Nonobese subjects of *Bacteroides* and *Prevotella* enterotypes responded differentially to calorie restriction intervention

Hua Zou[1,2, 6,*], Dan Wang[1,2,3, 6,*], Huahui Ren[2,3], Chao Fang[2,3,5], Zhun Shi[2,3], Pengfan Zhang[1,2], Peishan Chen[2.3], Jian Wang[2,4], Huanming Yang[2,4], Kaiye Cai[2,3,6#], Huanzi Zhong[2,3,5,6#]

[1] BGI Education Center, University of Chinese Academy of Sciences, Shenzhen 518083, China

[2] BGI-Shenzhen, Shenzhen 518083, China

[3] China National Genebank, BGI-Shenzhen, Shenzhen 518120, China

[4] James D. Watson Institute of Genome Sciences, Hangzhou 310058, China

[5] Laboratory of Genomics and Molecular Biomedicine, Department of Biology, University of Copenhagen, 2100 Copenhagen Ø, Denmark

[6] Shenzhen Key Laboratory of Human commensal microorganisms and Health Research

[*]These authors contributed equally to this work

[#]**Corresponding authors:**

Kaiye Cai. E-mail: caikaiye@genomics.cn;

Huanzi Zhong. E-mail: zhonghuanzi@genomics.cn

Abstract

Background: Today, growing numbers of normal-weight people prefer low-calorie food or snacks. However, to our knowledge, the effect of calorie restriction (**CR)** on different enterotypes was poorly studied in nonobese individuals.

Results: A total of 41 nonobese volunteers received a 3-week CR intervention and their fecal samples before and after the intervention were collected and sequenced. Before the intervention, these 41 subjects could be robustly grouped into two enterotypes: *Bacteroides* (ETB, n=28) and *Prevotella* (ETP, n=13). After the CR intervention, we

found that ETP subjects had a significantly larger body mass index (BMI) loss ratio than ETB subjects. In addition, changes on composition and functional potential of gut microbiome were also different between ETB and ETP subjects after the intervention. Despite of the above different responses to CR intervention, a convergent shift in gut microbiome was observed between ETB and ETP subjects after the intervention.

Conclusions: Our work highlights the potential application for microbiome stratification in nutrition intervention.

keywords: gut microbiota, enterotype, calorie restriction intervention

Background

The gut microbiota containing trillions of microbes is a complex microbial ecosystem and carries out functions that are important to host health, including dietary energy extraction as well as development and maintenance of the immune system [1]. Enterotype which is a way of stratifying the complex human gut microbial community was first described in 2011 [2]. Plenty of studies have shown that individuals with different enterotypes might have different clinical parameters and gut microbiota responses under the same diet [3-7].

Calorie Restriction (**CR**) without malnutrition has been demonstrated to extend lifespan and retard age-related diseases in many studies [8-10]. For instance, in a 2-year nonobese human trail, CR had larger decreased in Cardiometabolic risk factors [9]. Another CR intervention that lasted 2 years reduced BMI and improved mood and sleep duration on health-related quality of life in healthy nonobese adults [10].

The association between CR intervention and gut microbiota has been explored in many studies, particularly among overweight and obese individuals [11, 12]. A 10-week CR diet plus physical activity changed the composition of the gut microbiota in overweight adolescents [11]. A shorter CR intervention that lasted 4 weeks improved gut barrier integrity and reduced systemic inflammation on gut microbial diversity in obese women [12]. Compelling evidences suggest that CR intervention may improve the ability of gut microbiota for synthesis of short-chain fatty acids (SCFAs) which are involved in energy supplement for host [13, 14].

