The Signature Microbiota Driving Rumen Function Shifts in Goat Kids Introduced Solid Diet Regimes

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Running title: signature associated with diet and rumen function

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Abstract

The feeding regime of early supplementary solid diet improved rumen development and ruminant production. However, the signature microbiota linking dietary regimes to rumen function shifts and hosts are still unclear. We analyzed the rumen microbiome and functions affected by supplementation of solid diet using a combination of machine learning algorithms. The volatile fatty acids (i.e., acetate, propionate and butyrate) fermented by microbes increased significantly in the supplementary solid diet groups. The predominant genera altered significantly from unclassified *Sphingobacteriaceae* (non-supplementary group) to *Prevotella* (supplementary solid diet groups) RandomForest classification model revealed signature microbiota for solid diet that positively correlated with macronutrient intake, and linearly increased with volatile fatty acids production. The nutrient specific bacteria for carbohydrate and protein were also identified. According to FishTaco analysis, a set of intersecting core species contributed with rumen function shifts by solid diet. The core community structures consisted of specific signature microbiota and their symbiotic partners are manipulated by extra nutrients from concentrate and/or forage, and then produce more volatile fatty acids to promote rumen development and functions eventually host development. Our study provides mechanism of microbiome governing by solid diet and highlights the signatures microbiota for animal health and production.

Importance

Small ruminants are essential protein sources for human, so keeping them health and

increasing their production are important. The microbial communities resided in rumen play key roles to convert fiber resources to human food. Moreover, rumen physiology experience huge changes after birth, and understanding its microbiome roles could provide insights for other species. Recently, our studies and others have shown that diet changed rumen microbial composition and goat performance. In this study, we identified core community structures that were affected by diet and associated to the rumen development and goat production. This outcome could potentially allow us to select specific microbiome to improve rumen physiology and functions, maintain host health and benefit animal production. Therefore, it gives a significant clue that core microbiome manipulation by feeding strategies can increase animal products. To our knowledge, we firstly used FishTaco for determination of link between signatures abundances and rumen function shifts.

Keywords: goats, rumen microbiota, solid diet, rumen development, neutral detergent fibers, volatile fatty acids

Introduction

With the development of next generation sequencing, the roles of gut microbiome have been dramatically understood. The early life diet, especially introduction of solid diet, is an important driver in shaping long-term and adult gut microbiome profiles due to the novel alteration of diet components and macronutrient levels as well as gut anatomical development (1). Goat with rapid physiological changes (non-rumination, transition and rumination) could be proposed as an appropriate animal model for studying the gut microbial ecosystems development by early diet intervention and providing a means for prevention of metabolic diseases (2, 3). Young ruminants receiving only milk or fluid diet (milk replacer) have limited metabolic activity in the rumen epithelium and minimal absorption of volatile fatty acids (VFA) (4). Early supplementary feeding solid diet has been widely used in lamb production to improve rumen and body development since it can stimulate microbial proliferation and VFA production that initiates epithelial development (5). A solid concentrate diet (starter) containing high concentration of carbohydrate has been widely used to rear pre-weaned ruminants (4, 6, 7). Compared with breast milk-fed lambs, the community structure and composition of rumen microbiota of started-fed lambs tends to mature easily and quickly (8). Lin and their colleagues (3) analyzed rumen microbiota in lambs fed starter vs breast-milk. They found that acetate and butyrate increased in starter-feeding lambs, as well as increases of 5 genera including *Mitsuokella*, *Sharpea*, *Megasphaera*, *Dialiste*, and unclassified *Bifidobacteriaceae*. An extra alfalfa supplementation on the basis of concentrate diets improves rumen

development to the next level. Previous studies reported that increases of growth performance and changes of ruminal microbiota during the pre- and post-weaning periods were found in lambs fed starter plus alfalfa compared with lambs fed fluid-diet and starter (9, 10). In addition, some studies have summarized the significant changes of microbiota in solid feeding regime and evenly calculated the correlation between macronutrient intake and rumen bacterial abundances. Wang et al. (11) found the correlation between bacterial genera in lambs rumen tissue and functional variables at d42. Yang et al. (10) sequenced rumen samples from *Hu* lambs fed milk replacer from d5 to d38 and supplied with solid diet (starter and alfalfa). They observed the effect of solid diet on microbial composition, and a set of taxon correlated with CP, NDF and body weight.

Until now, although these studies remarkably extend the effects of solid diet on the development of rumen functions and microbial communities in lambs, they mainly focus on the microbiota at weaning day (around d 40) or at genus level. Many key questions remain unclear. For example, does goat kids have similar pattern by solid diet since lambs and goats belong to different genus? What are the signature microbiota for supplementary regimes? How does the regime supplemented starter plus alfalfa affect rumen microbiota manipulation? How does the signature microbiota associated with other members in solid diet regime maintain equilibrium and improve function? To address these questions, a study that feeds goats with solid supplement to investigate microbiome and their association with experimental factor and rumen function using more machine leaning algorithms is urgent. RandomForest, an

ensemble learning method for classification and regression, can be used to rank the importance of predictor variables in a regression or classification problem in a natural way (12). FishTaco, a computational framework for comprehensively computing taxon-level contribution to detected functional shifts and identifying key taxa, was introduced by (13). Network analysis that identify the microbial interaction allows us to characterize how the "core" microbiota impacts the overall composition and function (14).

Therefore, the objectives of this work was to assess the rumen fermentation, microbiome community and function shift influenced by supplementary solid diet fed until to d 60 (rumination phase). We addressed above questions by deeply analysis of microbial data with a combination of above three algorithms. Also, the correlation between phonotype, such as macronutrients intake and rumen fermentation parameters, and microbiome were identified. We observed that extra solid diet intake in early life could change the rumen microbial community structure towards maturated level by increasing signature microbiota qualitatively or quantitatively, and then fermentation environment and functions.

Results

Rumen fermentation parameters

The rumen fermentation parameters affected by the different dietary regimes were observed (Figure 1 & Table S3). The MRO group had greater concentration of NH_3-N ($P<0.05$) compared with the MRC and MCA group, while the opposite pattern

of ruminal microbial protein was found. The more concentration of total VFA, acetate, propionate, butyrate and valerate in supplementary solid diet regimes (MRC, MCA) as compared with MRO was observed ($P<0.05$), and except propionate and valerate, the acetate, butyrate and Total VFA were higher in MCA than MRC ($P<0.05$).

Since the key factors of diets influenced goat rumen environment and development were intake of nutrient including CP, NFC and NDF, thus, correlation between nutrient intake and rumen fermentation parameters was performed (Table S4). Regression analysis confirmed that pH and NH_3-N were negatively associated with average daily intake of CP, NFC and NDF, while rumen MCP and VFA (i.e., acetate, propionate, butyrate, and Total VFA) concentration had the strongly positive association with nutrient intake.

The diversity and core bacteria in rumen microbiome

After quality control, filtering, and OTUs clustering steps, 64,1197 high quality sequencing reads across all samples and an average of 3,5622 sequence reads for each sample were generated. Firstly, we analyzed all the rumen content microbiome at community level. Although diversity (Shannon Index) was not different (p=0.372), significance of microbial richness was observed among MRO, MRC and MCA rumen samples (p=0.012) (Figure 2 A&B). The MRO rumen microbiota had significantly higher observed species than both MRC and MCA samples (p=0.045, p=0.005), and there was no difference between MRC and MCA (p=0.180). The observed species of rumen microbiome was negatively correlated with nutrient average daily intake

147 including CP (r=-0.65, p=0.003), NFC(r=-0.73, p=0.001) and NDF (r=-0.74,
148 p=0.0003) (Table S5). Negative association between microbial richness and MCP and
149 VFA including acetate, propionate, butyrate, valerate and Total VFA was also
150 observed. Regarding the beta diversity measurements, significant cluster in
151 community structure among 3 regimes were detected (Weighted Unifrac ANOSIM,
152 R=0.68, P<0.05; UnWeighted Unifrac ANOSIM, R=0.69, P=0.001). The MRO
153 formed a distinct cluster (green dots) on the left side, while the MRC and MCA were
154 closely clustered (red and blue dots) on the right side of PCoA plot (Figure 2 C&D).

155 We next examined the rumen core microbiome among three treatments. At genus
156 level, a total of 152 genera were observed, and *Prevotella* followed by unclassified
157 *Prevotellaceae*, unclassified *Sphingobacteriaceae* and unclassified *Bacteroidetes*
158 accounted for 63.29% of the total sequences were the predominant genera with
159 abundance over 5% across all samples(Figure S1). The top genera in MRO were
160 unclassified *Sphingobacteriaceae* (30.32%), unclassified *Prevotellaceae* (16.92%),
161 unclassified *Bacteroidetes* (11.77%) and *Prevotella* (8.91%). In MRC, *Prevotella*
162 (56.02%) were the predominant bacteria, followed by *Roseburia* (4.49%), unclassified
163 *Prevotellaceae* (4.29%), *Selenomonas* (3.82%) and unclassified *Lachnospiraceae*
164 (3.73%). However, in MCA, the abundance of the predominant genus *Prevotella*
165 (44.02%) decreased compared with MRC, and other dominant genera were
166 unclassified *Prevotellaceae* (11.63%), *Fibrobacter* (7.01%), *Treponema* (5.35%),
167 *Succinivibrio* (4.74%) and unclassified *Lachnospiraceae* (4.50%)

168 At OTU level, there were 281 OTUs that were significantly different between 3

groups (Table S6), and 16 taxa in top 30 were significant. The top 30 most abundant bacterial taxa accounting 57.77% of all reads are displayed on stacked bar charts (Figure 3). Among top 30 OTUs, 14 belong to genus *Prevotella*, and 4 was owned by genus unclassified *Prevotellaceae*. The OTUs belong to unclassified *Sphingobacteriaceae* (OTU1 and OTU5), unclassified *Prevotellaceae* (OTU4 and OTU30) and OTU24 *Cloacibacillus* were greater in MRO. The OTUs affiliated with *Prevotella* (OTU2, OTU6, OTU13,) in top 30 had higher abundance in MRC and MCA. The MRC were abundant with OTU10-*Roseburia* OTU20-*Olsenella* and OTU21-*Prevotella*. The bacteria belong to genera of *Prevotella* (OTU6 and OTU13) *Succinivibrio* (OTU9), unclassified *Prevotellaceae* (OTU15), *Succiniclasticum* (OTU22) had the highest relative abundance in MCA.

The signature microbiota differentiating MRO, MRC and MCA supplementary regimes

To identify the rumen important microbiome that differentiate MRO, MRC and MCA, we performed an updated RandomForest classification model to differentiate these 3 supplementary regimes. The regimes-associated bacterial features were listed based on their MDA and the representatively selected microbiota were presented in Figure 4. All 3 groups were analyzed together, and optimal features with an AUC (area under the curve) of 1.00 (specificity 1.00, sensitivity 1.00) were selected from AUCRF model (Table S7; Figure S2). High AUC (0.931) was still observed at 50th feature suggesting those signatures being able to accurately predict whether goats was

191 fed concentrate plus alfalfa. Among top 50 features, only 3 core OTUs such as OTU5
192 (unclassified *Sphingobacteriaceae*), OTU24 (*Cloacibacillus*) and OTU6 (*Prevotella*)
193 were identified as regime-associated bacteria (Figure 4). Forty of top 50 bacteria were
194 more abundant in MRO. OTU5 associated with MRO predominant genus had more
195 relative abundance and prevalence (11.13%; 6/6) as compared with MRC (0.03%, 2/6)
196 and MCA (0.04%, 2/6). OTU24 as qualitative signatures had more abundance 2.16%
197 in MRO. Other species associated with *Prevotella* that was enriched genus in solid
198 diet groups were also found more abundant in MRO, including OTU119, OTU42 and
199 OTU60. For MCA microbiome, OTU6 and OTU104 affiliated with predominant
200 *Prevotella* increased. We observed the relative abundances of OTU6 was 0.01% 1.35%
201 and 5.89% in MRO, MRC and MCA (prevalence 2/6, 6/6 and 6/6). OTU87
202 (*Butyrivibrio*) and OTU83 (unclassified *Bacteroidales*) were significantly enriched in
203 MCA and extremely low abundance in MRO and/or MRC. Similar patterns could be
204 found in other MCA predictors such as OTU93 and OTU74 (*Treponema*), OTU539
205 (unclassified *Clostridiales*), OTU396 (unclassified *Proteobacteria*), OTU221
206 (*Pseudobutyrivibrio*) and OTU110 (unclassified *Prevotellaceae*) (Figure S3-1 &
207 S3-2).

