Diversity and assembly of active bacteria and their potential function along soil aggregates in paddy field

Authors

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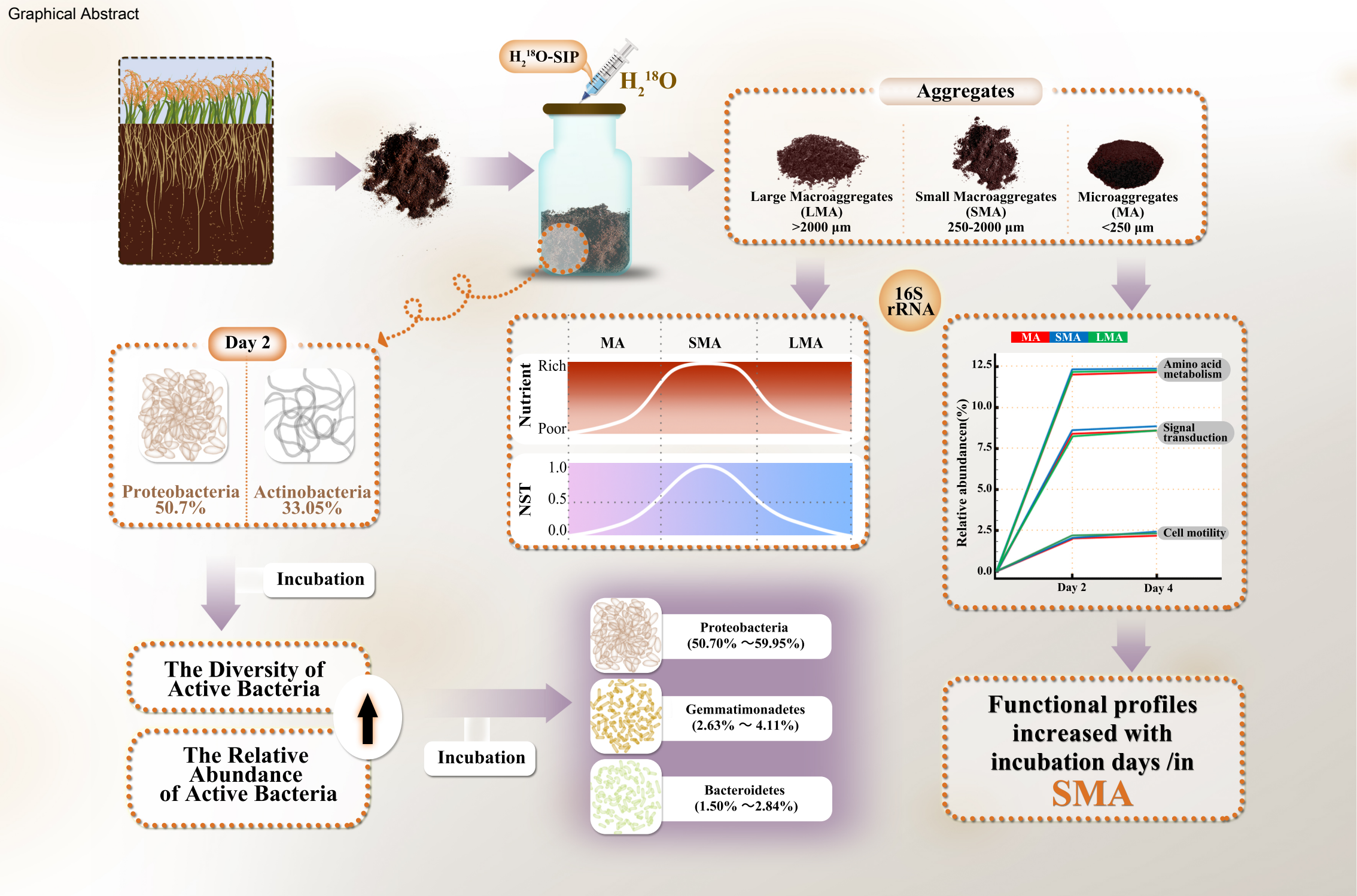
Graphical Abstract



Highlights

- More stochastic processes were found in active bacteria compared to total bacteria,
- Soil nutrients influenced active bacterial community in aggregates.
- Soil nutrients determined the assembly processes of active bacteria in aggregates.
- Potential functions of active bacteria increased obviously in small macroaggregates.

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Abstract:

Numerous studies have found that soil microbiomes differ at the aggregate level providing a spatially heterogeneous habitat for microorganisms to develop. However, assembly processes and the functional profile of microbes at the aggregate level remain largely rudimentary, particularly for those active members in soil aggregates. In this study, we investigated the diversity, co-occurrence network, assembly process and predictive functional profile of active bacteria at the aggregate level using $H_2{}^{18}O$-based DNA stable isotope probing (SIP) and 16S rRNA gene sequencing. The active microbial community belonged mostly of Proteobacteria and Actinobacteria, with a relative abundance of 55.32% and 28.12%, respectively. Assembly processes of community in the total and active bacteria were dominated by deterministic processes because of neutral pH and low soil organic matter. Furthermore, metabolism was the most important function in both Proteobacteria and Actinobacteria. After incubation, the diversity and relative abundance of active bacteria of certain phyla increased, such as Proteobacteria (50.70% to 59.95%), Gemmatimonadetes (2.63% to 4.11%), and Bacteroidetes (1.50% to 2.84%). In small macroaggregates (SMA: 0.25-2mm), the bacterial community and its assembly processes differed from that of other soil aggregates (MA: microaggregates, <0.25mm; LMA: large macroaggregates, 2-4mm) due to different aggregate characteristics. For functional profiles, the relative abundance of important functions, such as amino acid metabolism, signal transduction and cell motility, increased with incubation days and/or in SMA compared to other aggregates. This study provides robust evidence that nutrients of soil are not a

45 negligible factor in terms of community of active bacteria and its assembly processes
46 in soil aggregates, and suggests that dominant active bacteria (such as Proteobacteria)
47 show important functional profiles in soil ecosystem.

48 **Key Words:** active bacteria, aggregates, bacterial composition, assembly processes,
49 functional profiles, stable isotope probing

50

51 **1. Introduction**

52 Microorganisms are fundamental components of soil ecosystems and contribute
53 significantly to ecosystem processes (Bahram et al., 2018). Although thousands of taxa
54 exist in soil ecosystems, a large proportion of this diversity is composed of dormant or
55 inactive individuals (Del Giorgio and Gasol, 2008; Jones and Lennon, 2010; Luna et
56 al., 2002; Roesch et al., 2007). In order to identify the active microbiome in soil, stable
57 isotope probing (SIP) using $^{13}CH_4$, $^{13}CO_2$, $^{15}NO_2$ and $H_2{}^{18}O$ has been successfully used
58 (Aanderud and Lennon, 2011, Dumont and Hernández, 2019). Recently, studies have
59 further revealed that most taxa in soils are metabolically active when incubated with
60 $H_2{}^{18}O$ (Papp et al., 2018a, 2018b). Compared to ^{13}C- and/or ^{15}N-, $H_2{}^{18}O$-based SIP has
61 three advantages in linking microbial community with their function. Firstly, the
62 addition of a single ^{18}O atom increases the degree of physical separation between
63 labelled and unlabelled fractions during isopycnic centrifugation, compared to a single
64 ^{13}C or ^{15}N atom (Aanderud and Lennon, 2011). Secondly, pervasive requirement of
65 water for cellular maintenance and biosynthesis enables $H_2{}^{18}O$-SIP to identify all active
66 growing microorganisms (Schwartz, 2007). Finally, $H_2{}^{18}O$ can identify active microbes

67 in soils without additional material that more closely maintains the situ conditions.

68 Rice paddy ecosystems constitute the largest wetlands on Earth, and host diverse
69 microbial communities responsible for many important ecosystem functions and
70 services (Leff et al., 2004; Bardgett and Van Der Putten, 2014). Paddy soils are
71 developed by long-term flooding, taking advantage of the inherent feature of $H_2{}^{18}O$.
72 The driving factors of assembly processes to microbial community in paddy soil have
73 been discussed (Hou et al., 2020; Liu et al., 2020a), and both deterministic and
74 stochastic processes have been found contributing to the assembly of species (Chase,
75 2010; Ofiteru et al., 2010, Huber et al., 2020). The relative contributions of
76 deterministic and stochastic processes in microbial community can be calculated by
77 null and neutral models (Stegen et al., 2012, 2015; Vellend et al., 2014; Zhou and Ning,
78 2017). The basis for neutral theory is stochastic processes, such as ecological drift, and
79 dispersal (Hubbell, 2005). Under frequent flooding that facilitates dispersal,
80 Stochasticity is an enduring strength in paddy soil microbial communities (Liu et al.,
81 2020a; Liu et al., 2021). Besides, environmental factors are found to mediate the
82 deterministic processes based on niche-based theory (Tripathi et al., 2018).
83 Determinism increased with agriculture development and corresponded with an
84 increase in soil nutrients in paddy soil, especially for abundant bacterial
85 subcommunities (Liu et al., 2020a; Hou et al., 2020). Liu et al. (2022a) find the
86 assembly processes of active methane-oxidizing bacteria are governed by stochastic
87 processes, while the assembly processes of paddy soil bacteria are found more
88 determined compared to other soils (Li et al., 2021). Some researchers suggest that pH

and organic matter content are the main regulators of bacterial community composition in soils (Kuramae et al., 2012; Fierer, 2017). Nutrient availability and physicochemical conditions change with aggregate size, further affecting bacterial communities (Briar et al., 2011; Jiang et al., 2017; Trivedi et al., 2017; Vos et al., 2013). Nevertheless, the understanding of assembly processes at different aggregates are unclear yet.