Although plenty of studies have focused on the association between CR intervention and gut microbiota (as demonstrated above), to our knowledge, no studies have evaluated the impact of CR on nonobese individuals of different enterotypes. As the low-calorie diet becomes trendy, studying the effect of CR on human gut microbiota for nonobese people becomes essential. Under these circumstances, we designed our study (Figure 1), in which a CR intervention were conducted on 41 nonobese subjects (Body Mass Index (BMI) < 28 for Chinese [15]). All subjects underwent a 1-week normal calorie intake diet and 3-week calorie restriction intake diet (Figure 1). BMI values and fecal samples were collected before and after the CR intervention. We then conducted taxonomic and functional profiling of all the fecal samples *in silico*. Based on their taxonomic profiles before the intervention, subjects can be robustly clustered into two enterotypes: *Bacteroides* (ETB, n = 28) and *Prevotella* (ETP, n = 13). After the 3-week CR intervention, we found that ETB and ETP subjects responded differently to the CR intervention. Firstly, BMI loss ratio of ETP subjects drops more significantly than ETB subjects. Secondly, changes on composition and functional potential of gut microbiome were also different between ETB and ETP subjects after the intervention. Despite of the above different responses to CR intervention, a convergent shift on gut microbiome composition and functional potential was observed between ETB and ETP subjects after the intervention, which suggests that the same diet may make the gut microbiome more similar.

Results

Enterotyping and characterization of subjects before CR intervention

Our subjects can be robustly stratified into two enterotypes (See Methods): *Bacteroides* (ETB, n = 28) and *Prevotella* (ETP, n = 13). The two enterotypes are visualized in Figure 2A.

After finishing enterotyping of subjects, we firstly compared the phenotypes between ETB and ETP subjects using Wilcoxon rank-sum test or Pearson's chi-squares test (See Methods). The result revealed that the before-intervention phenotypes, including sex distribution (female/male ratio), age, BMI and weight, showed no significant difference between ETB and ETP subjects (Table 1). The significance threshold was set at $p < 0.05$ in this paper.

On the contrary, composition and functional potentials of gut microbiome showed significant differences between these two enterotypes before the intervention. In general, genus profiles between ETB and ETP before-intervention fecal samples were hugely different with $p < \mathbf{0.0001}$ using permutational analysis of variance (PERMANOVA) (See Methods). In detail, we performed Wilcoxon rank-sum test on the relative abundances of common genera or species (See Methods) between ETB and ETP subjects. Among the common genera, we identified a total of six genera with significantly different relative abundances between two enterotypes before the intervention: *Prevotella* and *Paraprevotella* were more abundant in samples belonging to ETP while relative abundances of *Bacteroides*, *Fusobacterium*, *Eggerthella*, and *Anaerostipes* were significantly higher in ETB samples (Wilcoxon rank-sum test, $p < 0.05$; Figure 2B). Among the common species, *Prevotella copri*, *Paraprevotella xylaniphila*, *Dorea longicatena* and other five bacteria were significantly more abundant in ETP samples (Wilcoxon rank-sum test, $p < 0.05$; Figure 2C). Meanwhile, the relative abundances of 28 common species, such as *Bacteroides dorei*, *Bacteroides uniformis*, *Parabacteroides distasonis* and *Bacteroides ovatus*, were significantly larger in ETB samples (Wilcoxon rank-sum test, $p < 0.05$; Figure 2C).

On the function level, differentially enriched KEGG pathways between ETB and ETP samples were identified according to their reporter Z-scores [16]. A reporter score $|Z| > 1.96$ (95% confidence according to the normal distribution) was used as the detection threshold for significantly differentiating pathways (See Methods). We identified a total of 29 KEGG pathways which were differentially enriched between ETB and ETP samples before the intervention ($|Z|>1.96$; Figure 2D). For example, nine pathways for carbohydrate metabolism, such as propanoate and butanoate metabolism, were highly enriched in ETB samples before the intervention (Figure 2D). For another instance, several pathways belonging to membrane transport, metabolism of cofactors and vitamins and xenobiotics biodegradation and metabolism were also enriched in ETB samples (Figure 2D). Only four pathways including phenylalanine, tyrosine and tryptophan biosynthesis, methane metabolism, peptidoglycan biosynthesis and terpenoid backbone biosynthesis were enriched in ETP samples before the intervention (pathways highlighted in blue in Figure 2D).