208 Then, we performed pair wise AUCRF comparisons to validate these predictors.
209 The results confirmed that most of the classified biomarkers could also be listed
210 (Figure S4-S6). Moreover, MRC were enriched with OTU148 (unclassified
211 *Lachnospiraceae*) and OTU114 (*Megasphaera*) compared with MRO, whereas it had
212 more abundance of OTU643 (*Neisseria*), OTU177 (*Campylobacter*) and OTU314

(*Blautia*) as compared with MCA.

Phenotypes and rumen microbiota

To find the relationship between rumen microbiota with major nutrients of diet for better understanding how supplementary feeding regimes influenced microbial communities. Firstly, we performed RandomForest regression model by using CP, NFC and NDF intake as outcomes and all taxa as independent variables. Then, the Pearson correlations were calculated between selected top 50 bacterial abundances and dietary CP, NFC and NDF intake respectively (Figure 5). On other hand, the impacts of alteration of rumen microbiota on rumen VFA were also estimated using same approaches.

The rumen microbiota had high prediction accuracy (>73%) to explain nutrients intake (Table S8). Among CP, NFC and NDF, 31 shared bacteria were observed, and 30 of 31 were the predictors identified by RandomForest classification model. In these shared bacteria, 27 as MRO-associated predictors had negative correlation with intake of CP, NFC and NDF, such as OTU5, OTU24. For other 3 shared features, OTU327 (*Clostridium XlVa*) negatively correlated with intake of CP, NFC and NDF, OTU148 (unclassified *Lachnospiraceae*) had no correlation ($p>0.05$), and OTU6 (*Prevotella*) was positively and moderately correlated with them ($r=0.53, 0.48, 0.49$, $p=0.023, 0.043, 0.041$). Regarding to CP and NFC intake, OTU165 (unclassified *Prevotellaceae*) was the shared OTUs increasing abundances. The OTU396 (unclassified *Proteobacteria*) and OTU27 (unclassified *Prevotellaceae*) were

235 specifically and positively correlated with CP intake (r=0.55, 0.63, p=0.019, 0.005).
236 When NDF intake was observed, the abundances of its associated microbiota went up.
237 For example, OTU464 (unclassified *Burkholderiales*) increased with more NDF
238 intake (r=0.65, p=0.003), while others identified as predictors for MCA (i.e., OTU87,
239 OTU83, OTU93, and OTU539) also linearly increased abundance with increase of
240 NDF intake (r=0.53, 0.52, 0.49, 0.50 and 0.48). Interestingly, OTU74 (*Treponema*)
241 identified as MCA signature had no significant association with NDF intake (r=0.38,
242 p=0.124). Moreover, the OTU396 and core significant *Succiniclasticum* (OTU22)
243 tended to moderately correlate with NDF intake (r=0.46, p=0.051; r=0.41, p=0.09).
244 Although the regression prediction of MCP and NH_3-N was not high (50.97% and
245 44.94%), OTU6 and OTU27 correlated with CP were associated with NH_3-N, and
246 other regime-associated signature (OTU148) significantly correlated with rumen
247 nitrogen indexes (File S1). Moreover, OTU152, OTU268 and OTU322 had
248 significant correlation with MCP. The rumen microbiota also had accurate prediction
249 for VFA concentration (Table S8). Shared OTUs were also found in the list of
250 between RandomForest classification and VFA regression (i.e., 39 acetate, 17
251 propionate, 24 butyrate, 25 valerate, 36 Total VFA). Those shared OTUs were most
252 of MRO-associated signatures and negatively correlated with VFA. For the bacteria
253 positively correlated with Total VFA, they were also observed within 1 or 2 of acetate,
254 propionate or butyrate regression models, for example, OTU6 within Total VFA and
255 butyrate; OTU396 within Total VFA and propionate and butyrate. Considered the
256 major VFA (acetate, propionate and butyrate), OTU83 was the only common

257 microbes were correlated positively with all of them (r=0.63, 0.54, 0.56; p=0.005,
258 0.020, 0.017). When increasing acetate was observed, the abundances of OTU122
259 (*Ruminobacter*), OTU143 (*Fibrobacter*) and OTU204 (unclassified *Bacteroidetes*)
260 tends to increase. Regarding to propionate, positive correlation were found in the
261 bacteria, such as OTU13 (*Prevotella*), OTU93 (*Treponema*), OTU165 (unclassified
262 *Prevotellaceae*), OTU258 (*Olsenella*), OTU120 (Megasphaera), OTU532
263 (unclassified *Bacteroidetes*), OTU322 (*Allisonella*), OTU604 (*Eubacterium*) and
264 OTU530 (*Mitsuokella*). The butyrate-associated bacteria were OTU6, OTU13,
265 OTU539, OTU15 (unclassified *Prevotellaceae*), OTU17 (unclassified
266 *Lachnospiraceae*), OTU114 (*Megasphaera*) and OTU205 (unclassified *Firmicutes*).
267 Notably, higher ensemble prediction score (70%) in valerate regression indicated that
268 rumen microbiota explained it better as well. When valerate increased**,** unclassified
269 *Lachnospiraceae* (OTU148 and OTU391), *Olsenella* (OTU20, OTU258),
270 *Megasphaera* (OTU114, OTU120 and OTU173), unclassified *Clostridiales*
271 (OTU311), *Mitsuokella* (OTU152), unclassified *Bacteria* (OTU52), *Prevotella*
272 (OTU186) and unclassified *Porphyromonadaceae* (OTU47) linearly increased.
273
274 **Rumen microbiota driving function shifts**
275 To predict how rumen microbiota associate with solid diet supplementary
276 regimes, PICRUSt based on OTUs' level was used to predict the abundances of
277 functional categories the KEGG. In the level 3, nutrient pathways were the most
278 popular in this study (Figure S7). Many bacterial genes in all 3 groups could

279 potentially trigger pathway function of same nutrient metabolism, but different
280 treatments participated different reaction modules. For example, carbohydrate
281 metabolism found in all groups had the specific reaction of pyruvate metabolism and
282 citrate cycle in MRO; Fructose, mannose Starch and sucrose metabolism in MRC; and
283 glyoxylate and dicarboxylate metabolism in MCA. Moreover, some cellular process
284 pathways were found in goat supplied with solid diet. MRC was enriched membrane
285 transport (ABC transporters) and Insulin signaling pathway. The pathways of
286 transcription factors and machinery were found in MRC and MCA.

287 The FishTaco was performed to identify the corresponding microbiota driving
288 the functional shifts between supplementary regimes. There were no differences of
289 normalized abundance of functions between MRC as control and MCA as case based
290 on Wilcoxon rank-sum test. When MRO as control and case defined as MRC and
291 MCA separately, 31 and 37 significant pathways were found (File S2-S3). Notably,
292 21 shared functions were observed between 2 comparisons, including metabolism of
293 nutrient (lipid, amino acid, carbohydrate, vitamin, peptidoglycan, terpenoids and
294 polyketides), and the pathway of endocrine system and cellular processes. (Figure 6 &
295 S8-S9). To better understand the driver OTUs function, we get all sequences identifier
296 with the highest scores on NCBI BALSTN database (File S4). Across all significant
297 functions enriched in MRC, a set of *Prevotella* bacteria including OTU3 (*Prevotella*
298 *copri DSM*), OTU2 (*Prevotella brevis strain GA33*), OTU14 (*Prevotella histicola*)
299 and OTU16 (*Prevotella ruminicola*) (occurrence 100%, 83.3%, 60% and 13.3%) were
300 the main drivers (Figure S8-S9). While in MCA, the function shifts were driven by a

301 convoluted outcome of *Fibrobacter* and *Prevotella* including OTU11 (*Fibrobacter*
302 *succinogenes*), OTU2, OTU3, OTU7 (*Prevotella ruminicola*) and OTU13 (*Prevotella*
303 *brevis strain GA33*) (their occurrence 100%, 100%, 100%, 30.3% and 15.2%).
304 Although selenocompound metabolism pathway was enriched in MRC and MCA
305 compared with MRO, the set of OTUs drove this enrichment of 2 regimes and the
306 level of contribution of each specie differed, with OTU2, OTU3 and OTU14 driving
307 the shift in MRC and a set of OTU2, OTU3, OTU11, and OTU13 in MCA (Figure 6).
308 These enrichments were attenuated by greatly different bacteria in MRC (OTU20,
309 OTU16, OTU8, OTU10, OTU12 and OTU14) and MCA (OTU15, OTU6, OTU7,
310 OTU17, OTU22 and OTU9). Other highlight pathways such as lipid and carbohydrate
311 metabolism, Biosynthesis of unsaturated fatty acids and transporters had similar
312 pattern. In addition, the MRO enriched microbiota including unclassified
313 *Sphingobacteriaceae* (OTU1 and OTU5 *Olivibacter sitiensis*) and *Cloacibacillus*
314 (OTU24 *Cloacibacillus porcorum*) were strongly depleted by solid diets.
315
316 **Network analysis of regime associated microbiota**
317 Network analysis revealed core sun-community structure within communities
318 that consisted of a set of bacteria associated with the phenotypes and rumen functions
319 in the supplementary regimes. We detected respectively 4, 7 and 8 main subnetworks
320 in MRO, MRC and MCA (Figure 7). The species that were observed as
321 regime-associated features and identified as functions drivers formed the main
322 subnetwork. In MRO, the predictors, OTU60 (violet cluster), OTU42 and OTU111

(green cluster), OTU99, OTU79, OTU55 (yellow cluster) and OTU33, OTU94, OTU89 (pink cluster) formed the main subnetwork, showing significant correlations with a large number of other members of MRO community. For MRC rumen microbiota, OTU2, OTU6 OTU16 within palegreen cluster, OTU3 within pink cluster and OTU14 within yellow cluster, as dominate species associated with other members, consisted of the main subnetworks. Within MCA, OTU104, OTU11, OTU2, OTU6, OTU13, OTU87, OTU74, OTU83 and OTU3 recognized as main drivers or signatures were the main members of subnetworks. Their partners interacted with these microbiota may associate with fermentation, such as OTU7 and OTU27 in MCA. Moreover, OTU79 (*Snodgrassella alvi*) and OTU99 (*Elusimicrobium minutum*) as hub nodes in MRO connected yellow and blue cluster, whereas OTU87 (*Butyrivibrio hungatei*), OTU15 (*Metaprevotella massiliensis*) and OTU31 (*Fibrobacter succinogenes subsp. Elongates*) served as a bridge to link three clusters.