The living environment of soil microorganisms is controlled by soil aggregates in different size and shape, and possess different characteristics (Lavelle et al., 2006). Soil characteristics are important factors affecting microbial diversity (Pacchioni et al., 2014). Some studies showed that the higher contents of organic carbon and nutrients are associated to microaggregates (< 0.25 mm) (Yan et al., 2018), while others have found that as aggregates became larger, content of soil organic matter increased (Guo et al., 2008; Lin et al, 2019). The biomass and activity of microorganisms in microaggregates may be higher (<0.25mm) (Jiang et al, 2013; Zhang et al., 2013a), but they were also found to be higher in macroaggregates (>0.25mm) (Helgason et al., 2010; Li et al., 2015; Zhang et al., 2015). Liu et al. (2014) found that microbial biomass in the 1- to 2- mm aggregate fractions was the most active and contained the most nutrients in farmland. For functional profiles, many functional genes, such as carbon degradation, organic remediation and other categories have been detected in paddy soils. Metabolism genes, such as amino acid metabolism and carbohydrate metabolism were predicted more compared to other functional genes (Barq et al., 2021). Revealing patterns of these genes will facilitate understanding and prediction of relative functional processes performed by them (Bai et al., 2013; Zhang et al., 2013b). Since soil microorganisms

111 are deeply engaged in biogeochemical processes of nutrients and soil fertility, the
112 functions of different community structures are different (Bai et al., 2017; Philippot et
113 al., 2013; Ofek-Lalzar et al., 2014). Although many studies have found that the diversity
114 of bacteria among soil aggregates are different, the active bacterial community and the
115 functional profiles are still unknown.

116 In this study, we explore the active bacterial community, assembly processes and
117 functional profiles at the soil aggregate level by using $H_2{}^{18}O$ SIP 16S ribosomal RNA
118 (rRNA) gene sequencing. Based on previous studies, we hypothesize that (1) the
119 assembly processes of active bacteria are more stochastic compared to total bacteria;
120 and (2) the diversity of active bacteria is higher and contains higher relative abundance
121 of important functional profiles in SMA compared to other soil aggregates.

122

123 **2. Materials and methods**

124 **2.1. Soil sampling and physicochemical properties**

125 Soil samples were taken from a paddy field at Changxing, Zhejiang province
126 (31°00′ N, 119°55′ E). The climate of this region is subtropical, with an annual
127 precipitation of 1309 mm and an annual temperature of 15.6 °C. Soil samples from 0
128 to 20 cm depth were taken on 14 December 2014 at five random locations with three
129 plots (2×2m) using a soil core sampling. The five soil cores from plots were mixed to
130 form a single composite sample, and stored at 4 °C through a 4-mm sieve until use.
131 Some soils were air-dried and their physicochemical properties were analyzed. The
132 physicochemical properties of soil were estimated according to methods described

133 previously and were provided in Table S1 (Supplemental materials, Table. S1) (Li et
134 al., 2019; Liu et al., 2019a).

135 **2.2. $H_2{}^{18}O$-labelled incubation and aggregate fractionation**

136 $H_2{}^{18}O$ labelled microcosms were setup as described previously (Schwartz, 2007;
137 Papp et al., 2018a) with minor modifications. Briefly, $H_2{}^{18}O$ (99 atom%, Sigma Aldrich,
138 St. Louis, MO) labelled water (^{18}O) and natural-abundance water (unlabelled control,
139 ^{16}O) were constructed for microcosm incubation. Soils were incubated with $H_2{}^{16}O$ was
140 as control at 25 °C in the dark with 100% maximum water-holding capacity of $H_2{}^{18}O$
141 for 4 days in triplicate. Microcosm uses 120-ml serum bottles containing wet soil (~ 6
142 g dry soil), and then sealed with rubber stoppers and aluminum caps.

143 Sampling took place in triplicate microcosms at day-2 and day-4, while day-0 was
144 used as control. Soils were prepared under sterile conditions for soil aggregate
145 separation using the previously described "optimal moisture" method to standardize soil
146 water content and minimize disturbance to microbial communities (Bach et al., 2018).
147 Soils were dried for eight hours to reach a stable moisture content (~10%), and the
148 fllowing aggregate fractions were separated by shaking through two sieves (2000 μm
149 and 250 μm): large macroaggregates (> 2000 μm, LMA), small macroaggregates (250-
150 2000 μm, SMA) and microaggregates (< 250 μm, MA), avoiding submersion in water
151 (Jiang et al., 2014). Soils were vibrated up and down 60 times every 2 min to go through
152 2000 μm sieve. The soils passed through the 2000 μm sieve was transferred to the next
153 smaller sized sieve (250 μm) for further screening, resulting in three aggregate fractions.
154 The aggregate fractions were stored at -80 °C for DNA extraction (Fig. S1).

155 **2.3. Nucleic acid extraction and SIP fractionation**

156 FastDNA SPIN kit for soil (MP Biomedicals; Solon, OH, USA) was used to extract
157 DNA from 0.5g soil. Nanodrop® ND-2000 UV-vis spectrophotometer (NanoDrop
158 Technologies, Wilmington, DE, USA) were used to estimate the concentrations and
159 quality of DNA.

160 Bulk DNA extracted from soil aggregates under $H_2{}^{18}O$ and $H_2{}^{16}O$ treatments as
161 described by Liu et al. (2019b) was centrifuged by density gradients and modified on a
162 small scale. DNA was blended with gradient buffer CsCl solution in Beckman ultra-
163 centrifuge tubes. After centrifugation at 177,000 gav for 44 h at 20 °C in a Vti65.2
164 vertical rotor (Beckman Coulter, Palo Alto, CA, USA), the DNA was divided into 14
165 equal fractions (Zhang et al., 2019a). The isolated DNA was purified and dissolved in
166 TE buffer.

167 **2.4. Quantitative PCR and sequencing processes**

168 To measure the growth and efficiency of ^{18}O incorporation into the bacterial
169 community genomic DNA, quantitative PCR (qPCR) was performed on a
170 LightCycler® 480II (Roche, Germany) for each buoyant density of DNA gradient
171 fraction based on 16S rRNA genes. According to the 16S rRNA genes, the primer pair
172 515F and 806R was used for the qPCR of bacteria (Walters et al., 2016). The
173 amplification efficiencies of all genes ranged from 89 to 105%, and R values ranged
174 from 0.992 to 0.999.

175 Bacterial 16S rRNA genes were amplified in bulk DNA and in DNA gradient
176 fractions foe each buoyant density from soil aggregates with $H_2{}^{18}O$ treatments using

177 primer pair 515F and 806R with 12bp barcode (Walters et al., 2016). Sequence libraries
178 were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (New
179 England Biolabs, MA, USA) according to the manufacturer recommendations. The
180 libraries were sequenced on an IlluminaHiseq2500 Platform (Illumina, San Diego, CA,
181 USA) by Guangdong Magigene Biotechnology Co. Ltd. (Guangzhou, China).

182 Raw fastq files were quality-filtered using Trimmomatic (REF) and merged using
183 FLASH (REF) according to the Liu et al. (2020b) described previously. Operational
184 taxonomic units (OTUs) were clustered using UPARSE (REF, version 10
185 http://drive5.com/uparse/) with a similarity cutoff of 97%. The most frequently
186 occurring sequences were extracted as representative sequences for each OTU and the
187 Silva (https://www.arb-silva.de/) database was used to filter taxonomic annotations.
188 The sequencing reads of the 16S rRNA genes were stored in the Genome Sequence
189 Archive (GSA, China) database with the accession number CRA005780.

190 **2.5. Statistical analysis**

191 All data analysis was performed in the R environment (v3.6.3; http://www.r-
192 project.org) (Hamilton and Ferry, 2018).

193 To identify OTUs associated with ^{18}O assimilation, the R package DESeq2 was
194 used to analyze. The abundance of differential genes with negative binomial
195 distributions in high-density gradient fractions based on $H_2{}^{18}O$ labelled treatments
196 relative to corresponding gradient fractions of non-labelled control [^{16}O] (Love et al.,
197 2014; Kong et al., 2019). Log2-fold changes above zero with padj value (FDR-adjusted
198 P-value) of less than 10% were selected as ^{18}O labelled OTUs. Scatter plots were

199 performed to visualize differentially labelled OTUs in heavy DNA fractions using the
200 plotMA function.