ETB and ETP subjects responded differentially to CR Intervention

After the 3-week CR intervention, BMI of both ETB and ETP subjects decreased significantly (Figure 3A; paired Wilcoxon rank-sum test, $p<0.05$, See Methods). Subsequent analysis revealed that ETP subjects had a significantly larger BMI loss ratio compared to ETB subjects (Wilcoxon rank-sum test, $p <0.05$; Figure 3B). The BMI loss ratio of a subject was calculated based on the following equation:

$$BMI\ loss\ ratio = \frac{BMI_{after} - BMI_{before}}{BMI_{before}}$$

where BMI_{before} and BMI_{after} is the respective BMI of a subject before and after the intervention.

What's more, gut microbiota of ETB and ETP subjects changed differentially to CR intervention for both microbiome composition and functional potentials (Figure 3C, 3D). In terms of common species, relative abundances of *Enterobacter cloacae*, *Enterobacter hormaechei/cloacae* and *Klebsiella oxytoca* significantly increased and *Collinsella aerofaciens* significantly decreased only in ETP samples (Figure 3C, species in blue; Paired Wilcoxon rank-sum test). Whereas, relative abundances of ten species significantly changed in ETB samples specifically (Figure 3C, species in orange; Paired Wilcoxon rank-sum test). Among these ten species, the relative abundances of seven species, like *Eubacterium rectale, Bacteroides plebeius, Ruminococcus lactaris, Prevotella copri*, and *Eggerthella lenta etc.*, significantly increased while the remaining three species (*Bacteroides stercoris, Bacteroides coprocola* and *Veillonella parvula*) significantly decreased in ETB subjects.

On the function level, we observed a total of 49 KEGG pathways which changed differentially between ETB and ETP samples (Figure 3D). Among these 49 pathways, levels of 31 pathways (in orange in Figure 3D) changed significantly only in ETB samples, 17 pathways (in blue in Figure 3D) changed significantly only in ETP samples and 1 pathway (in green in Figure 3D) changed significantly in both ETB and ETP samples (decreased in ETB samples while increased in ETP samples). In particular, for carbohydrate metabolism, levels for propanoate and butanoate metabolism significantly

increased in ETB samples only (Figure 3D). A significant decrease of metabolism levels for amino sugar, nucleotide sugar, fructose and mannose were observed in ETP samples only (Figure 3D). For energy metabolism, three pathways including methane metabolism, carbon fixation pathways in prokaryotes and nitrogen metabolism decreased in ETP samples only (Figure 3D). Four pathways for the lipid metabolism significantly changed in ETB samples only: an increase of levels for fatty acid and glycerolipid metabolism *etc.* and a decrease of levels for fatty acid biosynthesis. For metabolism of cofactors and vitamins, abundances of six out of the seven pathways shown in Figure 3D decreased in either ETB or ETP samples, such as biotin and riboflavin metabolism.

ETB vs. ETP gut microbiota after CR intervention

After finding the different responses, we then compared microbiota of ETB and ETP after-intervention samples. Firstly, we compared the relative abundances of common genera using Wilcoxon rank-sum test. As shown in Figure 4A and Figure 2B, the six common genera (marked with an asterisk in Figure 4A and Figure 2B) which were significantly different between ETB and ETP before-intervention samples remained significantly different after the intervention. In addition, relative abundances of ten genera which showed no inter-enterotype difference before the intervention became significantly different between ETB and ETP after-intervention samples, including nine genera (*Parabacteroides*, *Ruminococcus*, *Clostridium*, *Blautia*, *Bilophila*, *Coprobacillus*, *Campylobacter*, *Staphylococcus* and *Anaerococcus*) became more abundant in ETB samples and one genus (*Enterobacter*) got more abundant in ETP samples after the intervention (Figure 4A). On the species level, the relative abundances of a total of 42 species became significantly different between ETB and ETP samples after the intervention, including 27 species (marked with an asterisk in Figure 4B and Figure 2C) which already showed significant differences between ETB and ETP samples before the intervention (Figure 4B). 15 species that showed no significant inter-enterotype difference before the intervention became significant different between ETB and ETP samples: 13 species (in orange with no asterisk in Figure 4B) became significantly more abundant in ETB samples while 2 species, *Enterobacter cloacae* and *Ruminococcus flavefaciens* (in orange with no asterisk in Figure 4B) got more abundant in ETP samples after the intervention. In addition,

the relative abundances of 9 species (with no asterisk in Figure 2C), including *Bacteroides intestinalis*, *Bacteroides salanitronis* and *Prevotella timonensis*, etc., became no longer significantly different between ETB and ETP samples after CR intervention.