Discussion

Early supplementary feeding with solid diet has a positive impact on rumen development by influencing rumen microbial population and composition, environment alteration and functional achievement. However, lack of information of microbial predictors for supplementary regimes leads to unclear mechanism of manipulation of rumen microbiota and function shifts. This study confirmed that rumen VFA especially acetate, propionate and butyrate increased significantly with the supplementation of solid diet, and promoted rumen weight and functions. The

345 predominate genera changed from unclassified *Sphingobacteriaceae* to *Prevotella*
346 when goat kids were supplied solid diet. The signature microbiota in corresponding
347 feeding regimes significantly correlated with phonotypes such as major nutrients
348 intake and VFA concentration. For example, the biomarkers for MCA (OTU6,
349 OTU87, OTU83, OTU93 and OTU539) were positively correlated with NDF intake
350 and VFA production. The improved rumen function in goats supplied solid diet were
351 caused by the core bacteria, such as OTU3 (*Prevotella copri DSM*), OTU2 (*Prevotella*
352 *brevis strain GA33*), OTU14 (*Prevotella histicola*) and OTU11 (*Fibrobacter*
353 *succinogenes*). All these signatures and/or core microbiome formed main
354 sub-communities to response solid diet feeding and drive function shifts.

355 The VFAs that are products of the fermentation of diets are essential to the
356 rumen papillae development and nutrient source for host requirements (15). In
357 ruminants, VFA produced in the rumen meets 70–80% of the energy requirement for
358 the rumen epithelia, and 50–70% of the energy requirement for the body (16). In this
359 study, rumen microbial proteins and VFA concentration increased in supplementation
360 of solid diet. Other studies also revealed that early starter and alfalfa consumption
361 facilitated rumen development and changed the pattern of ruminal fermentation (9, 11,
362 17). Moreover, we found the rumen microbial proteins and VFAs were positively
363 correlated with intake of CP, NDF and NFC. Previous study reported that ruminal
364 NH_3-N increased linearly in response to increasing dietary CP (18). This study
365 confirmed that microbiota in goats fed solid diet had more strong ability for
366 biosynthesis of microbial proteins and VFA. Moreover, except the physical

367 stimulation from solid diet, the chemical effects of nutrient intake may be another
368 reason leading to increase of VFA. Therefore, early supplementation of solid diet
369 leading to high nutrient intake increases rumen VFA production and nitrogen
370 utilization efficiency, which reflects that microbiome experienced solid diets had a
371 strong ability to utilize nutrient.

372 In pace with the change of rumen environment, this study also observed that the
373 membership and structure of microbiota also altered by supplied with concentrate or
374 forage compared with only fluid diet groups. Significant lower alpha diversity in
375 starter feed-lambs and distinct beta diversity between starter feed- and breast milk-fed
376 lambs were also reported (11). High bacterial richness in fluid diet groups might be
377 temporary phenomenon at d60. Others confirmed that rumen microbiota at d70 had a
378 lower richness compared with it at d42 (8). As we know, rumen is in rumination
379 phase after 8 weeks and in transition phase in 3-8 weeks. In this study, compared with
380 MRO, rumen microbiota in goats supplied with solid diet at d60 may have a more
381 mature rumen function and stable microbiome structure at the same age. Another
382 reason for reduction of richness in solid feeding regimes might be due to high
383 concentration of VFA and low pH (19). In addition, the rumen microbiota in solid
384 supplementary regimes had similar alpha and beta diversity. The similar pattern also
385 could be observed in rumen fermentation parameters. This might be due to less feed
386 intake of alfalfa and similar concentrate intake. In animal trial, the MCA goats had *ad*
387 *libitum* access to concentrate and alfalfa pellets in separately troughs. Based on feed
388 intake results, the goats preferred concentrate. Thus, future studies have to increase

389 roughage intake for its effects on rumen microbiota or detect the microbiome after
390 weaned milk replacer.

391 RandomForest is a powerful tool capable of delivering performance that is
392 among the most accurate methods (20). It has been widely used in human microbiome
393 studies to find the signatures for disease or healthy (21, 22). This study identified
394 important signatures from 838 OTUs using RandomForest, which could provide more
395 effective and accuracy information how diet supplementary regimes affected
396 microbial composition. A higher AUC value (AUC=1.00) indicates the features are
397 more efficiently classified. RandomForest not only gave an important score to the
398 significant species but also find the accurate bacteria for experimental factors. For
399 example, the low abundances of OTU87, OTU83, OTU93 and OTU539 were
400 identified as the important predictor for regimes, which indicated that low abundance
401 bacteria may also paly critical roles in function drifts. Therefore, previous literature (3,
402 10) only focused on the genera with significantly different abundances may not
403 provide the best conclusion. RandomForest regression is useful and robust method for
404 correlation applications because of its ability of automatically producing accuracy
405 estimation and measuring the variable importance. Using it to select microbiota with
406 high important scores would be a better and corrected method for finding precise
407 signatures. The percent explained variance is a measure of how well out-of-bag
408 predictions explain the target variance of the training set. High % explained variance
409 (over 70%) in this study were found in CP, NFC and NDF model, which indicated
410 that those top microbiota were more important to the responders. It helps to figure out

411 the relationship between specific nutrient and microbiota. In addition, using FishTaco
412 to link the microbiota abundances and rumen function shift caused by supplement of
413 solid diet is a creative attempt (13). We used it to integrate comprehensively the
414 significant species and function shifts. Compared with the original PICRUSt result,
415 there was an improved result of significant pathways. Its process rely on a
416 permutation-based approach, carefully designed normalization and scaling schemes to
417 preserve overall community taxonomic characteristics and to account not only for
418 variation induced by each bacteria but also for the way this variation correlated with
419 community-wide context. Our results identified that a set of core bacteria were the
420 main taxon drivers since low taxonomic abundance profiles were filtered and
421 normalized. Finally, we observed subnetworks formed by these signature microbiota
422 and their partners. In a word, more algorithms gave insights how microbiota had
423 impacts on function shifts.

424 Rumen microbiota degrade fibers polysaccharides and proteins in diet, and yield
425 VFAs and microbial proteins, which offer nutrients to meet the host's requirement for
426 maintenance and growth (23, 24). Based on these machine leaning algorithms, we
427 analyzed microbiome to link supplementary regime to alteration of rumen
428 environment and function. Supplementary solid diet altered the core microbiota from
429 unclassified *Sphingobacteriaceae* to *Prevotella*. The representative OTU5 associated
430 with unclassified *Sphingobacteriaceae* as MRO predictors were negatively with
431 macronutrients intake and VFA production. OTU5 classified as *Olivibacter sitiensis*
432 function is not clear, but it decreased with pH reduction when intake high concentrate

433 diet (25). The species affiliated with *Prevotella* (OTU2, OTU3, OTU6 and OTU13) in
434 top 30 increased in solid supplementary regimes. Other studies also reported that the
435 abundances of the genus *Prevotella* that was predominent in starter-fed lambs
436 positively correlated with acetate, propionate and urea nitrogen concentration (3, 8,
437 11). This genus is good at utilization of proteins and carbohydrates (either fiber- or
438 non-fiber-carbohydrate) (26). Importantly, OTU6 (*Prevotella oralis*) was identified as
439 signature species for solid diets, correlated with macronutrient intake and butyrate
440 concentration. Another microbiota, OTU13 (*Prevotella brevis strain GA33*), was not
441 classified as predictors for MCA, but we observed it had high abundance in MCA,
442 positive association with propionate and butyrate, driving function shifts and
443 interaction with other core microbiome. Therefore, increased abundance of these 2
444 species represented as *Prevotella* in rumen accessed solid diets promoted the
445 improvement of rumen digestibility and function by yielding more VFA products.
446 OTU2 (*Prevotella brevis*) and OTU3 (*Prevotella copri*) were the main drivers for
447 functions shifts by solid diet. De Filippis et al. detected distinct strains of *Prevotella*
448 *copri* by metagenome studies and showed that fiber-rich diets were linked to these
449 strains with improved potential for carbohydrate catabolism (27). Broadly,
450 introduction of solid fiber-rich diet to goat before weaning, *Prevotella* proliferated,
451 which was also observed in other large domestic animals (28). They could be used as
452 potential microbiota to utilize solid diet, maintain rumen community balance and
453 prevent metabolic disease caused by dysbiosis. Another core genus increased in both
454 solid diet regimes was unclassified *Lachnospiraceae*. The family *Lachnospiraceae*

455 contains many known plant degrading species and most of the butyrate-producers (29).
456 In our results, OTU148-*Lachnospiraceae* (*Kineothrix alysoides*) enriched in MRC
457 was significant associated with NH_3-N and valerate, although it was identified by
458 regression model for nutrients intake and fermentation parameters. Regarding to
459 others dominant bacteria, whereas *Roseburia* and *Selenomonas* specifically increased
460 in concentrate diet regime. The abundances of *Fibrobacter*, *Treponema* and
461 *Succinivibrio* arose became greater in extra supplementation of alfalfa. *Succinivibrio*,
462 a saccharolytic bacteria, can yield acetate and lactate (30). The OTUs associated with
463 these genera were not observed well in our study. For example, OTU10-*Roseburia*
464 and OTU9-*Succinivibrio* formed main structure with other members; OTU74
465 affiliated with *Treponema* predicted MCA well but no relation with phonotypes; and
466 OTU11-*Fibrobacter* drove the enriched functions of MCA while
467 OTU143-*Fibrobacter* increased with acetate. These microbes either at genus or OTUs
468 level had significant abundances in different regimes, however, they were not
469 correlated with phonotypes very well. The reasons might be they were symbiotic with
470 other microbiota. For example, *Treponema* does not utilize fiber, but it helps other
471 bacteria to digest cellulosic materials (31).

472 The MCA-associated features, OTU87 (*Butyrivibrio hungatei*), OTU83
473 (*Prevotellamassilia timonensis*), OTU539 (*Abyssivirga alkaniphila*), and OUT93
474 (*Treponema pectinovorum*), correlated positively with NDF intake. Nevertheless,
475 OTU539 associated with butyrate production and OTU93 related with propionate
476 while OTU83 correlated with all 3 major VFA. The *Butyrivibrio hungatei* is the

primary butyrate producers in the rumen and degraded effectively hemicellulose (32). *Prevotellamassilia timonensis* is a hemicellulose-degrading bacteria (33). *Abyssivirga alkaniphila* ferments saccharides, peptides and amino acids (34). *Fibrobacter* and *Treponema* synergetically break down the fiber components (35, 36). The function of OTU396 (*Pelobacter propionicus*) and OTU165 (*Marseilla massiliensis*) may be similar with OTU6. We observed their association with macronutrient and major VFA. Those MCA signatures cooperatively digest carbohydrate or protein and produce VFA. Increase of butyrate that is as an important regulator and stimulator of rumen development. Gorka et al. (37) revealed that the supplementation of alfalfa (NDF) could improve rumen development by increasing abundance of these synergistic bacteria. In addition, the OTU27 (*Prevotella falsenii*) might be nitrogen-specific bacteria since it correlated with CP intake and NH_3-N very well though it was not classified as MCA signatures. A review reported some strains in *Prevotella* can degrade dietary proteins (31). Therefore, those signatures for regimes supplied with alfalfa contribute to both protein and carbohydrate utilization, and yield more nitrogen materials and VFA for host development. By contrast, the MRO signature microbiota cannot promote rumen functions at ruminant phase, however, they still may provide some baseline information of rumen in non-ruminant stage. Except *Sphingobacteriaceae*, OTU24 (*Cloacibacillus*) was another important specie for goats used only milk replacer as nutrient source. *Cloacibacillus* is a novel bacterium that degrades amino acids and produced VFAs (38). These MRO-associated signatures could be considered as passengers that contributed rumen development at specific

499 time. Although little was known about their contribution of these bacteria, they were
500 important for digestion of milk replacer and could be the primary strains impacted on
501 late bacterial colonization.