201 Diversity indexes include Shannon, Simpson, Richness were estimated using
202 vegan package (Dixon, 2003). The differences of beta diversity were calculated by
203 Principal coordinate analysis (PCoA) with the Bray-Curtis distance of bacterial
204 community profiles with vegan and ggplot2 packages (Lozupone et al., 2011), and two-
205 way permutational multivariate analysis of variance (PERMANOVA) was used to
206 quantitatively measure the effects of the incubation time and aggregate treatment.

207 OTUs were also used to evaluate the main species phylum among aggregates and
208 time of incubation, and to calculate the relative abundance of the top ten abundant
209 phylum by using amplicon and reshape2 packages. To ascertain the changes of
210 interactions between bacterial groups as the incubation days and soil aggregates
211 changed, co-occurrence networks among bacteria were described using the igraph
212 package and the Gephi 0.9.2 platform (Bastian et al., 2009; Chen et al., 2020). Setting
213 the filtering threshold of spearman correlation coefficients >0.6 and p-values <0.05, the
214 significance of edges between nodes were determined. Prior to network analysis, excess
215 OTUs were removed when they were present in less than ~90% of samples or when
216 their relative abundance was less than 0.01% (Liu et al., 2022b). The network was
217 further used to calculate topology property parameters. According to the nodes and
218 links in the network, the main phylum species in the network are determined.

219 To assess the assembly processes of bacteria in days with different aggregates and
220 to assess responses to environmental factors, we used the normalized stochastic ratio

(NST) to evaluate the underlying mechanisms of bacterial community assembly (Ning et al., 2019). NST was assessed based on different distance metrics and different null model algorithms [with 50% as the boundary, more stochastic (>50%) or more deterministic (<50%)]. NST indexes, such as cao, mGower, gower and binomial were calculated based on Jaccard matrix, which is suggested to estimate the stochastic effects in community assembly (Ning et al., 2019). Statistically significant differences in alpha diversity indices, NST indices among incubation days and soil aggregates were determined by two-way analysis of variance (ANOVA), accompanied with least significant difference (LSD) test for multiple comparisons. Moreover, if the observed variances were heterogeneous, the group variance was calculated by nonparametric Kruskal-Wallis test.

To compare different functional profiles in different incubation days and soil aggregates, we used Tax4Fun to predict functional profiles of bacterial community from 16S rRNA gene sequences (Wemheuer et al, 2020; Ahauer et al, 2015). Data were then compared with KEGG (Kyoto Encyclopedia of Genes and Genomes) functional database at level 2, and the biological metabolic pathways could be significantly identified. After calculating the relative abundance of each functional profile, we used ANOVA as well as by LSD test for multiple comparisons among soil aggregates and days for functional profiles, printing those which were significantly different among incubation days and soil aggregates ($p<0.05$). By using Z scores, we printed functional profiles in the Tidyverse and reshape2 packages. Correlation analysis among main species phylum in network and functional profiles of ^{18}O labelled soil were performed

by using Z scores based on Pearson correlations.

3. Results

3.1. Labelling of active bacteria with $H_2{}^{18}O$

On day 2 and 4, DNA obtained from $H_2{}^{16}O$ and $H_2{}^{18}O$ microcosms was separated by isopycnic ultra-centrifugation to isolate ^{18}O-labelled DNA from unlabelled DNA. Compared to that of $H_2{}^{16}O$ control microcosms, 16S rRNA gene copy numbers buoyant density gradient throughout DNA from $H_2{}^{18}O$ treatment showed shift to relative higher buoyant density, with detection of ^{18}O-DNA at buoyant density of 1.723-1.744 g ml^{-1} (the 6th – 8th fractions), irrespective of the incubation time (Fig. 1). Thus, 6th, 7th and 8th fractions were selected as representatives of heavy DNA fractions (^{18}O-DNA) for 16S rRNA gene sequencing individually.

3.2. Diversity and taxonomic composition of active bacteria in soil aggregates

For alpha diversity, Shannon and Simpson indexes of total bacteria showed significant differences across aggregate fractions ($p < 0.001$, $p<0.001$) or incubation time ($p = 0.029$, $p = 0.003$), while not for integrated effects of aggregates and incubation time ($p = 0.473$, $p = 0.144$) (Fig. 2a, c). Shannon and Simpson diversity of active bacteria also differed among aggregate fractions ($p = 0.016$, $p = 0.008$) (Fig. 2b, d). For example, Shannon and Simpson indexes of SMA are shown to be separated from other aggregates in active bacteria, especially for day 2. Compared to total bacteria, aggregate fractions and incubation days showed clear synergies on Shannon and Simpson indexes of active bacteria ($p = 0.038$, $p = 0.014$). Richness index of total bacteria also differed

265 in incubation days ($p = 0.001$) (Fig. S2). The Simpson index of active bacteria showed
266 significant differences ($p = 0.001$) between incubation time while no significant
267 difference ($p = 0.139$) was observed for Shannon indices (Fig. 2).

268 In terms of beta diversity, 49.56% of the variation in total bacterial community and
269 80.62% of variation in the active bacteria were explained by the first two axes of the
270 PCoA (Fig. 2e, f). In total bacterial community, two-way PERMANOVA showed that
271 incubation time explained 44.15% of variation in bacterial community while aggregates
272 only explained ~9.04% of that (Fig. 2e). Interestingly, aggregates explained 19.56%
273 variation of active bacterial community, close to the extent of incubation time (26.42%)
274 (Fig. 2f).

275 Taxonomic composition of total and labelled bacteria is calculated based on the
276 OTUs. The bacterial community of total bacteria were dominated (OTUs>50000) by
277 Proteobacteria (38.41%), Chloroflexi (14.99%), and Acidobacteria (9.45%), while the
278 labelled bacterial community were dominated (OTUs>50000) by Proteobacteria
279 (54.80%) and Actinobacteria (28.57%) (Fig. 3, Fig. S3). The relative abundance of
280 Proteobacteria (54.80%) and Actinobacteria (28.57%) increased in active bacterial
281 communities compared to those in the total communities (38.41% and 5.81%,
282 respectively) (Fig. 3, Fig. S3). Additionally, the distribution of bacterial diversity
283 indicated changes under aggregates and incubation time in labelled community than
284 that of total community (Fig. 3, Fig. S3). In active bacteria, the relative abundance of
285 Proteobacteria increases with incubation time (49.70% at day 2 to 60.03% at day 4),
286 while that of Actinobacteria decreases (33.05% at day 2 to 22.21% at day 4). The

relative abundance of other taxa (except Proteobacteria and Actinobacteria) in SMA (26.48%) are much higher than that of MA (8.65%) and LMA (11.07%) isolated from 2-day incubation soil in active bacteria, while similar result is also found in 4-day incubation soil (Fig. 3b, S3b).

3.3. The network and assembly processes of active bacteria in soil aggregates

The network of bacteria at OTU level between total and active bacterial community were different (Fig. 4). Among pairs of bacterial phylum, more of them were clustered in the active than in the total bacterial community (Fig. 4). The total number of nodes, the number of links, the average degree and the modularity of the active bacterial community were higher than the total bacterial community (Table S2). In active bacterial community, there were more negative correlations compared to that of total bacterial community (Table S2). Together, networks of the labelled community showed more correlations and a compact network structure than in the total community.

The NST explained the changes in ecological community assembly processes at incubation time and aggregate scales (Fig. 5). In total bacterial community, the value is less than 50%, suggesting that deterministic processes dominate bacterial community assembly. Incubation days significantly influence ecological community assembly processes in total bacterial community ($p < 0.05$) (Fig. 5a, c; Fig. S4c). However, aggregates show more significant effects on ecological community assembly processes of active bacterial community ($p < 0.05$) than incubation time (Fig. 5b, d; Fig. S3d). For instance, NST of SMA is different from NST of MA and LMA in active bacterial community. Especially at day 2, the ecological community assembly processes of SMA

309 in active bacterial community is dominated by stochastic processes. Synergies of days
310 and aggregates influenced the ecological community assembly processes in active
311 bacterial community ($p < 0.05$) (Fig. 5b, d).

312 **3.4. Predictive functional profiles of active bacteria**

313 The prediction map showed the results with significant differences among
314 aggregates ($p < 0.05$). Predicted KEGG pathways at level 1 for both total (73.22%) and
315 active bacteria (61.87%) among aggregates are dominated by metabolism. However,
316 compared to total bacteria, functional profiles in active bacteria are significantly
317 different among aggregates (Fig. 6).

