=3) and Zymobiomics (N=3) methods.

Figure S2 – Rarefaction curves with α-diversity measures: "Observed", "Shannon", and "Simpson" for the intermediate pellet (N=6) and supernatant (N=6) fractions from subway air samples (N=6) processed separately with the MetaSUB method.

Figure S3 – Proportion of total DNA and 16S rRNA gene copy yield found in the supernatant fractions, referencing the total yield in the combined pellet and supernatant fractions, from subway air samples (N=24) where the intermediate pellet and supernatant fractions were processed separately with the MetaSUB method.

Figure S4 – The 20 fungal species that were among the top 100 species from the random forest classification analysis of subway air samples (N=3) that were split and processed with the MetaSUB (N=3) and Jiang (N=3) methods, where *Z*-score distributions were compared with linear models.