502 The first limitation of this study is small sample size (6 per groups). However, it
503 still showed good results between fluid diet and supplement of solid diet, providing
504 some insights for future large scale studies. Second, the alfalfa in MCA groups were
505 provide *ad libitum* resulting in less intake and less significant effects of adding alfalfa,
506 but we did observed many bacteria related with fiber digestion due to significant fiber
507 effects. Despite limitations, we confirm that signature microbiota for supplementary
508 solid diet plays important roles in the promotion of rumen functions. Moreover, most
509 of the MRO signatures function were not well described and are required for the
510 identification of their functions by longitudinal measurements in further studies.

511

512 **Conclusions**

513 Rumen fermentation and microbial composition were altered by supplementation
514 of concentrate or concentrate+alfalfa, particularly the latte, in early life. The
515 concentration of rumen VFA especially acetate, propionate and butyrate increased
516 significantly when goats intake more nutrients from solid diet, and positively
517 correlated with intake of crude protein, non-fiber carbohydrate and neutral detergent
518 fiber. The membership and structure of rumen microbiota were altered. This study
519 identified a set of signatures for supplementary solid diet regimes and validated their
520 association with macronutrient intake and rumen fermentation. Also, it was the first

time to use FishTaco for determination of link between those signatures' abundances and rumen function shifts. Then, we performed network analysis to detect the interaction of signatures. By comprehensive integration, many members of bacteria having symbiotic relationship with signatures were classified. Therefore, for goat kids, extra nutrient from concentrate and/or forage manipulated core community structures by specific signature microbiota and their symbiotic partners, and then more volatile fatty acids were produced, and eventually rumen development and functions were promoted.

Our study answers several key questions in rumen microbiome affected by supplementary solid diet, and offers a foundation for studies aimed at improving ruminant health and production.

Materials and Methods

Goat kids, treatments and management

The experimental procedure was approved by the Chinese Academy of Agricultural Sciences Animal Ethics Committee, and humane animal care and handling procedures were followed throughout the experiment. This animal trial was conducted using Haimen goat kids at a commercial farm in the Jiangsu province, China.

A total of 72 Haimen goat kids (20 days old and average body weight 4.54± 0.51kg) were separated from their dams, and randomly allotted to three groups based on their following diets: milk replacer only (**MRO**), milk replacer + concentrate (**MRC**), milk replacer + concentrate + alfalfa pellets (**MCA**). Each group had six

543 replicates and four kids per pen were as a replicate.

544 Goat kids remained with their mother and received breast milk from 0 to 20 days.
545 During 20 to 60 days of age, they were separated with their dams and the above 3
546 kinds of diets were provided to corresponding groups. Other feeding management
547 including vaccination, cleaning and disinfection of pens followed farm normal policy.
548 All animals were fed with milk replacer from 20 to 60 days. Feeding amount of milk
549 replacer were 2% body weight. Goat kids were fed 4 times a day (0600, 1200, 1800
550 and 2200) at 20-30 days, and thrice daily at 30-60 days (0600, 1200 and 1800). The
551 milk replacer was dissolved with hot water cooled to 65-70 °C after boiling, and
552 offered to goat kids when it was cooled to 40 ± 1 °C. The ratio of milk replacer to
553 water was 1:6 (weight (g)/ volume (ml)). The milk replacer (China patent products
554 ZL02128844.5) used in the experiment was provided by Beijing Precision Animal
555 Nutrition Research Center. The concentrate with ingredients of corn, soybean etc. was
556 purchased from Cargill Feed company, Nanjing. The alfalfa pellets purchased from
557 Baofa Agriculture and Animal Husbandry Co. Ltd, Gansu, China had same diameter
558 (4 mm) with concentrate diet. During the animal trial, all the goat kids had *ad libitum*
559 access to water, the MRC and MCA kids were freely to access concentrate, and the
560 MCA goats had extra free choice of alfalfa pellets. The nutritional levels of milk
561 replacer, concentrate and alfalfa pellets are shown in Table S1.

562 ***Sample collection and Chemical analysis***

563 Daily feed intakes were recorded in animal trial. Feed samples were collected,
564 dried in a forced-air oven at 65°C for 48 h and analyzed for crude protein (CP),

565 non-fiber carbohydrate (NFC), and neutral detergent fiber (NDF) according to the
566 Association of Official Analytical Chemists (39). Then, average daily intake of CP,
567 NFC and NDF were calculated. Only data of table S1 (dietary composition) and table
568 S2 (growth performance) were published in a Chinese journal paper (40), and other
569 data, such as rumen fermentation parameters and microbiome analysis, were not
570 published and used for the current draft.

571 Six goat kids (healthy and BW close to the average BW of the corresponding
572 groups) from each group were selected and slaughtered for rumen samples collection.
573 At 60 days of age, the goat kids were taken to an on-farm slaughterhouse,
574 anesthetized using sodium pentobarbitone, and slaughtered by exsanguination from
575 the jugular vein. Then, the rumen organs were taken out, and the ruminal content pH
576 was measured immediately using pH electrode (PB-10; Sartorius, Goettingen,
577 Germany). Around 10 ml rumen content were sampled from the whole mixed rumen
578 digesta and stored at -80°C for sequencing. The rumen fluid around 10 ml filtered
579 through four layers of gauze was placed in a 15 ml centrifuge tube immediately frozen
580 at -20°C for measurement of rumen fermentation. Determination of rumen fluid
581 NH_3-N concentration by phenol-sodium hypochlorite colorimetric method after the
582 liquid was thawed at 4°C. The microbial proteins were analyzed according to the
583 method described by (41). The volatile fatty acids (VFA) in rumen fluid were
584 quantified by gas chromatography (42) using methyl valerate as internal standard in
585 an Agilent 6890 series GC equipped with a capillary column (HP-FFAP19095F-123,
586 30 m, 0.53 mm diameter and 1 mm thickness).

587 ***DNA extraction and 16S rRNA gene sequencing***

588 Rumen fluid samples were thawed on ice and microbial DNA was extracted
589 using a commercial DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to
590 manufacturer's instructions. Total DNA quality analysis using a Thermo NanoDrop
591 2000 UV spectrophotometer and 1% agarose gel electrophoresis. The V3-V4 region
592 of the bacteria 16S ribosomal RNA genes were amplified by PCR (95 °C for 3 min,
593 followed by 30 cycles at 98°C for 20 s, 58 °C for 15s, and 72 °C for 20 s and a final
594 extension at 72 °C for 5 min) using indexes and adaptors-linked universal primers
595 (431 F:ACTCCTACGGGRSGCAGCAG; 806R: GGACTACVVGGGTATCTAATC).
596 PCR reactions were performed in 30 μL mixture containing 15 μL of 2 × KAPA
597 Library Amplification Ready Mix, 1 μL of each primer (10 μM), 50ng of template
598 DNA and ddH_2O. All PCR products were normalized and quantified by a Qubit 2.0
599 Fluorometer (Thermo Fisher Scientific, Waltham, US). Amplicon libraries were
600 mixed using all qualified products and sequenced with Illumina HiSeq PE250
601 plateform at Realbio Technology Genomics Institute (Shanghai, China).

602 ***Sequencing Data Processing***

603 Raw sequences were filtered through a quality control pipeline using the
604 Quantitative Insight into Microbial Ecology (QIIME) tool kit (43). The chimeras and
605 singletons were detected and removed by Usearch software, and the high quality
606 sequences were clustered into operational taxonomic units (OTUs) at the 97%
607 similarity level. Samples were normalized to 24136 sequencing reads. The
608 representative sequence was classified based on the Ribosomal Database Project

609 (RDP) database (44) at the default confidence threshold of 0.8, trained on the SILVA
610 reference database (release 111) (45). The alpha diversities (Shannon Index and
611 Observed species), and beta diversity (Unweighted and Weighted Unifrac distance)
612 were calculated. The ANalysis Of SIMilarity (ANOSIM) test was used to examine the
613 statistical significance of differences in beta diversity. The datasets in the current
614 study are available in the NCBI BioProject database with the BioProject ID
615 PRJNA544381 (https://www.ncbi.nlm.nih.gov/sra/PRJNA544381).
616
617 ***Data Analysis***
618 Rumen fermentation parameters were shown using bar charts made in R (v3.6.0)
619 by 'ggplot2' package. The Anova test was used for significance calculation after
620 detection of homogeneity of variance. After the global test was significant, a post-hoc
621 analysis (Tukey's HSD test) was performed to determine which group of the
622 independent variable differ from each other group.
623 Alpha diversity of the rumen microbial data among three treatments was tested
624 using Kruskal–Wallis test and a post-hoc Dunn Kruskal-Wallis multiple comparison
625 with Bonferroni adjustment to evaluate differences between two groups, and boxplots
626 were made in R ('ggpubr' packages). Beta diversity was visualized with PCoA plot
627 through R.
628 RandomForest classification model was performed to identify the top
629 microbiome signatures to differentiate 3 supplementary feeding regimes. R package
630 'AUCRF' (v.1.1) was used to process RandomForest model and select optimal

631 variables based on the area-under-the receiver operator characteristic curve (AUC) of
632 the RandomForest method (AUCRF) (46). The relative abundances of all the
633 microbiota were included for predictors selection. The 'ntree' parameters was set at
634 10,000 in the model. For calculation the probability of each selected variable, a
635 10-fold cross validation analysis and 20 times repetitions of cross validation were
636 performed. The model accuracy, including AUC, sensitivity and specificity of
637 variables, was calculated using the 'pROC' package (v.1.13). Thus, variables
638 importance plot was generated based on the importance scores (Mean Decrease in
639 Accuracy, MDA) of optimal features and their boxplots of selected features were
640 drawn in R.

641 RandomForest regression model was used to select the rumen microbiota that
642 were important for average daily intake of major nutrients (i.e., CP, NDF and NFC)
643 and rumen fermentation parameters. The model was run in R software using
644 'RandomForest' package (v 4.6-14). The percent variance explained was reported for
645 the estimation of accuracy of regression model. The top 50 selected features were then
646 analyzed Pearson correlation with those macro indicators respectively.

647 Predictive function analysis was performed using the PICRUSt algorithm based
648 on the Kyoto Encyclopedia of Genes and Genomes (KEGG) classification using the
649 closed-reference OTUs (47). The Functional Shifts' Taxonomic Contributors
650 (FishTaco) software was used to find the rumen bacteria driving the functional shifts
651 between supplementary regimes in this study. A taxonomic abundance at OTUs' level
652 and functional abundance profile at levels 3 from the PICRUSt analysis were used. In

pairwise comparisons, we labeled MRO groups as control and MRC or MCA as case, and tested MRC as control vs MCA as case. Each functional shift was grouped into case-associated with driving case-enrichment or attenuating case-enrichment, and control-associated driving case-enrichment or attenuating case-enrichment. The output results visualization was performed in FishTacoPlot package in R (Version 3.6.0).

Network analysis was performed by calculating all possible Pearson rank correlation coefficients (ρ) between microbial pairs. To minimize the occurrence of spurious associations, we considered a valid co-occurrence between two different taxa if a correlation co-efficiency over 0.6 or less than 0.6 and statistically significant. The network was demonstrated by using the 'igraph' package in R with edges connecting nodes (bacterial taxa). The subnetworks in regimes were produced based on the betweenness cluster calculated by the Girvan-Newman algorithm (48).

Acknowledgements

This study was funded by grants from National Key R&D Program Projects(2018YFD0501902), National Natural Science Foundation of China (31872385) and National Technical System Construction of Mutton Sheep Industry(CARS-39).

Competing interests

The authors declare that they have no competing interests.

Supplemental material

Supplementary information is available at the ISME journal's website.