318 Among active bacteria, the functional profiles of the labelled soil bacterial
319 community on day 4 changed significantly compared to day 2 ($p < 0.05$) (Fig. S5).
320 Functional genes related to metabolism decreased (62.22% to 61.52%), except for
321 amino acid metabolism. However, the relative abundance of other important functional
322 profiles increased, such as for cell motility and signal transduction (Fig. S5). For
323 aggregates, the function of SMA differed from MA and LMA, in which amino acid
324 metabolism, cell motility, cell growth and death, and bacterial infectious disease were
325 the key functions (Fig. 6b). Correlations between major taxa and function genes also
326 differed in SMA and other aggregates (Fig. S6).

327

328 **4. Discussion**

329 **4.1. Identification of active bacteria with $H_2{}^{18}O$**

330 In this study, compared to total bacterial community, the composition, network

interactions, assembly processes and functional profiles in the active bacterial community was different (Fig. 3, Fig. 4, Fig. 5, Fig. 6). The results of this study are similar to that of previous study in which ^{18}O labelled bacteria community showed different patterns and more significant random phylogenetic distribution compared to total bacteria (Coskun et al., 2019). Proportion of active bacterial community increased when compared to total bacteria, especially in Proteobacteria (38.58% in total bacteria to 55.32% in active bacteria) and Actinobacteria (5.81% in total bacteria to 28.12% in active bacteria) (Fig. 3, Fig. S3). Dominance of Proteobacteria and Actinobacteria in paddy soil bacteria has been reported previously (Wu et al., 2011; Itoh et al., 2013). The obvious habitat preference of soil bacterial families is closely related to their respiratory characteristics, and these results are attributed to respiratory characteristics of different bacteria (Shen et al., 2021). Compared to aerobic bacteria belonging of Acidobacteria and Bacteroidetes, Proteobacteria and Actinobacteria grow quickly under flooded condition due to their anaerobic characteristic (Wang et al., 2012). In addition, the results showed in this study also indicate that $H_2{}^{18}O$ based DNA-SIP is an ideal approach to identify active microbes in soils without requirement of addition substrate other than water (Fig. 1).

Network analysis showed that there were more nodes, OTUs links, average degree, and modularity in active bacterial community compared to that in total bacteria (Fig. 4, Table S2), indicating more intensive interactions in active bacteria community. Positive links were dominated in all networks, indicating that microbial synergy plays an important role in bacterial community (Zhou et al, 2020). However, the proportion of

353 negative correlations of active bacteria is nearly twice (28.02%) than that of total
354 bacterial community (14.83%). As the negative links among nodes could be attributed
355 to competition and amensalism, these results suggest stronger competitive interaction
356 among active microbes (Faust and Raes, 2012). This might be due to heterotrophic
357 lifestyle and strong competition of Proteobacteria and Actinobacteria with other
358 bacteria (Dai et al., 2021). Additionally, assembly processes of bacterial community in
359 paddy fields are dominated by deterministic processes (Fig. 5). Deterministic processes
360 of abundant taxa have also been found in paddy soils (Hou et al., 2020). It is suggested
361 that soil pH and organic matter are deterministic factors driving assembly processes of
362 bacterial community (Tripathi et al., 2018; Dini Andreote et al., 2015). Higher soil pH
363 (>6.7) also leads to deterministic assembly of abundant community (Jiao and Lu, 2020).
364 In this study, deterministic processes may be attributed to the neutral pH (6.9 ± 0.08)
365 and low soil organic matter (13.5 ± 0.01 g kg^{-1}). Interestingly, stochasticity is more
366 important in governing soil active microbes than the total bacteria (Fig. 5). Flooding
367 conditions promoted by hydrologic mixing presumably enhanced the ability of active
368 microorganisms to migrate across geographical areas, which might explain why the
369 stochastic processes in active bacteria were more important than in total bacteria (Liu
370 et al., 2020a; Liu et al., 2022a). Besides, Jiao et al. (2021) have recently showed that
371 the richness of microbiome is closely linked to the community. In this study, the
372 stochastic processes increased with decreasing bacterial richness from total bacteria to
373 labelled bacteria (Fig. S2). This result may be attributed by stochastic assembly
374 processes induce synergy of microorganisms (Jiao et al., 2020), which may lead to more

375 species competition with species richness reduction (Grime, 1973; Rajaniemi, 2002).

376 For functional profiles, predicted KEGG pathways at level 1 for both total (73.22%)
377 and active bacteria (61.87%) are dominated by metabolism, and the relative abundance
378 of other functional genes, such as environmental information processing, in active
379 bacteria (19.39%) increased compared to that of total bacteria (11.22%) (Fig. 6). Similar
380 results have also confirmed that metabolic genes are dominant in anaerobic
381 environment (Lesniewski et al., 2012), and other studies have revealed that members
382 of the phylum Proteobacteria are key drivers of the important metabolic activities in
383 soil ecosystem (Salam and Obayori, 2019). The active community is more closely
384 related to functional profiles than total community (Bastida et al., 2016). Therefore, the
385 higher proportion of Proteobacteria and Actinobacteria, the more competitive
386 interactions, more stochasticity in assembly processes and more different functional
387 profiles are observed in ^{18}O labelled bacteria compared to total bacteria.

388 **4.2. Succession of active microbes and functional profiles along incubation time**

389 Previous studies have shown succession of bacterial community in paddy field,
390 for instance, Ding et al. (2017) showed succession of diversity and functional profiles
391 of active bacteria along incubation time. Furthermore, Yang et al. (2019) found bacterial
392 diversity was higher in flooded areas than in control areas, whereas other studies found
393 that soil bacterial diversity was lower in saturated water(Zhou et al., 2002; Kozdrój and
394 van Elsas, 2000). In our study, the diversity increases from day 2 to day 4 in active
395 bacteria (Fig. 2). It is found that some bacteria, which can survive periods of hypoxia,
396 would wake up from inactive states and thrive under flooded conditions (Berney et al.,

2014; Furtak et al., 2020; Fredrickson et al., 2008). Furthermore, flooded environments promote active bacterial colonization in soil, and bacteria actively use alternative electron acceptors for respiration to manage hypoxic to improve survival (Eggleston et al., 2015; Engelhardt et al., 2018; Yan et al., 2015). Similar to species diversity, the composition of active bacteria also differed with incubation time (Fig. 3b, Fig. S3b). It is known that flooded conditions can increase abundance of some communities, such as Proteobacteria, and Bacteroidetes (Afzal et al., 2019; de León-Lorenzana et al., 2017; Zhang et al., 2019b). In our study, the relative abundance of Actinobacteria decreased from day 2 (33.9%) to day 4 (22.34%), while Proteobacteria increases from day 2 (50.7%) to day 4 (59.95%) (Fig. 3). The variation of Actinobacteria and Proteobacteria in active bacteria may be due to different reproductive strategies. Actinobacteria are ubiquitous and usually predominant in arid habitats. Their drought tolerance may stem from their unique life-cycle characteristics (Lebre et al., 2017), including mycelium growth (Jones and Elliot, 2017) and arthrospore formation (Kämpfer et al., 2014). In contrast, Proteobacteria are more adapted to flooding condition and more competitive under such circumstances, and a similar trend has been observed in wet soil compared to dry soil (Na et al., 2019). Additionally, the relative abundance of Gemmatimonadetes increases from 2.63% in day 2 to 4.11% in day 4 (Fig. 3b, Fig. S3b). Growth of Gemmatimonadetes could be attributed to its ability to low-oxygen conditions (Debruyn et al., 2011).

As for functional genes of active microbes, after 4 days of incubation, the functional profiles changed significantly compared to that in day 2 (Fig. S5). Functional

419 genes related to metabolism still dominated even though it decreased from 62.22% in
420 day 2 to 61.52% in day 4. For metabolism, amino acid metabolism increases, while
421 others, such as carbohydrate metabolism decreases (Fig. S5). Studies from Salam (2019)
422 have found that amino acid metabolism is mainly predicted by Proteobacteria. More
423 amino acid metabolism functional genes are found with incubation, which is paralleled
424 by the increase in relative abundance of Proteobacteria (Fig. S3b; Fig.S5). For
425 carbohydrate metabolism, these results can be attributed to the submergence condition,
426 which decreases carbohydrate metabolism of bacteria in soil (Moreno-Espindola et al.,
427 2018; Ding et al., 2019). Less metabolism function is predicted in day 4 compared to
428 that in day 2, which could be due to the decrease of Actinobacteria, as Actinobacteria
429 are reported to contribute to the production of secondary metabolites (Yan et al., 2021)
430 (Fig. S3b). Furthermore, some studies showed that flooding increases nutrient
431 availability in soil (Oorschot et al., 2000; Shekiffu and Semoka, 2007). Qiu et al. (2020)
432 found that the addition of organic matter activates connections and closes relationships
433 among microorganisms with incubation progressed. We assume that flooded soils have
434 similar influence on active bacterial community, making more nutrients available,
435 hence the relative abundance of many KEGG pathways increases, including
436 environmental information processing, cellular processes, organismal systems and
437 human diseases. For example, the relative abundance of cell motility and signal
438 transduction of day 4 in active bacteria shows a significant increase compared to day 2
439 (Fig. S5). The relative abundance of active Bacteroidetes increases twice from 1.49%
440 in day 2 to 2.84% in day 4 (Fig. 3b, Fig. S3b). Bacteroidetes is highly effective at

secreting carbohydrate-active enzymes and immobilizing them to cell surface, and it is closely related to quickly slide across solid surfaces to increase cell motility (Larsbrink and Mckee, 2020). Signal transduction of soil microbiomes is promoted by environmental stresses (Sun et al., 2020), thus incubations in day 4 showed a higher relative abundance of signal transduction compared to day 2. In brief, as diversity of active bacteria increases, composition become complexed from day 2 to day 4, and the relative abundance of many functional profiles also increase. A large proportion of functional gene species are significantly altered due to changes in biodiversity and composition (Jung et al, 2016).