We further studied inter-enterotype differences on functional potentials after the intervention. There were a total of 28 KEGG pathways showing differential enrichment between ETB and ETP samples after the intervention (Figure 4C), including 24 pathways (marked with an asterisk in Figure 4C and Figure 2D) were already significantly different before the intervention. 4 pathways (with no asterisk in Figure 4C) began to show differential inter-enterotype enrichment after the intervention: valine, leucine and isoleucine degradation and glutathione metabolism got more enriched in ETB subjects while valine, leucine and isoleucine biosynthesis and d−glutamine and d−glutamate metabolism became more enriched in ETP subjects. 5 pathways (with no asterisk in Figure 2D) which showed differential inter-enterotype enrichment before the intervention were no longer significantly different between ETB and ETP samples and these five pathways were lysine degradation, phenylalanine, tyrosine and tryptophan biosynthesis, flagellar assembly, methane metabolism and bisphenol degradation.

A convergent shift on gut microbiota of ETB and ETP subjects

Interestingly, from the results shown in Figure 3C, we observed that in fecal samples belonging to the *Bacteroides* enterotype, the relative abundance of one *prevotella* bacteria (*Prevotella copri*) significantly increased while two *Bacteroides* bacterium (*Bacteroides stercoris* and *Bacteroides coprocola*) significantly decreased. Additionally, three species (*Bacteroides intestinalis*, *Bacteroides salanitronis* and *Prevotella timonensis*) of the *Bacteroides* or *Prevotella* genus which were initially significantly different between ETB and ETP samples showed no inter-enterotype difference after the intervention. On the functional level, we found five pathways which changed simultaneously in ETB and ETP samples (Figure 3E). All these above observations provided us a thought that the same 3-week diet may reduce the gut microbiota dissimilarity between ETB and ETP subjects.

To confirm our hypothesis, we then calculated the inter-enterotype Bray-Curtis distances of gene, genera and species profiles before and after the intervention (Figure 5 A-C). The results revealed that the inter-enterotype distances in gene, genera and species profiles had

significantly decreased after the intervention (paired Wilcoxon rank-sum test, $p < 0.05$; Figure 5 A-C). But it should be noted that the inter-enterotype distances in gene, genera and species profiles were still significantly larger than that of intra-enterotype after the intervention (Wilcoxon rank-sum test, $p < 0.05$; Figure 5 D-E).

Discussion

The most important finding in our study is that ETB and ETP subjects responded differentially to CR intervention in both BMI and gut microbiota. As for BMI, although CR intervention resulted in a BMI loss for both ETB and ETP subjects, subjects of ETP enterotype had a significantly larger BMI loss ratio than ETB subjects. This finding is consistent with a previous study that high P/B group had higher weight loss than low P/B group when participants received the New Nordic Diet [6]. For gut microbiota, both microbiome composition and functional potentials changes differentially between ETB and ETP samples. Particularly, we observed that relative abundances of species and functional pathways (*Eubacterium rectal*, butanoate and propanoate metabolisms) which are related to SCFA-synthesis and involved in energy supplement for host significantly increased in ETB gut microbiomes only [17]. In addition, relative abundances of pathways for metabolism of cofactors and vitamins changed differentially between ETB and ETP samples: biotin metabolism, folate, pantothenate and CoA biosynthesis levels only decreased in ETB samples while riboflavin (vitamin B2), nicotinate and nicotinamide metabolism levels decreased in ETP samples only. Despite of these different responses, both ETB and ETP gut microbiome exhibited decreased levels for cofactors and vitamins metabolism, which suggested that extra supplements for cofactors and vitamins may be needed for low-calorie diet. Riboflavin (vitamin B2) metabolism level decreased after CR in fecal samples of ETP was also reported in a previous study [18].