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831

832 Figure Legends

833 **Figure 1** Effects of early supplementary solid diet on rumen fermentation parameters
834 in goat kids

835 The Anova test was used for significance calculation after detection of homogeneity
836 of variance. After the global test was significant, a post-hoc analysis (Tukey's HSD
837 test) was performed to determine which group of the independent variable differ from
838 each other group. High dietary nitrogen conversion ratio was found in MRC and
839 MCO (P<0.05). The total VFA, acetate propionate and butyrate had the highest values
840 in MCA (P<0.05), and they were significantly higher in MRC than in MRO (P<0.05).
841 MRO=milk replacer only, MRC= milk replacer + concentrate , MCA= milk replacer +
842 concentrate + alfalfa. VFA: Volatile fatty acids. Statistical significance was accepted
843 at P<0.05.

844

845 **Figure 2.** The early supplementary solid diet impacted on Alpha and Beta diversity of
846 rumen microbiome in goat kids. (A-B) The Shannon Index and Observed species.
847 Alpha diversity of the rumen microbial data was tested using Kruskal-Wallis test and
848 a post-hoc Dunn Kruskal-Wallis multiple comparison with Bonferroni method for p
849 value correction. Principal coordinate analysis (PCoA) of the community membership
850 based on the weighted (C) and unweighted (D) UniFrac distance, with the green
851 cycles as MRO, red cycles as MRC and blue cycles as MCA.

852 Although diversity (Shannon index) was not different (p=0.372), significance of
853 microbial richness was observed among MRO, MRC and MCA rumen samples

854 (p=0.012). Significances in community structure among 3 group were detected
855 (Weighted Unifrac ANOSIM, R=0.68, *P*<0.05; UnWeighted Unifrac ANOSIM,
856 R=0.69, *P*=0.001). The MRO formed a distinct cluster in on the left side, while the
857 MRC and MCA were closely clustered on the right side of PCoA plot.
858 MRO=milk replacer; MRC= milk replacer + concentrate; MCA= milk replacer +
859 concentrate + alfalfa; ANOSIM: Analysis of similarity
860
861 **Figure 3** The top 30 OTUs in 3 supplementary regimes. Each bar shows the average
862 relative abundance of MRO, MRC and MCA. Each color represents the relative
863 abundance of a bacterial taxon on the stacked bar chart.
864 MRO=milk replacer, MRC= milk replacer + concentrate, MCA= milk replacer +
865 concentrate + alfalfa.
866
867 **Figure 4.** The highlight signature microbiota identified by AUCRF for differentiating
868 MRO, MRC and MCA
869 All the OTUs abundances were tested using Kruskal–Wallis test and a post-hoc Dunn
870 Kruskal-Wallis multiple comparison with Bonferroni method for p value correction.
871 The black dots within each bar were values from individual animal, and the black
872 lines within each bar represented the medians.
873 MRO=milk replacer; MRC= milk replacer + concentrate; MCA= milk replacer +
874 concentrate + alfalfa; AUCRF: RandomForest based on optimizing the area-under-the
875 receiver operator characteristic curve (AUC);

876

877 **Figure 5** Correlation analysis between nutrient (CP, NFC and NDF) intake and rumen
878 microbes in goat kids.
879 We performed the RandomForest regression model across all samples between dietary
880 average daily CP, NFC and NDF intake and all the genera with high prediction
881 accuracy (Table S6). Then, using the abundances of top 50 features to calculate the
882 pearson correlation with intake of CP, NFC and NDF was carried out. We consider
883 $p<0.05$ as a significant correlation and yellow crosses indicated non-significant. The
884 Pearson coefficients were labeled black values inside cycles, red dots represented
885 negative correlation, and blue dots indicated a positive correlation. The bacteria from
886 up to bottom followed the descending order of mean square error.
887 CP: Crude protein average daily intake; NDF: Neutral detergent fibers average daily
888 intake; NFC: Non-fibrous carbohydrates average daily intake.

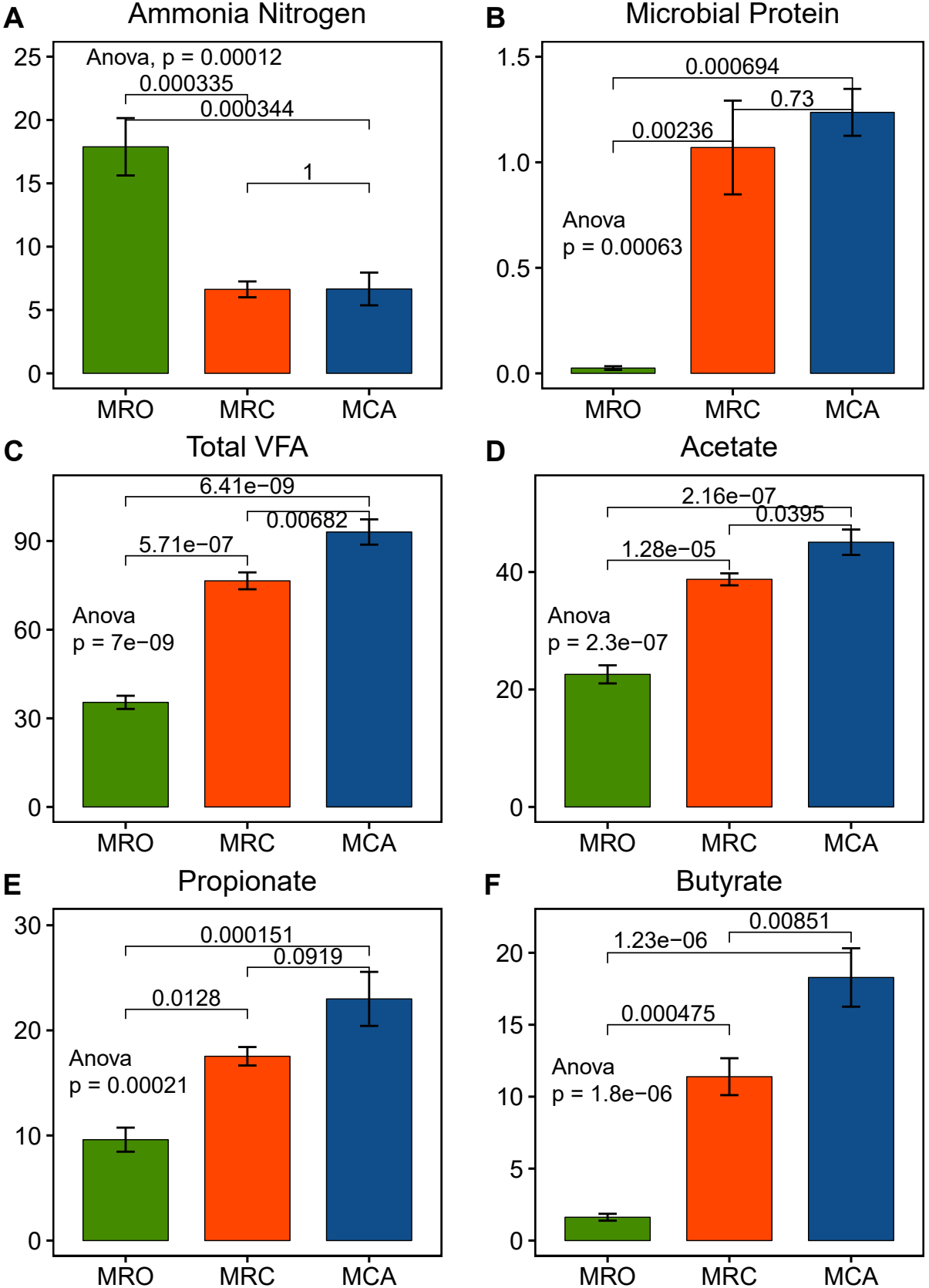
889

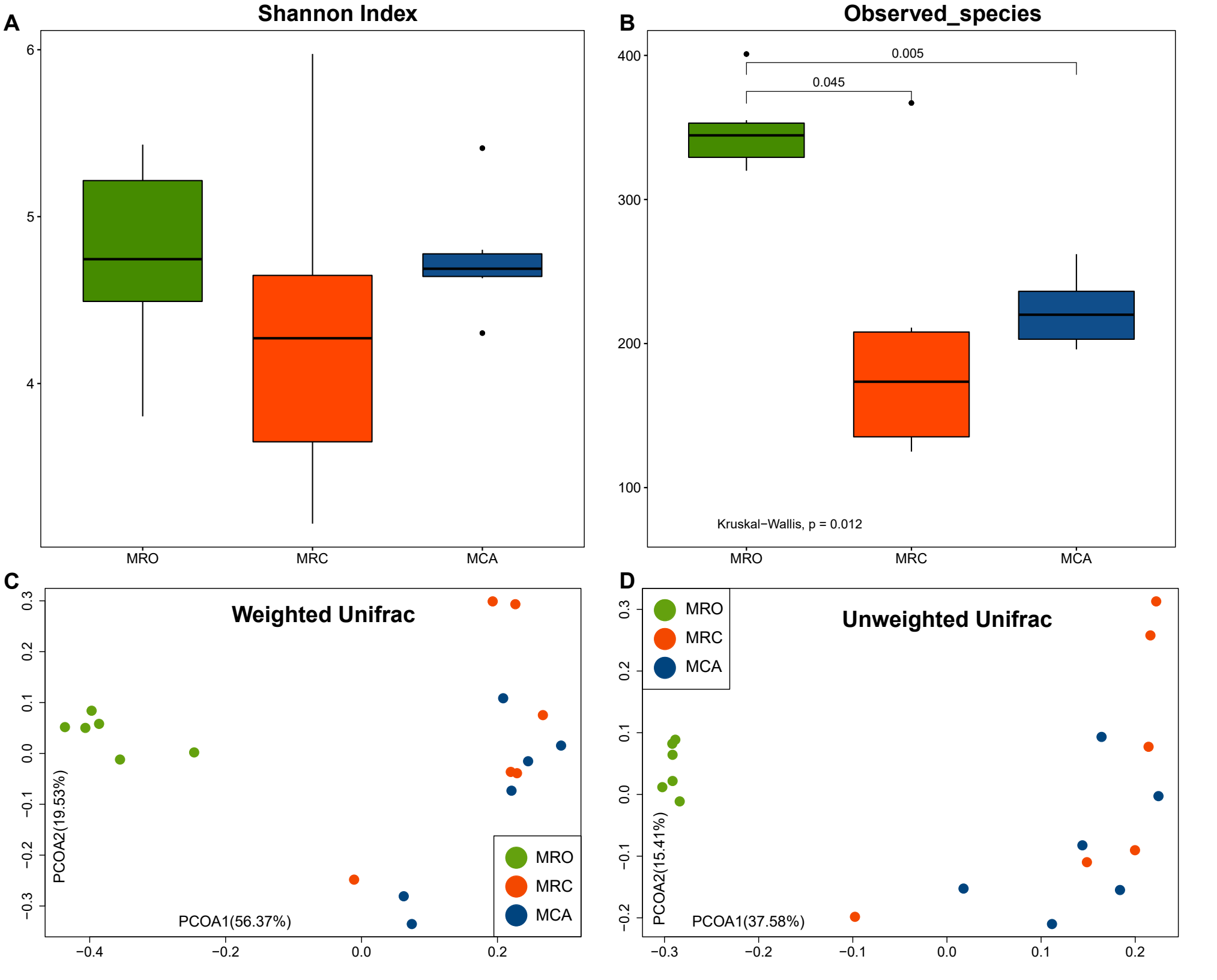
890 **Figure 6** Comparing taxon-level contribution profiles of functional shifts: A: MRO
891 control and MRC case, B: MRO control and MCA case.
892 Taxon-level shift contribution profiles for case-associated functional modules by
893 FishTaco. The horizontal axis represents rank and statistic scores, and the vertical axis
894 represents related pathways. For each functional pathway, the bar on the top-right of Y
895 axis represents case-associated bacteria driving the enrichment in the functional
896 module; the bar on the top-left of Y axis indicates case-associated bacteria attenuating
897 functional shift; the bar on the bottom-right of Y axis represents bacteria depleted in