4.3. Diversity, assembly processes and functional profiles of active bacteria at aggregate level

Some researchers found the bacterial among different aggregates were different (Bailey et al., 2013; Trivedi et al.,2017). Especially for active bacteria, it can be showed that there were more differences among aggregates compared to total bacteria (Fig. 2; 4). Diversity of bacteria tends to increase with increasing aggregate size (Lupwayi et al., 2001), while bacterial biomass and diversity are higher in small aggregates with more stable structure (Hemandez and López-Hernández, 2002; Ling et al., 2014). In our study, the diversity of active bacteria in SMA are higher than MA and LMA, and nearly all active bacteria were more enriched in SMA compared to MA and LMA (Fig. 2, Fig. 3). These results can be attributed due to SMA provides more nutrients compared to MA and LMA (Wang et al., 2014; Ling et al., 2014). It is found that soil organic matter and total nitrogen increase as aggregates become larger (from MA to SMA) (Lin

et al., 2019; Zheng et al., 2021). LMA is poor in nutrient, while SMA shows the opposite (Tang et al., 2022; Zhang et al., 2021). While nutrient contents of aggregates lead to differences in bacteria diversity, it has also crucial effects on assembly processes. Liao et al. (2022) found that bacterial assembly processes in macro- and micro-aggregates are mainly affected by total carbon and soil organic carbon. Better nutrient situation in SMA compared to MA and LMA may make stochastic processes dominate the assembly (Fig. 4) (Lin et al., 2019; Tang et al., 2022; Zheng et al., 2021). In our study, we found that active bacteria in SMA are dominated by stochasticity, while active bacteria in other aggregates showed more deterministic processes (Fig. 4). Our study is consistent with previous reports showing that environment of restrictive nutrient is dominated by deterministic processes, while the stochastic processes are more likely to be dominant in the environment with nutrient redundancy (Chase, 2010; Wang et al., 2015). It is also worth noting that nutrients and structure of aggregates were not measured in this study as the amount of soil samples obtained were insufficient after aggregate fractionation.

Differentiated communities among aggregates in active bacteria suggest different community functions. The functional prediction map shows that function of SMA differed from MA and LMA. Amino acid metabolism, cell motility, cell growth and death, and bacterial infectious disease are key functions found in ^{18}O labelled SMA (Fig. 6b). Functional genes of amino acid metabolism in SMA showed a relatively high abundance compared to those in MA and LMA, in which Proteobacteria dominates (Salam and Obayori, 2019). The results from our study, also showed a significant positive correlation of Proteobacteria in SMA to amino acid metabolism than that in

MA and LMA (Fig. S6). As the most important phylum (Fig. 3; Fig. S3), Proteobacteria determined cell motility genes by means of flagella movement in active bacteria (Anderson et al., 2010; Beeby, 2015). Compared to that in MA (58.99%) and LMA (59.61%), the relative abundance of Proteobacteria in SMA (61.25%) was higher at day 4 (Fig. 3, Fig. S3). Hence the relative abundance of cell motility genes is higher in SMA compared to MA and LMA. For cell growth and death, SMA provides more nutrients compared to MA and LMA (Lin et al., 2019; Tang et al., 2022; Zhang et al., 2021). Therefore, the relative abundance of cell growth and death genes are higher in SMA compared to MA and LMA. Proteobacteria also contributes to bacterial infectious diseases, for example, Salmonella and Vibrio of Proteobacteria will lead to infectious diseases. Besides, symbiotic relationship between Gemma-proteobacteria and invertebrates like nematode as found in previous research (Williams et al., 2010), suggesting its interaction with parasitic, thus the relative high abundance of bacterial infectious disease is found in SMA other than in MA and/or SMA. Together, the diversity and composition of active bacteria in SMA is more complex, assembly processes in SMA are more deterministic, and higher relative abundance of key functional profiles are predicted.

5. Conclusion

We applied $H_2{}^{18}O$ based DNA-SIP to identify active bacterial community in paddy soil aggregates. The results showed that higher microbial diversity, different composition, more complexed network and more stochastic processes were shown in

active bacteria compared to those in total bacteria. Active bacterial community and functional profiles altered significantly along the incubation days and soil aggregates. Compared to other soil aggregates with poorer nutrient, the assembly processes of active bacteria in SMA were more stochastic with richer nutrient. In summary, this research improves our understanding of ^{18}O labelled active bacteria community and their assembly processes among soil aggregates in paddy field.

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934 **Figure Legends**

935 **Fig. 1**. Distribution of the relative abundance of 16S rRNA genes in aggregates (MA,
936 SMA, LMA) retrieved from the 100% maximum water-holding capacity of $H_2{}^{18}O$
937 and 100% maximum water-holding capacity of $H_2{}^{16}O$ treatments in the 2-day and
938 4-day DNA-SIP microcosms.

939 **Fig. 2.** Diversity measurements of Shannon (a, b), Simpson (c, d) index of total and ^{18}O
940 labelled bacteria in the different aggregates and incubation days treatments.
941 Different letters and asterisks indicate significant differences ($P < 0.05$) based on
942 two-way of variance (ANOVA) as well as by LSD test for multiple comparisons.
943 Composition measurements of principal coordinate analysis (PCoA) based on
944 Bray-Curtis distances. Each point of total bacteria (e) and labelled bacteria (f)
945 corresponds to a different sample shaped by days and colored by aggregates. The
946 percentage of variation indicated in each axis corresponds to the fraction of the
947 total variance explained by the projection. Two-way permutational multivariate
948 analysis of variance (PERMANOVA) was employed to quantitatively assess the
949 effects of the day and aggregate treatment. Single, double and three asterisks
950 represent significance at $P < 0.05$, $P < 0.01$, and $P < 0.001$ respectively.

951 **Fig. 3.** Relative abundance of the soil bacterial community composition in both (a) total
952 bacteria and (b) active bacteria among days and aggregates.

953 **Fig. 4.** Network analysis revealing the associations among 16S rRNA OTUs in (a) Total
954 MA, (b) Total SMA, (c) Total LMA, (d) Labelled MA, (e) Labelled SMA, (f)
955 Labelled LMA. Colored nodes signify corresponding OTUs assigned to major

phylum. The size of nodes represents the number of links between the OTUs and others. Red and blue lines represent the positive and negative links between OTUs.

Fig. 5. Boxplot of (a) NST_{cao} and (c) NST_{mGower} values of total bacteria in nine treatments and boxplot of (b) NST_{cao} and (d) NST_{mGower} values of labelled bacteria in six treatments. Different letters and asterisks indicate significant differences ($P < 0.05$) based on two-way analysis of variance (ANOVA) as well as by LSD test for multiple comparisons.

Fig. 6. The functional profiles are divided into day 2 and day 4 with significant differences showed by letters among aggregates (ANOVA). The result of functional profiles that Z scores is showed in the heatmap. The relative abundance of function profiles and the significant differences are printed on the histogram. (a) The main functional differences in total bacteria are metabolism, cellular processes, organismal systems, human diseases and genetic information processing, (b) while in labelled bacteria are metabolism, environmental information processing, cellular processes, organismal systems and human diseases.