Another interesting finding in our study is that a convergent shift of the ETB and ETP gut microbiota was observed after this short-term CR intervention, which agreed with a published finding that human gut microbiome functions were driven convergence by dietary intervention [19]. Inter-enterotype Bray-Curtis distances of gene/genus/species profiles decreased significantly after CR intervention. The relative abundances of several species including *Bacteroides intestinalis*, *Bacteroides salanitronis* and *Prevotella*

timonensis showed no significant inter-enterotype differences after the intervention. Some previous studies also showed that diet history contributed to enterotype clustering and metagenomics content and enterotype of some persons even switched to other enterotypes because of dietary interventions [3, 20-22].

Our study provides new evidences for the potential feasibility of gut microbiota stratification for personalized BMI loss guidance with CR intervention. Future work can utilize more information, such as human genome and metabolome, to conduct a systematic study on CR intervention.

Conclusions

Our study reveals that BMI and gut microbiota of ETB and ETP individuals changed differentially to a short-term calorie-restriction intervention. Moreover, a convergent shift on ETB and ETP gut microbiota was observed after the intervention. Our results highlight the potential application for microbiome stratification in nutrition intervention.

Methods

Subject enrollment

Volunteer-wanted posters were propagated at the China National Gene Bank in Shenzhen from March to April 2017. A volunteer was considered if his/her BMI was less than 28 kg/m^2. In addition, recruited volunteers should meet all the following criteria: 1) had not taken antibiotics in the recent 2 months; 2) had no hypertension, diabetes mellitus and intestinal disease; 3) had regular eating and lifestyle patterns; 4) could follow the controlled feeding trial. Finally, 41 subjects (24 females and 17 males aged 30 ± 6 years old) met all the criteria and were recruited in this study (Table 2).

Study design

As shown in Figure 1, all subjects underwent a 1-week normal-calorie diet and 3-week low-calorie diet. During this 4-week study, all participants ate the meals at our study center. The low-calorie diet, which was of ~50% calories of the normal-calorie diet (female, 1000kcal/day; male, 1200kcal/day), was designed according to the Dietary Guidelines for Chinese Residents (2016) [23] and nutritionally balanced. The respective proportion of protein, carbohydrates and fat was approximately 35%, 50% and 15%. Before the CR

intervention, BMI and fecal samples were collected at our study center (Figure 1). In the last week of CR intervention, BMI values for each subject were collected multiple times and an averaged BMI values was used in our study (Figure 1). Fecal samples were also collected after the CR intervention (Figure 1).

Fecal DNA extraction and metagenomic sequencing

Fecal samples were transferred to the laboratory on dry ice and kept frozen at -80°C. The stool DNA was extracted following the MetaHIT protocol as described previously [24]. The DNA concentration was estimated by Qubit (Invitrogen). Sequencing was performed based on the BGISEQ-500 protocol in the SE100 mode.

Data processing, gut microbiota profiling and enterotyping

Raw reads from BGISEQ-500 were trimmed by an overall accuracy (OA) control strategy for quality adjustment [25]. 98.15% of the raw reads remained as high-quality reads by using this approach. To remove human reads, the high-quality reads were aligned to hg19 using SOAP2.22 [25]. The retained clean reads were aligned to the integrated non-redundant gene catalog (IGC) using SOAP2.22 (identity $\geq$ 0.95) [26]. As shown in Supplemental Table 1, the clean reads reached an average IGC mapping rate of 80.18% and an average unique mapping rate of 65.76%. The relative abundances of genes, genera, species and KOs were annotated by IGC reference [26]. For enterotyping, we applied a universal classifier (http://enterotypes.org/) which circumvents many of the problems in enterotyping (no standardization, such as sample size is small and clustering a single sample is impossible) and provides a more comparable result [27, 28]. From the classification result, all our samples fitted the models of this classifier with 28 subjects belonging to ETB and 13 subjects belonging to ETP. The enterotype for each subject was shown in Supplemental Table 2.