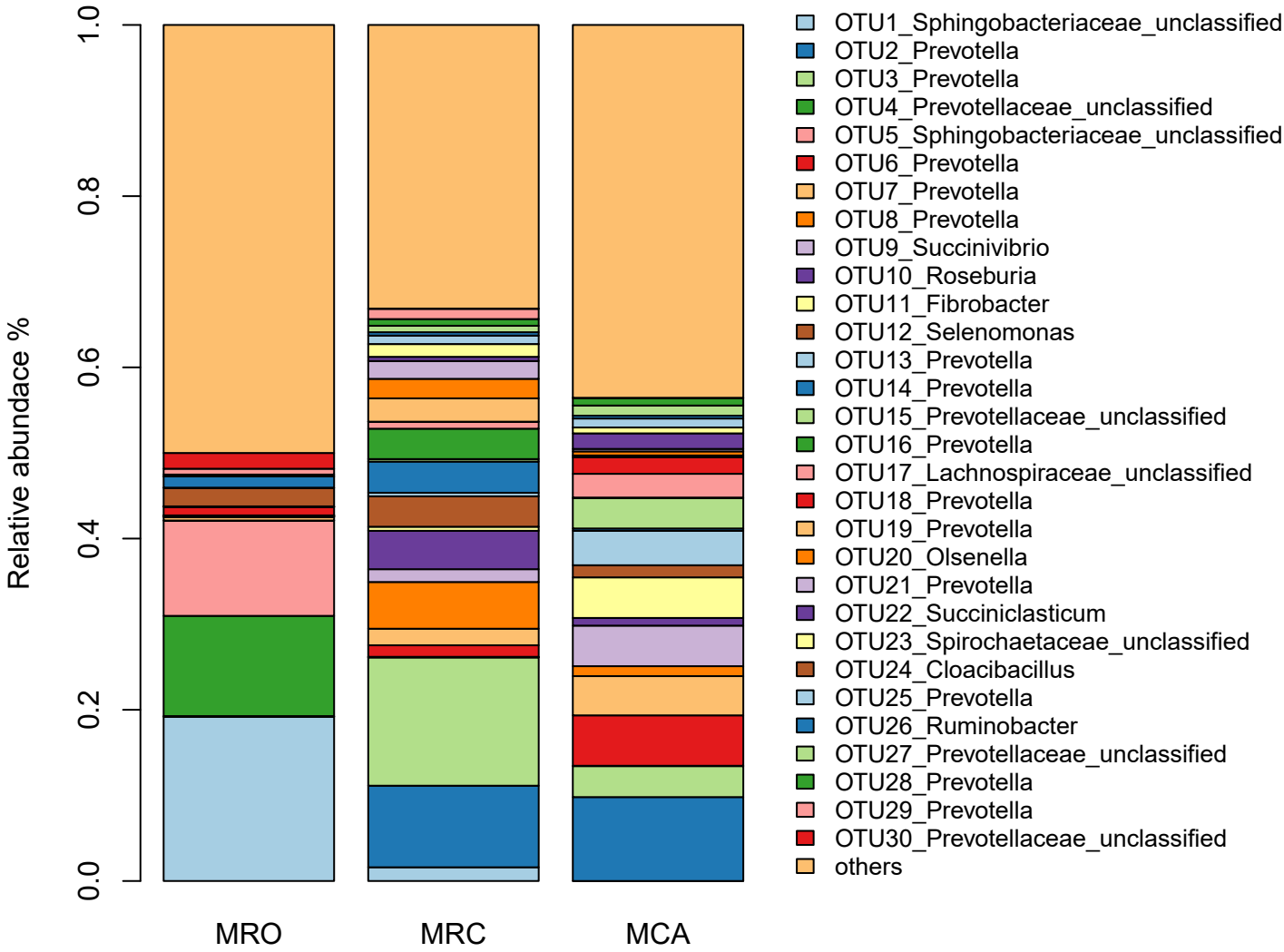
898 control driving functional shift; the bar on the bottom-left of Y axis shows bacteria
899 depleted in control attenuating functional shift. White diamonds represent
900 bacterial-based functional shift scores.
901 The abundances of main drivers were displayed on the right side. OTU2 and OTU3,
902 the shared drivers of enrichments of MRC and MCA were abundant in solid diet
903 regimes. OTU1 enriched in MRO was strongly depleted by solid diets. OTU11, OTU7
904 and OTU13, driving mainly MCA function shifts, increased abundance with
905 supplementation of solid diet.
906 FishTaco:Functional Shifts' Taxonomic Contributors; MRO=milk replacer; MRC=
907 milk replacer + concentrate, MCA= milk replacer + concentrate + alfalfa;
908
909 **Figure 7** Network analysis of the interactions between bacterial taxa at MRO (A),
910 MRC (B) and MCA (C).
911 The OTUs accounting for >0.5% of the total sequences were selected to network
912 analysis. Each node denotes a particular OTU within the network and each line (edge)
913 a significant co-efficiency relationship (Pearson rank correlation coefficient >0.6 or
914 <−0.6. The table under corresponding figures contained the highlight OTUs and their
915 sequence identifiers with the highest scores from NCBI database (other OTUs
916 identifiers were shown in File S4).
917 MRO=milk replacer; MRC= milk replacer + concentrate; MCA= milk replacer +
918 concentrate + alfalfa;
919

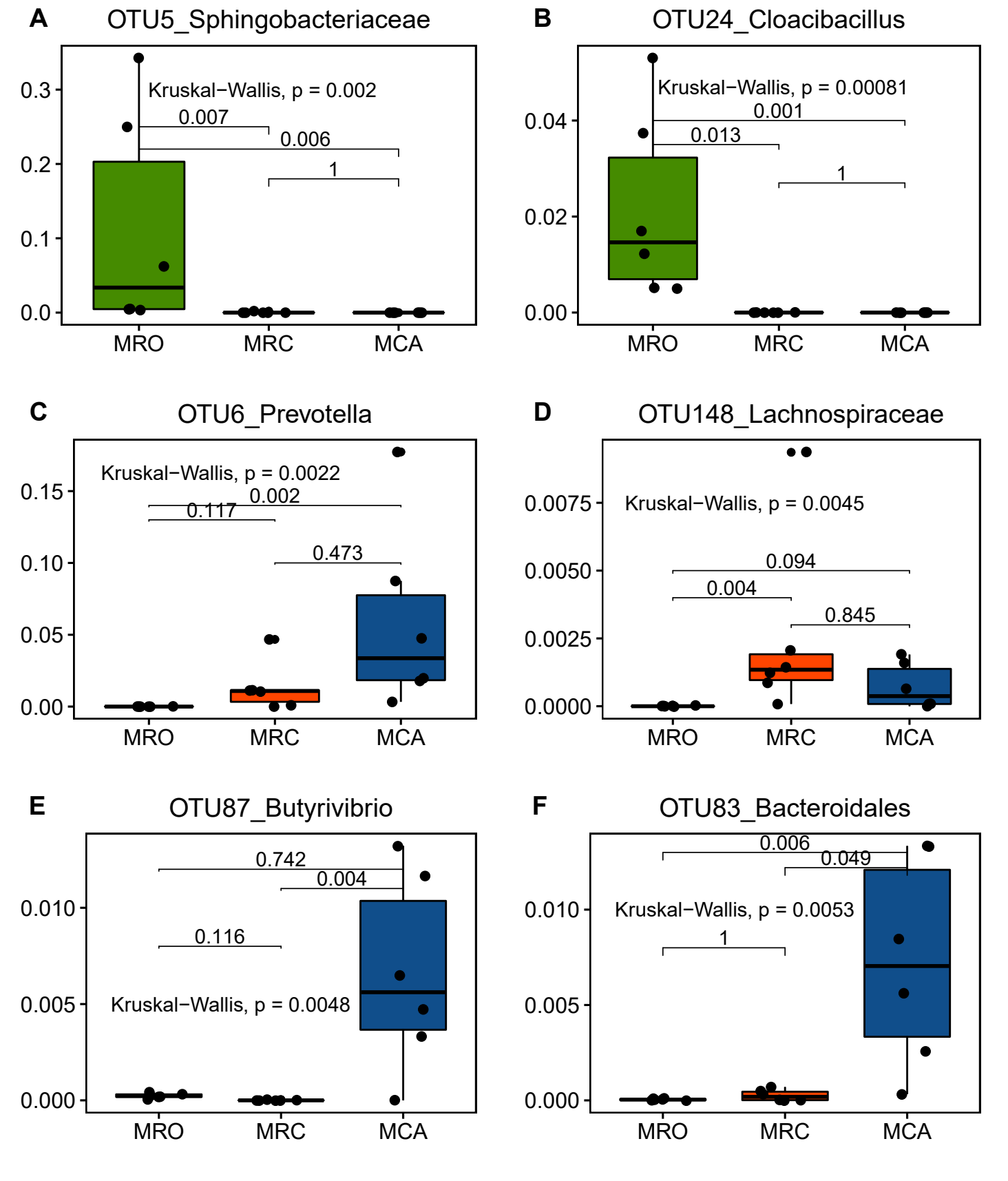
920 legend for supplemental materials

921


A
Ammonia Nitrogen
Anova, p = 0.00012
0.000335
0.000344
1
25
20
15
10
5
0
MRO
MRC
MCA
B
Microbial Protein
0.000694
0.73
0.00236
Anova
p = 0.00063
1.5
1.0
0.5
0.0
MRO
MRC
MCA
C
Total VFA
6.41e−09
0.00682
5.71e−07
Anova
p = 7e−09
90
60
30
0
MRO
MRC
MCA
D
Acetate
2.16e−07
0.0395
1.28e−05
Anova
p = 2.3e−07
40
20
0
MRO
MRC
MCA
E
Propionate
0.000151
0.0919
0.0128
Anova
p = 0.00021
30
20
10
0
MRO
MRC
MCA
F
Butyrate
0.00851
1.23e−06
0.000475
Anova
p = 1.8e−06
20
15
10
5
0
MRO
MRC
MCA


A
Shannon Index
6
5
4
MRO
MRC
MCA
B
Observed_species
400
300
200
100
0.005
0.045
Kruskal−Wallis, p = 0.012
MRO
MRC
MCA
C
Weighted Unifrac
PCOA2(19.53%)
PCOA1(56.37%)
MRO
MRC
MCA
D
Unweighted Unifrac
MRO
MRC
MCA
PCOA2(15.41%)
PCOA1(37.58%)


Relative abundace %
0.0
0.2
0.4
0.6
0.8
1.0
MRO
MRC
MCA
OTU1_Sphingobacteriaceae_unclassified
OTU2_Prevotella
OTU3_Prevotella
OTU4_Prevotellaceae_unclassified
OTU5_Sphingobacteriaceae_unclassified
OTU6_Prevotella
OTU7_Prevotella
OTU8_Prevotella
OTU9_Succinivibrio
OTU10_Roseburia
OTU11_Fibrobacter
OTU12_Selenomonas
OTU13_Prevotella
OTU14_Prevotella
OTU15_Prevotellaceae_unclassified
OTU16_Prevotella
OTU17_Lachnospiraceae_unclassified
OTU18_Prevotella
OTU19_Prevotella
OTU20_Olsenella
OTU21_Prevotella
OTU22_Succiniclasticum
OTU23_Spirochaetaceae_unclassified
OTU24_Cloacibacillus
OTU25_Prevotella
OTU26_Ruminobacter
OTU27_Prevotellaceae_unclassified
OTU28_Prevotella
OTU29_Prevotella
OTU30_Prevotellaceae_unclassified
others