Fig.1

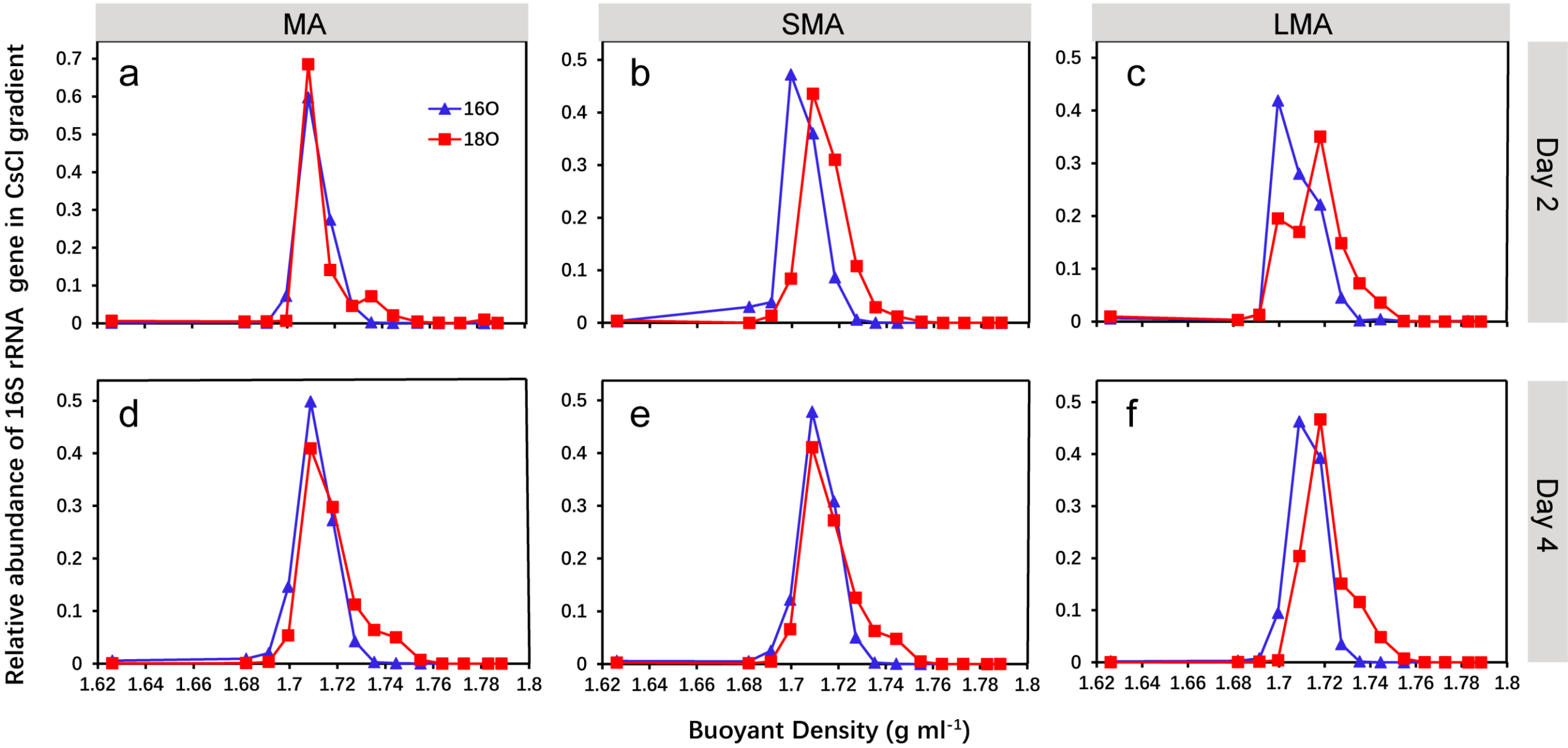


Fig.2

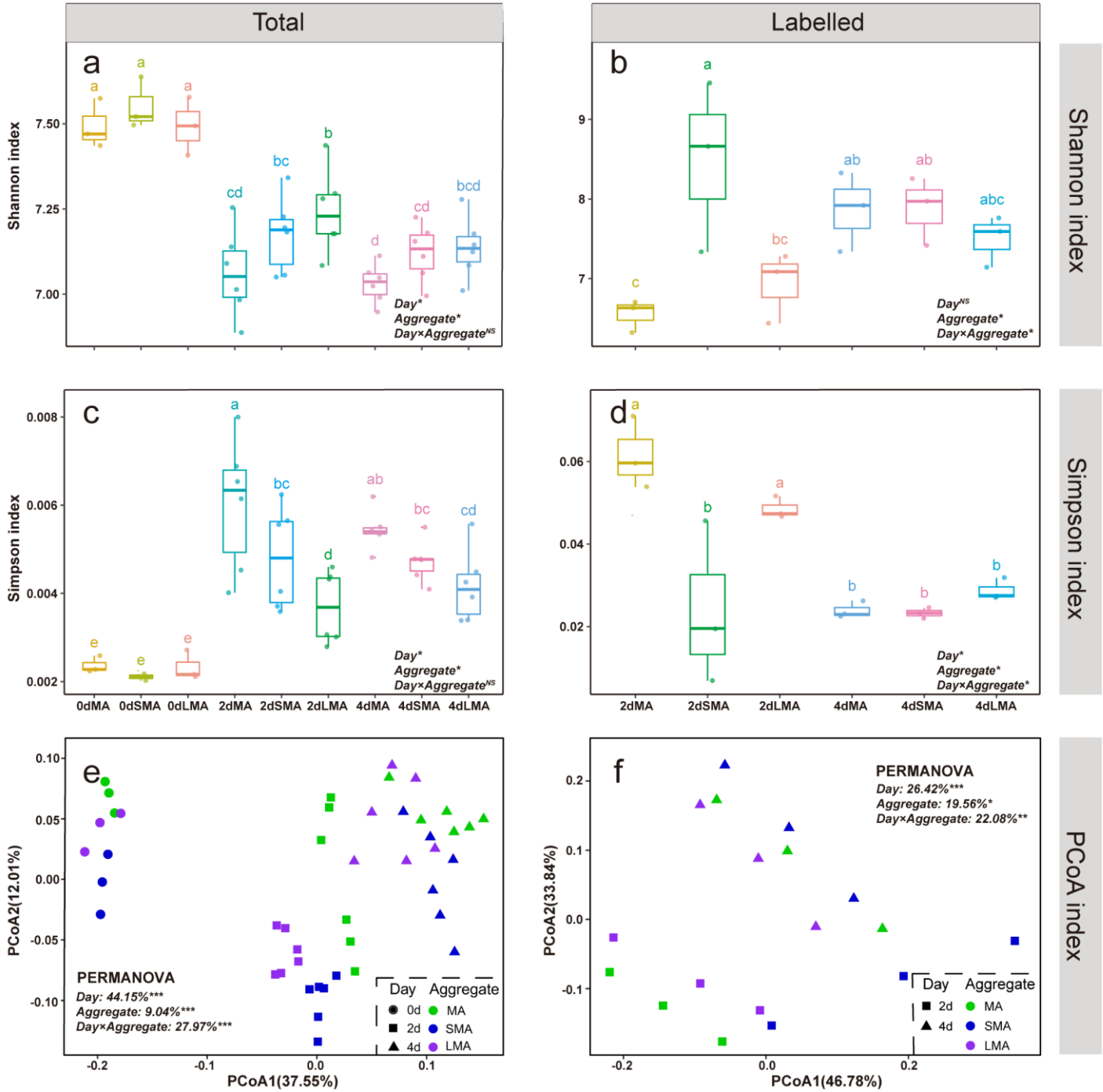

Total
Labelled
Shannon index
Simpson index
PCoA index
a
b
c
d
e
f
Shannon index
Simpson index
Day*
Aggregate*
Day×Aggregate^NS
Day^NS
Aggregate*
Day×Aggregate*
0dMA 0dSMA 0dLMA 2dMA 2dSMA 2dLMA 4dMA 4dSMA 4dLMA
2dMA 2dSMA 2dLMA 4dMA 4dSMA 4dLMA
PERMANOVA
Day: 44.15%***
Aggregate: 9.04%***
Day×Aggregate: 27.97%***
PERMANOVA
Day: 26.42%***
Aggregate: 19.56%*
Day×Aggregate: 22.08%**
Day Aggregate
0d MA
2d SMA
4d LMA
Day Aggregate
2d MA
4d SMA
LMA
PCoA1(37.55%)
PCoA2(12.01%)
PCoA1(46.78%)
PCoA2(33.84%)