Wilcoxon rank-sum test

Wilcoxon rank-sum test was performed by a R built-in function named wilcox.test with default parameters. Paired Wilcoxon rank-sum test was also calculated by the wilcox.test function with the parameter *paired* = T. The R version we used in this study is version 3.5.0.

Pearson's chi-square test

To evaluate whether the female/male ratio was significantly different between subjects of the two enterotypes, Pearson's chi-square test was performed by R *chisq.test* function with default parameters.

Permutational multivariate analysis of variance (PERMANOVA)

To evaluate the correlation level between genus profiles and enterotypes, we performed a permutational multivariate analysis of variance (PERMANOVA) with 9,999 permutations on enterotypes and the Bray-Curtis distance matrix calculated from the genus profiles (R *vegan* package).

Common genus or species

Genus or species with occurrence rate > 80% and median relative abundance > 1e-6 in all samples was defined as common genus or species. The detailed information for common genus/species is shown in Supplemental Table 3-4.

Reporter score

Differentially enriched KEGG pathways were identified according to their reporter Z-score [29]. A reporter score |Z| > 1.96 (95% confidence according to normal distribution) was used as a detection threshold for significantly differentiating pathways.

List of abbreviations

CR: Calorie restriction; BMI: Body mass index; KO: Kyoto Encyclopedia of Genes and Genomes Orthology; PERMANOVA: permutational analysis of variance

Declarations

Ethics approval and consent to participate

The study protocol was approved by the institutional review board on bioethics and biosafety of BGI (NO. BGI-IRB 17020).

Consent for publication

Not applicable.

Availability of data and material

The metagenomics sequencing reads of this study can be found in the China National Genebank Nucleotide Sequence Archive (CNSA) (https://db.cngb.org/cnsa/) under the BioProject number CNP0000247. The scripts used to perform statistical analysis and figure plotting are available at https://github.com/HuaZou/NonobeseBP. The gut microbiota profiles are also available at https://github.com/HuaZou/NonobeseBP.

Competing interests

The authors declare that they have no competing interests.

Funding

This work is supported by National Key Research and Development Program of China (No.2017YFC0909703) and Shenzhen Municipal Government of China (No. CXB201108250098A).

Authors' contributions

P.C and K.C collected the samples and provided the fecal microbiome data. H.Z, D.W, Z.H.Z, H.R, Z.S, P.Z, C.F, H.Y and J.W established the concept and analysis framework of the study. H.Z and D.W performed the bioinformatic analysis. H.Z wrote the first draft of the manuscript and D.W and Z.H.Z revised and edited the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We are very grateful to acknowledge colleagues from the China National Genebank for collecting fecal samples and metadata and as well as DNA extraction, library construction, sequencing by colleagues in BGI-Shenzhen.

References

1. Sommer F, Bäckhed F: **The gut microbiota — masters of host development and physiology**. *Nature Reviews Microbiology* 2013, **11**(4):227-238.
2. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM *et al*: **Enterotypes of the human gut microbiome**. *Nature* 2011, **473**(7346):174-180.
3. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R *et al*: **Linking long-term dietary patterns with gut microbial enterotypes**. *Science* 2011, **334**(6052):105-108.
4. Vandeputte D, Falony G, Vieira-Silva S, Tito RY, Joossens M, Raes J: **Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates**. *Gut* 2016, **65**(1):57-62.
5. Kang C, Zhang Y, Zhu X, Liu K, Wang X, Chen M, Wang J, Chen H, Hui S, Huang L *et al*: **Healthy Subjects Differentially Respond to Dietary Capsaicin Correlating with Specific Gut Enterotypes**. *J Clin Endocrinol Metab* 2016, **101**(12):4681-4689.
6. Hjorth MF, Roager HM, Larsen TM, Poulsen SK, Licht TR, Bahl MI, Zohar Y, Astrup A: **Pre-treatment microbial Prevotella-to-Bacteroides ratio, determines body fat loss success during a 6-month randomized controlled diet intervention**. *Int J Obes (Lond)* 2018, **42**(3):580-583.
7. Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, Britt EB, Fu X, Wu Y, Li L *et al*: **Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis**. *Nat Med* 2013, **19**(5):576-585.
8. Fontana L, Partridge L, Longo VD: **Extending healthy life span--from yeast to humans**. *Science (New York, NY)* 2010, **328**(5976):321-326.
9. Ravussin E, Redman LM, Rochon J, Das SK, Fontana L, Kraus WE, Romashkan S, Williamson DA, Meydani SN, Villareal DT *et al*: **A 2-Year Randomized**

Controlled Trial of Human Caloric Restriction: Feasibility and Effects on Predictors of Health Span and Longevity. *J Gerontol A Biol Sci Med Sci* 2015, **70**(9):1097-1104.