A
OTU5_Sphingobacteriaceae
Kruskal−Wallis, p = 0.002
0.007
0.006
1
0.3
0.2
0.1
0.0
MRO
MRC
MCA
B
OTU24_Cloacibacillus
Kruskal−Wallis, p = 0.00081
0.001
0.013
1
0.04
0.02
0.00
MRO
MRC
MCA
C
OTU6_Prevotella
Kruskal−Wallis, p = 0.0022
0.002
0.117
0.473
0.15
0.10
0.05
0.00
MRO
MRC
MCA
D
OTU148_Lachnospiraceae
Kruskal−Wallis, p = 0.0045
0.094
0.004
0.845
0.0075
0.0050
0.0025
0.0000
MRO
MRC
MCA
E
OTU87_Butyrivibrio
0.742
0.004
0.116
Kruskal−Wallis, p = 0.0048
0.010
0.005
0.000
MRO
MRC
MCA
F
OTU83_Bacteroidales
0.006
0.049
Kruskal−Wallis, p = 0.0053
1
0.010
0.005
0.000
MRO
MRC
MCA


A
CP
0.48 OTU165_Prevotellaceae_unclassified
-0.7 OTU24_Cloacibacillus
-0.65 OTU313_Methanomassiliicoccus
-0.84 OTU154_Methanomassiliicoccus
-0.86 OTU39_Ruminococcaceae_unclassified
-0.79 OTU60_Prevotella
-0.58 OTU111_Methanomassiliicoccus
-0.6 OTU119_Prevotella
-0.8 OTU365_Neisseriaceae_unclassified
-0.54 OTU5_Sphingobacteriaceae_unclassified
-0.7 OTU79_Neisseriaceae_unclassified
-0.52 OTU99_Elusimicrobium
-0.73 OTU90_Bacteroidetes_unclassified
-0.56 OTU55_Sphaerochaeta
0.55 OTU396_Proteobacteria_unclassified
-0.52 OTU290_Bacteroidetes_unclassified
-0.61 OTU132_Pyramidobacter
-0.74 OTU296_Clostridiales_unclassified
-0.7 OTU330_Firmicutes_unclassified
-0.68 OTU169_Bacteroidetes_unclassified
0.53 OTU6_Prevotella
-0.38 OTU89_Bacteroidetes_unclassified
-0.56 OTU168_Methanomicrobium
-0.6 OTU298_Pyramidobacter
-0.61 OTU279_Clostridiales_unclassified
-0.56 OTU422_Porphyromonadaceae_unclassified
-0.75 OTU506_Bacteria_unclassified
-0.78 OTU387_Bacteroidetes_unclassified
-0.6 OTU548_Bifidobacterium
-0.65 OTU327_Clostridium.XlVa
-0.53 OTU217_Bacteroidetes_unclassified
-0.88 OTU75_Ruminococcaceae_unclassified
-0.58 OTU385_Moraxella
-0.52 OTU94_Clostridiales_unclassified
-0.4 OTU178_Bacteroides
-0.8 OTU411_Clostridia_unclassified
-0.61 OTU139_Bacteroidetes_unclassified
-0.76 OTU245_Clostridiales_unclassified
-0.55 OTU147_Bacteroidales_unclassified
-0.82 OTU487_Firmicutes_unclassified
-0.71 OTU287_Pasteurellaceae_unclassified
0.42 OTU13_Prevotella
-0.52 OTU219_Bacteroidetes_unclassified
-0.63 OTU392_Bilophila
-0.56 OTU208_Bacteria_unclassified
-0.7 OTU281_Bacteroidetes_unclassified
-0.73 OTU266_Porphyromonadaceae_unclassified
-0.65 OTU151_Ruminococcaceae_unclassified
0.63 OTU27_Prevotellaceae_unclassified
0.19 OTU148_Lachnospiraceae_unclassified
B
NFC
0.41 OTU165_Prevotellaceae_unclassified
-0.84 OTU365_Neisseriaceae_unclassified
-0.91 OTU39_Ruminococcaceae_unclassified
-0.87 OTU154_Methanomassiliicoccus
-0.78 OTU296_Clostridiales_unclassified
-0.55 OTU5_Sphingobacteriaceae_unclassified
-0.6 OTU111_Methanomassiliicoccus
-0.82 OTU60_Prevotella
-0.7 OTU24_Cloacibacillus
0.49 OTU6_Prevotella
-0.67 OTU313_Methanomassiliicoccus
-0.64 OTU119_Prevotella
-0.56 OTU55_Sphaerochaeta
-0.73 OTU79_Neisseriaceae_unclassified
-0.67 OTU222_Firmicutes_unclassified
0.36 OTU114_Megasphaera
-0.54 OTU94_Clostridiales_unclassified
-0.76 OTU90_Bacteroidetes_unclassified
-0.64 OTU279_Clostridiales_unclassified
-0.54 OTU290_Bacteroidetes_unclassified
-0.4 OTU178_Bacteroides
-0.6 OTU385_Moraxella
-0.72 OTU281_Bacteroidetes_unclassified
-0.77 OTU266_Porphyromonadaceae_unclassified
-0.57 OTU217_Bacteroidetes_unclassified
-0.46 OTU62_Prevotellaceae_unclassified
0.27 OTU148_Lachnospiraceae_unclassified
-0.51 OTU33_Bacteroidetes_unclassified
-0.91 OTU75_Ruminococcaceae_unclassified
-0.55 OTU99_Elusimicrobium
-0.77 OTU192_Methanimicrococcus
-0.39 OTU89_Bacteroidetes_unclassified
-0.86 OTU487_Firmicutes_unclassified
-0.61 OTU208_Bacteria_unclassified
-0.72 OTU330_Firmicutes_unclassified
-0.56 OTU422_Porphyromonadaceae_unclassified
-0.87 OTU477_Ruminococcaceae_unclassified
-0.67 OTU182_Bacteroidetes_unclassified
-0.7 OTU190_Sphaerochaeta
-0.8 OTU387_Bacteroidetes_unclassified
-0.57 OTU147_Bacteroidales_unclassified
-0.76 OTU506_Bacteria_unclassified
-0.63 OTU139_Bacteroidetes_unclassified
-0.77 OTU413_Clostridium.XlVb
-0.68 OTU270_Bibersteinia
-0.67 OTU392_Bilophila
-0.87 OTU411_Clostridia_unclassified
-0.8 OTU245_Clostridiales_unclassified
-0.69 OTU327_Clostridium.XlVa
-0.52 OTU255_Comamonas
C
NDF
-0.9 OTU39_Ruminococcaceae_unclassified
-0.66 OTU313_Methanomassiliicoccus
-0.84 OTU365_Neisseriaceae_unclassified
0.48 OTU6_Prevotella
-0.54 OTU5_Sphingobacteriaceae_unclassified
0.27 OTU148_Lachnospiraceae_unclassified
-0.65 OTU119_Prevotella
-0.82 OTU60_Prevotella
0.36 OTU114_Megasphaera
-0.85 OTU154_Methanomassiliicoccus
0.49 OTU539_Clostridiales_unclassified
-0.68 OTU24_Cloacibacillus
-0.78 OTU296_Clostridiales_unclassified
-0.59 OTU111_Methanomassiliicoccus
-0.55 OTU55_Sphaerochaeta
0.29 OTU104_Prevotella
-0.73 OTU79_Neisseriaceae_unclassified
0.5 OTU87_Butyrivibrio
0.46 OTU396_Proteobacteria_unclassified
0.53 OTU93_Treponema
-0.67 OTU270_Bibersteinia
-0.67 OTU132_Pyramidobacter
-0.59 OTU298_Pyramidobacter
-0.54 OTU99_Elusimicrobium
-0.73 OTU90_Bacteroidetes_unclassified
0.18 OTU57_Prevotella
-0.71 OTU281_Bacteroidetes_unclassified
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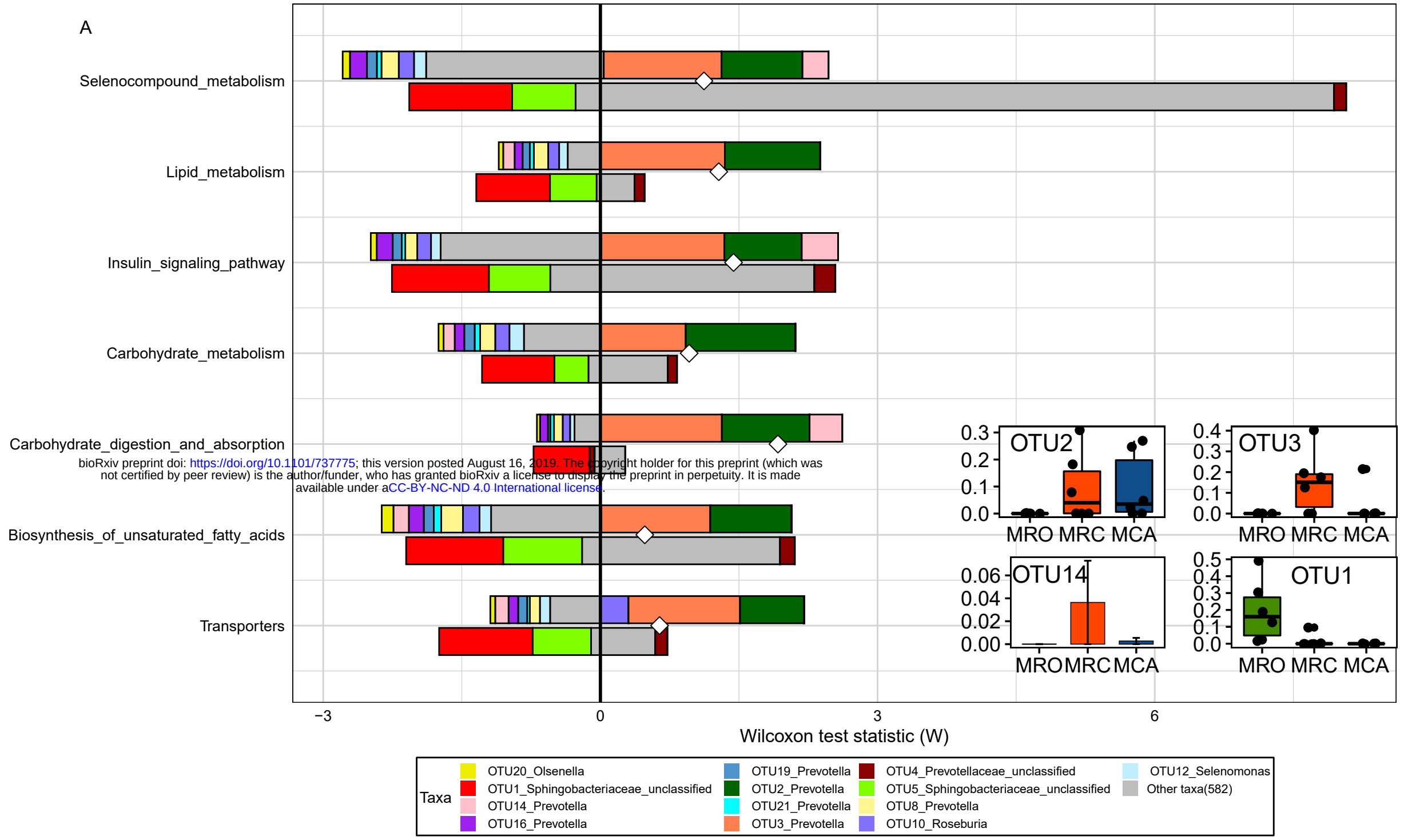
-0.62 OTU208_Bacteria_unclassified
-0.69 OTU327_Clostridium.XlVa
0.65 OTU464_Burkholderiales_unclassified
-0.8 OTU245_Clostridiales_unclassified
-0.56 OTU147_Bacteroidales_unclassified
-0.73 OTU506_Bacteria_unclassified
-0.72 OTU169_Bacteroidetes_unclassified
-0.39 OTU178_Bacteroides
-0.89 OTU75_Ruminococcaceae_unclassified
-0.85 OTU487_Firmicutes_unclassified
-0.77 OTU192_Methanimicrococcus