Fig.3

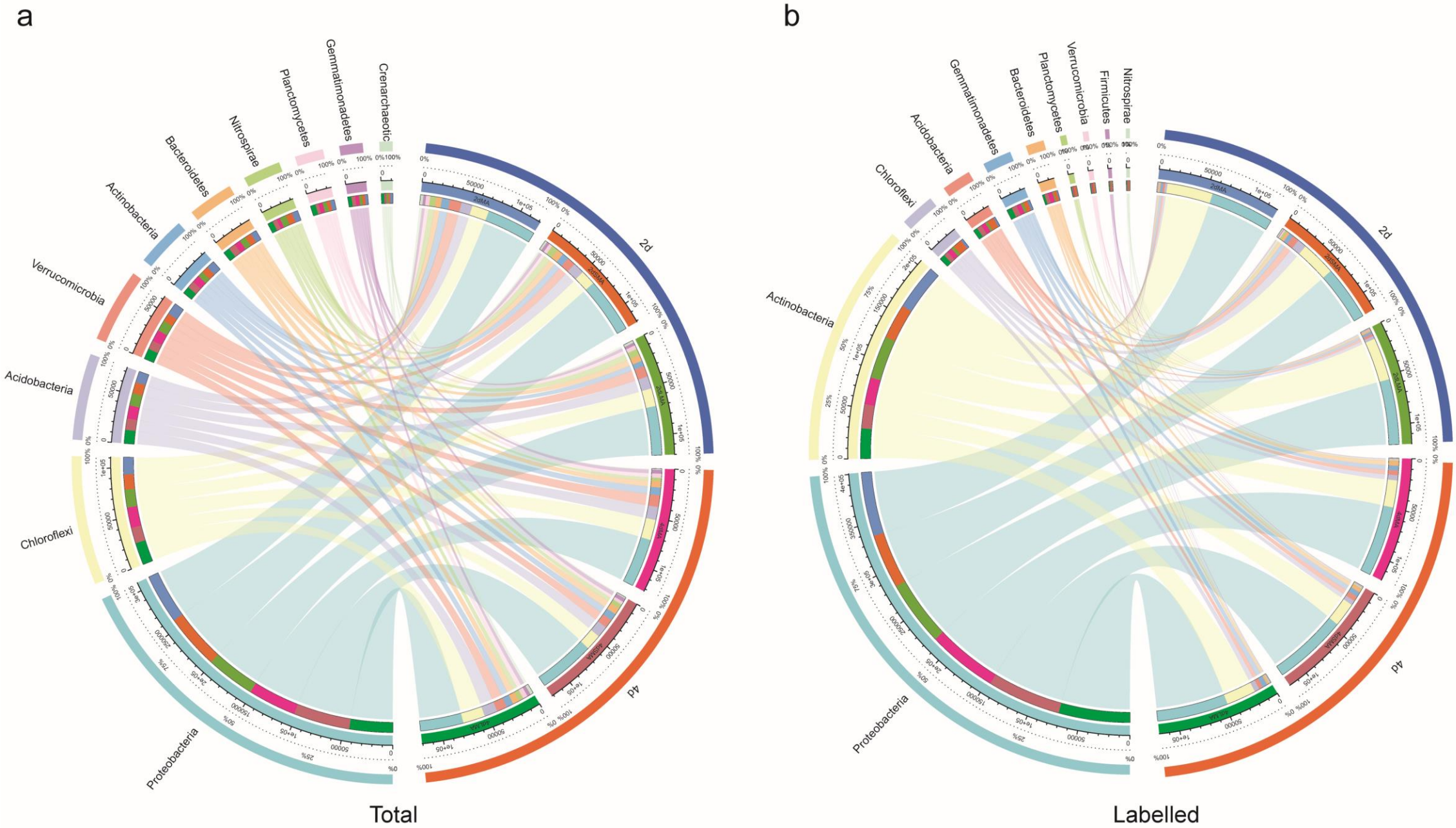

a
Crenarchaeotic
Gemmatimonadetes
Planctomycetes
Nitrospirae
Bacteroidetes
Actinobacteria
Verrucomicrobia
Acidobacteria
Chloroflexi
Proteobacteria
2d
4d
Total
b
Nitrospirae
Firmicutes
Verrucomicrobia
Planctomycetes
Bacteroidetes
Gemmatimonadetes
Acidobacteria
Chloroflexi
Actinobacteria
Proteobacteria
2d
4d
Labelled

Fig.4

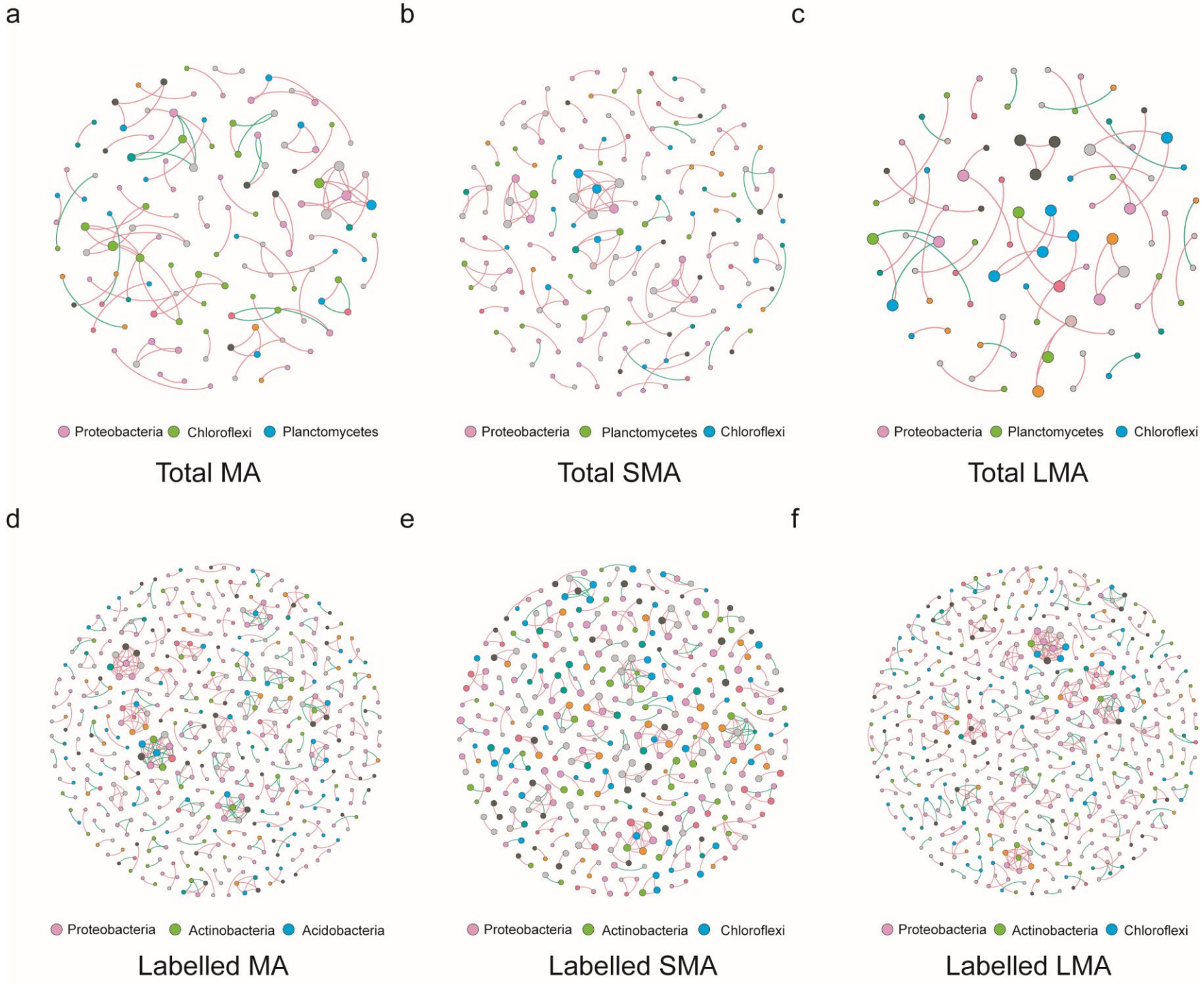

a
b
c
Proteobacteria Chloroflexi Planctomycetes
Total MA
Proteobacteria Planctomycetes Chloroflexi
Total SMA
Proteobacteria Planctomycetes Chloroflexi
Total LMA
d
e
f
Proteobacteria Actinobacteria Acidobacteria
Labelled MA
Proteobacteria Actinobacteria Chloroflexi
Labelled SMA
Proteobacteria Actinobacteria Chloroflexi
Labelled LMA