10. Martin CK, Bhapkar M, Pittas AG, Pieper CF, Das SK, Williamson DA, Scott T, Redman LM, Stein R, Gilhooly CH *et al*: **Effect of Calorie Restriction on Mood, Quality of Life, Sleep, and Sexual Function in Healthy Nonobese Adults: The CALERIE 2 Randomized Clinical Trial**. *JAMA Intern Med* 2016, **176**(6):743-752.
11. Santacruz A, Marcos A, Warnberg J, Marti A, Martin-Matillas M, Campoy C, Moreno LA, Veiga O, Redondo-Figuero C, Garagorri JM *et al*: **Interplay between weight loss and gut microbiota composition in overweight adolescents**. *Obesity (Silver Spring)* 2009, **17**(10):1906-1915.
12. Ott B, Skurk T, Hastreiter L, Lagkouvardos I, Fischer S, Buttner J, Kellerer T, Clavel T, Rychlik M, Haller D *et al*: **Effect of caloric restriction on gut permeability, inflammation markers, and fecal microbiota in obese women**. *Sci Rep* 2017, **7**(1):11955.
13. Cox Laura M, Blaser Martin J: **Pathways in Microbe-Induced Obesity**. *Cell Metabolism* 2013, **17**(6):883-894.
14. Heiman ML, Greenway FL: **A healthy gastrointestinal microbiome is dependent on dietary diversity**. *Mol Metab* 2016, **5**(5):317-320.
15. Potts M: **China's one child policy**. *BMJ* 2006, **333**(7564):361-362.
16. **Uncovering transcriptional regulation of metabolism by using metabolic network topology**.
17. Damms-Machado A, Mitra S, Schollenberger AE, Kramer KM, Meile T, Konigsrainer A, Huson DH, Bischoff SC: **Effects of surgical and dietary weight loss therapy for obesity on gut microbiota composition and nutrient absorption**. *Biomed Res Int* 2015, **2015**:806248.
18. Heinsen FA, Fangmann D, Muller N, Schulte DM, Ruhlemann MC, Turk K, Settgast U, Lieb W, Baines JF, Schreiber S *et al*: **Beneficial Effects of a Dietary Weight Loss Intervention on Human Gut Microbiome Diversity and Metabolism Are Not Sustained during Weight Maintenance**. *Obes Facts* 2016, **9**(6):379-391.
19. Muegge BD, Kuczynski J, Knights D, Clemente JC, González A, Fontana L, Henrissat B, Knight R, Gordon JI: **Diet drives convergence in gut microbiome**

functions across mammalian phylogeny and within humans. *Science (New York, NY)* 2011, **332**(6032):970-974.

20. Bergström A, Skov TH, Bahl MI, Roager HM, Christensen LB, Ejlerskov KT, Mølgaard C, Michaelsen KF, Licht TR: **Establishment of intestinal microbiota during early life: a longitudinal, explorative study of a large cohort of Danish infants**. *Applied and environmental microbiology* 2014, **80**(9):2889-2900.

21. Wang J, Linnenbrink M, Kunzel S, Fernandes R, Nadeau MJ, Rosenstiel P, Baines JF: **Dietary history contributes to enterotype-like clustering and functional metagenomic content in the intestinal microbiome of wild mice**. *Proc Natl Acad Sci U S A* 2014, **111**(26):E2703-2710.