A



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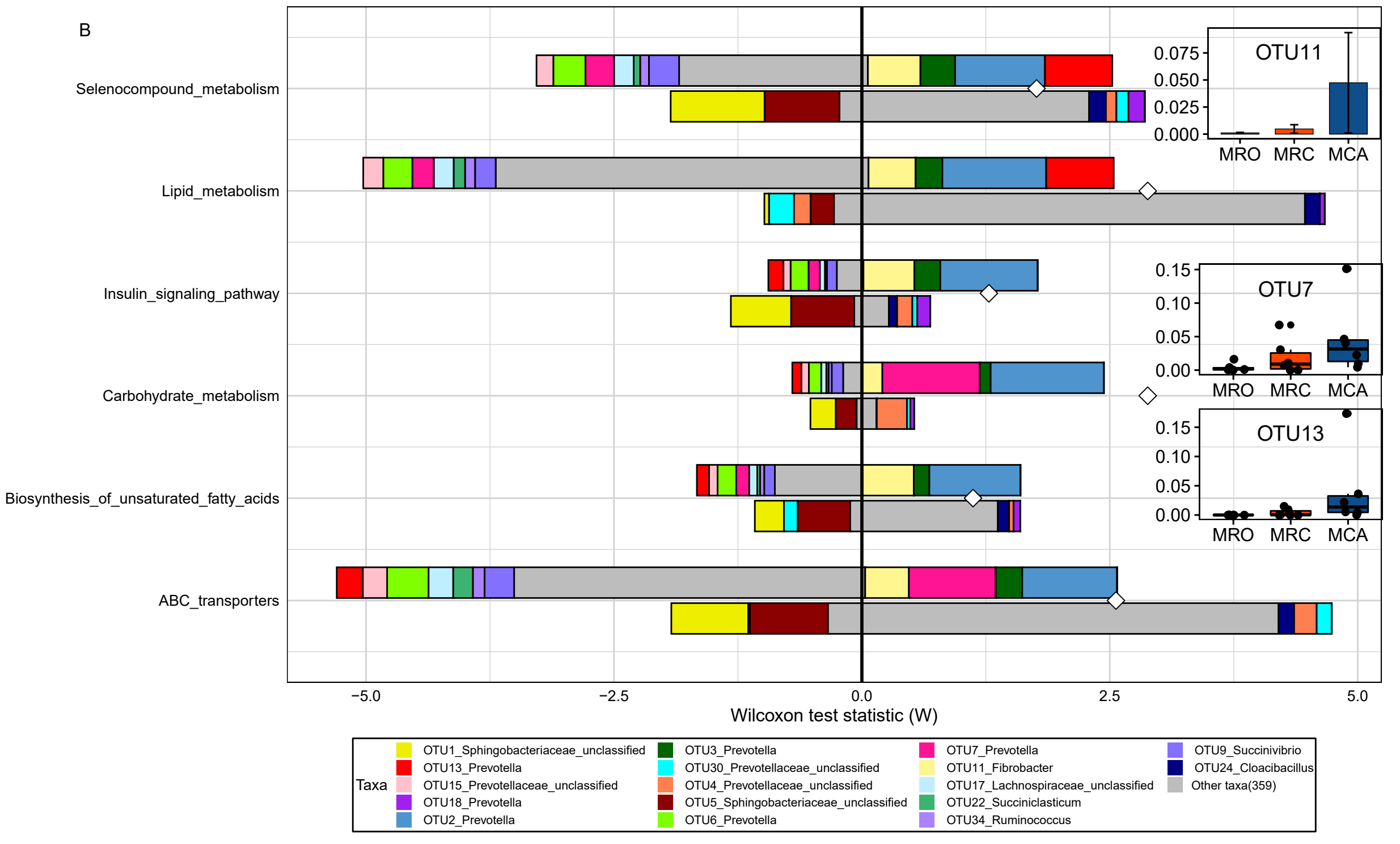

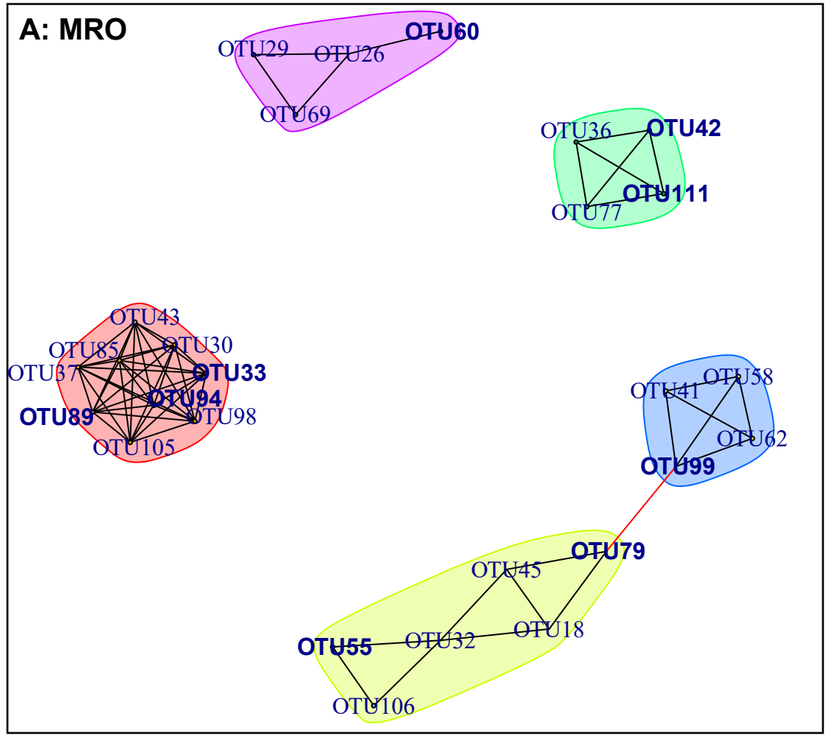

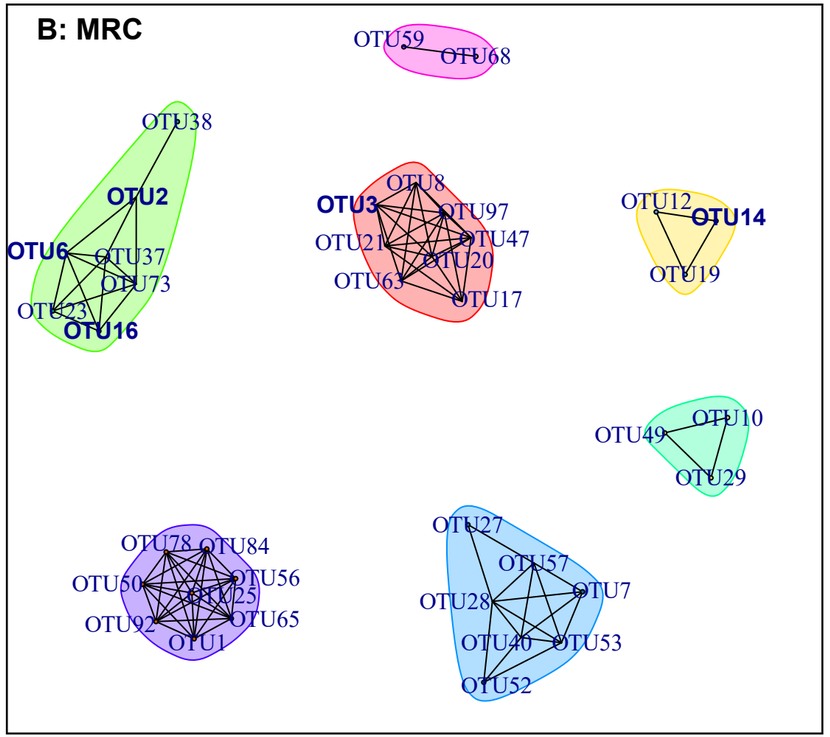

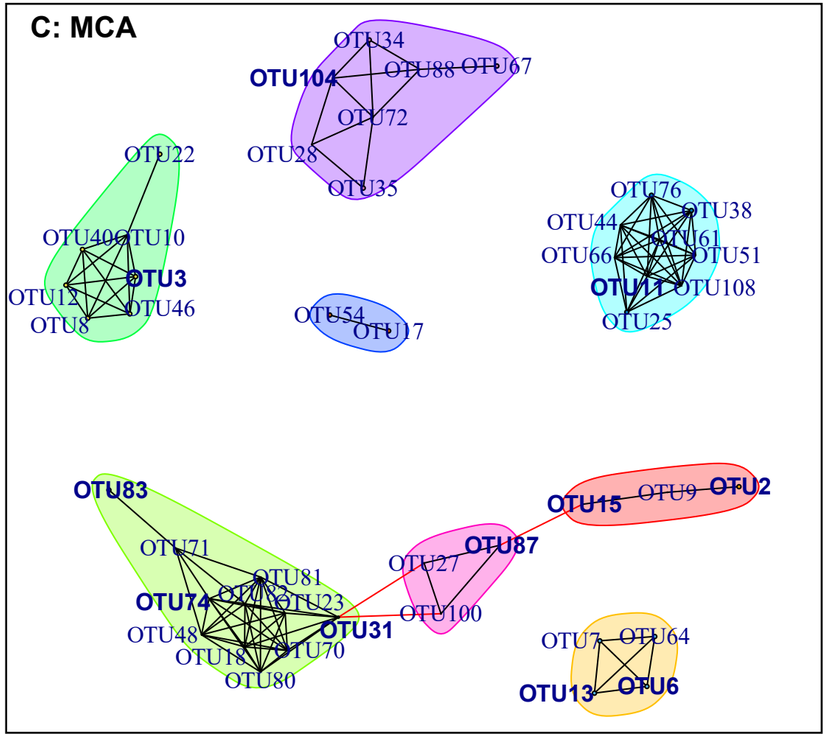

OTU60_Prevotella	Prevotella oralis
OTU42_Prevotella	Prevotella shahii
OTU111_Methanomassiliicoccus	Methanomassiliicoccus luminyensis
OTU99_Elusimicrobium	Elusimicrobium minutum
OTU79_Neisseriaceae_unclassified	Snodgrassella alvi
OTU55_Sphaerochaeta	Sphaerochaeta pleomorpha
OTU33_Bacteroidetes_unclassified	Porphyromonas pogonae
OTU94_Clostridiales_unclassified	Christensenella minuta
OTU89_Bacteroidetes_unclassified	Schleiferia thermophila

OTU2_Prevotella	Prevotella brevis
OTU6_Prevotella	Prevotella oralis
OTU16_Prevotella	Prevotella ruminicola
OTU3_Prevotella	Prevotella copri
OTU14_Prevotella	Prevotella histicola

OTU104_Prevotella	Prevotella ruminicola
OTU11_Fibrobacter	Fibrobacter succinogenes
OTU2_Prevotella	Prevotella brevis
OTU6_Prevotella	Prevotella oralis
OTU13_Prevotella	Prevotella brevis
OTU87_Butyrivibrio	Butyrivibrio hungatei
OTU74_Treponema	Treponema bryantii
OTU83_Bacteroidales_unclassified	Prevotellamassilia timonensis
OTU3_Prevotella	Prevotella copri
OTU15_Prevotellaceae_unclassified	Metaprevotella massiliensis
OTU31_Fibrobacter	Fibrobacter succinogenes