Fig.5

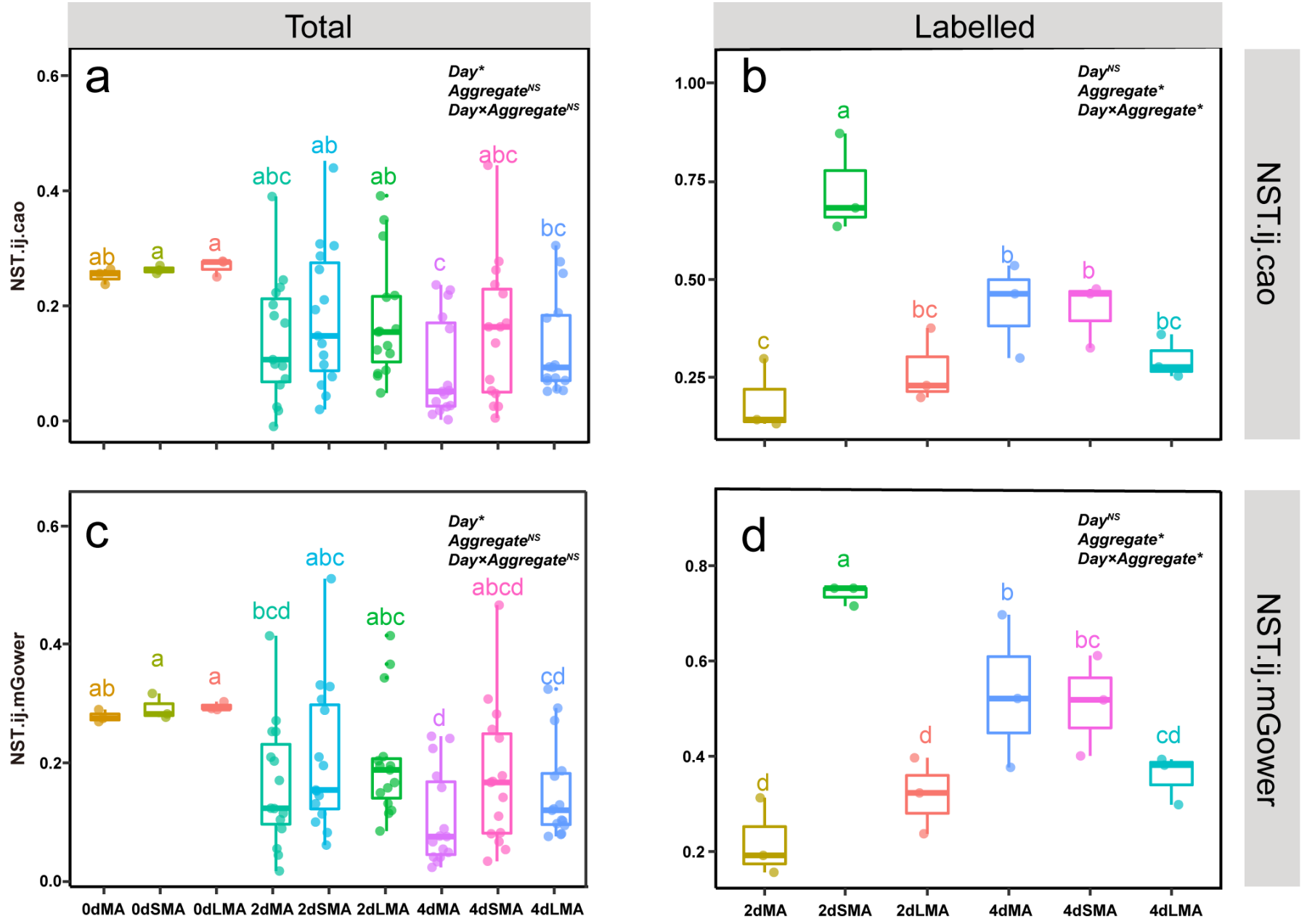
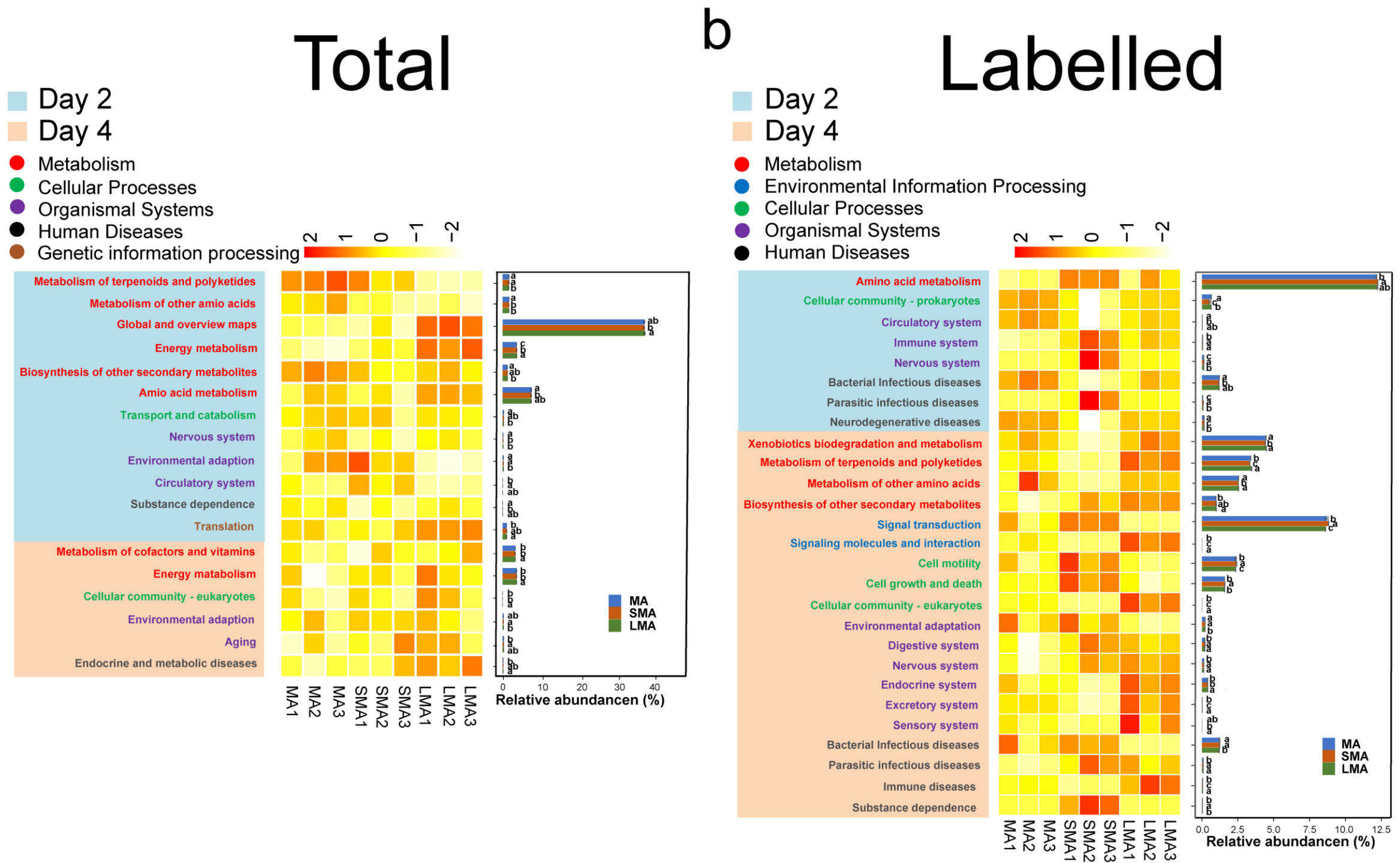

Total
Labelled
NST.ij.cao
NST.ij.mGower
a
b
c
d
Day*
Aggregate^NS
Day×Aggregate^NS
Day^NS
Aggregate*
Day×Aggregate*
0dMA
0dSMA
0dLMA
2dMA
2dSMA
2dLMA
4dMA
4dSMA
4dLMA

Fig.6


a
Total
Day 2
Day 4
Metabolism
Cellular Processes
Organismal Systems
Human Diseases
Genetic information processing
2 1 0 −1 −2
Metabolism of terpenoids and polyketides
Metabolism of other amio acids
Global and overview maps
Energy metabolism
Biosynthesis of other secondary metabolites
Amio acid metabolism
Transport and catabolism
Nervous system
Environmental adaption
Circulatory system
Substance dependence
Translation
Metabolism of cofactors and vitamins
Energy matabolism
Cellular community - eukaryotes
Environmental adaption
Aging
Endocrine and metabolic diseases
MA1 MA2 MA3 SMA1 SMA2 SMA3 LMA1 LMA2 LMA3
MA
SMA
LMA
0 10 20 30 40
Relative abundancen (%)
b
Labelled
Day 2
Day 4
Metabolism
Environmental Information Processing
Cellular Processes
Organismal Systems
Human Diseases
2 1 0 −1 −2
Amino acid metabolism
Cellular community - prokaryotes
Circulatory system
Immune system
Nervous system
Bacterial Infectious diseases
Parasitic infectious diseases
Neurodegenerative diseases
Xenobiotics biodegradation and metabolism
Metabolism of terpenoids and polyketides
Metabolism of other amino acids
Biosynthesis of other secondary metabolites
Signal transduction
Signaling molecules and interaction
Cell motility
Cell growth and death
Cellular community - eukaryotes
Environmental adaptation
Digestive system
Nervous system
Endocrine system
Excretory system
Sensory system
Bacterial Infectious diseases
Parasitic infectious diseases
Immune diseases
Substance dependence
MA1 MA2 MA3 SMA1 SMA2 SMA3 LMA1 LMA2 LMA3
MA
SMA
LMA
0.0 2.5 5.0 7.5 10.0 12.5
Relative abundancen (%)

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Author contributions:

Chenxiao Ding contributed to the experiment, the statistical analyses, and the writing of the manuscript.

Xinji Xu performed the experiment and the writing of the manuscript.

Yaowei Liu contributed significantly to analysis.

Xing Huang contributed significantly to analysis.

MengYuan Xi helped perform the analysis with constructive discussions.

Haiyang Liu contributed to analysis.

Elizabeth Deyett helped modify the manuscript.

Marc G. Dumont helped modify the manuscript.

Hongjie Di helped modify the manuscript.

Marcela Hernández helped modify the manuscript.

Jianming Xu helped modify the manuscript.

Yong Li helped modify the manuscript and contributed to the conception of the study.