22. Moeller AH, Ochman H: **Microbiomes are true to type**. *Proc Natl Acad Sci U S A* 2014, **111**(26):9372-9373.

23. Wang SS, Lay S, Yu HN, Shen SR: **Dietary Guidelines for Chinese Residents (2016): comments and comparisons**. *J Zhejiang Univ Sci B* 2016, **17**(9):649-656.

24. Qin J, Li Y, Cai Z, Li S, Zhu J, Zhang F, Liang S, Zhang W, Guan Y, Shen D *et al*: **A metagenome-wide association study of gut microbiota in type 2 diabetes**. *Nature* 2012, **490**(7418):55-60.

25. Fang C, Zhong H, Lin Y, Chen B, Han M, Ren H, Lu H, Luber JM, Xia M, Li W *et al*: **Assessment of the cPAS-based BGISEQ-500 platform for metagenomic sequencing**. *Gigascience* 2018, **7**(3):1-8.

26. Li J, Jia H, Cai X, Zhong H, Feng Q, Sunagawa S, Arumugam M, Kultima JR, Prifti E, Nielsen T *et al*: **An integrated catalog of reference genes in the human gut microbiome**. *Nat Biotechnol* 2014, **32**(8):834-841.

27. Costea PI, Hildebrand F, Arumugam M, Bäckhed F, Blaser MJ, Bushman FD, de Vos WM, Ehrlich SD, Fraser CM, Hattori M *et al*: **Enterotypes in the landscape of gut microbial community composition**. *Nature Microbiology* 2017, **3**(1):8-16.

28. Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, Almeida M, Arumugam M, Batto JM, Kennedy S *et al*: **Richness of human gut microbiome correlates with metabolic markers**. *Nature* 2013, **500**(7464):541-546.

29. Patil KR, Nielsen J: **Uncovering transcriptional regulation of metabolism by using metabolic network topology**. *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102**(8):2685-2689.

Figures, tables and additional files

Figure 1. Overview of Experimental design. CR intervention was conducted in the last three weeks of our study.

Figure 2. Gut microbiota of ETB vs. ETP before the intervention. A, NMDS plot of ETB and ETP samples. **B**, common genera whose relative abundances were significantly different between ETB and ETP samples before the intervention. **C**, common species whose relative abundances were significantly different between ETB and ETP samples before the intervention. **D**, KEGG pathways which were differentially enriched between ETB and ETP samples before the intervention. Genus/species/pathways marked with an asterisk are genus/species/pathways which remained significant inter-enterotype different before and after the intervention, which will be discussed later.

Figure 3. Differential responses of ETB and ETP to CR on BMI and gut microbiota. A, within-group changes on BMI. **B,** comparison of the BMI loss ratio between ETB and ETP subjects. **C**, common species whose relative abundances changed differentially between ETB and ETP samples. **D**, KEGG pathways whose levels changed differentially between ETB and ETP samples. **E**, KEGG pathways whose levels changed simultaneously between ETB and ETP samples. Species/pathways in orange are species/pathways which changed significantly in ETB samples only. Species/pathways in blue are species/pathways which changed significantly in ETP samples only. Species/pathways in green are species/pathways which changed significantly in both ETB and ETP samples. *, $p<0.5$; **, $p<0.01$

Figure 4. Gut microbiota of ETB vs. ETP after the intervention. A, common genera whose relative abundances were significantly different between ETB and ETP samples after the intervention. **B**, common species whose relative abundances were significantly different between ETB and ETP samples after the intervention. **C**, KEGG pathways which were differentially enriched between ETB and ETP samples after the intervention. Genus/species/pathways marked with an asterisk are genus/species/pathways which remained significant inter-enterotype different before and after the intervention.

Figure 5. Bray-Curtis distance of profiles for genes, genera and species. A-C, Comparison of inter-enterotype distance before and after the intervention on gene, genus and species level. **D-F**, Comparison of inter- and intra- enterotype distance after the intervention on gene.

Table 1. Comparison of phenotypes of ETB and ETP subjects before CR intervention

Table 2. Cohort description

Supplemental Table 1. Metagenomic sequencing statistics for fecal samples

Supplemental Table 2. Enterotyping results

Supplemental Table 3. Common genus

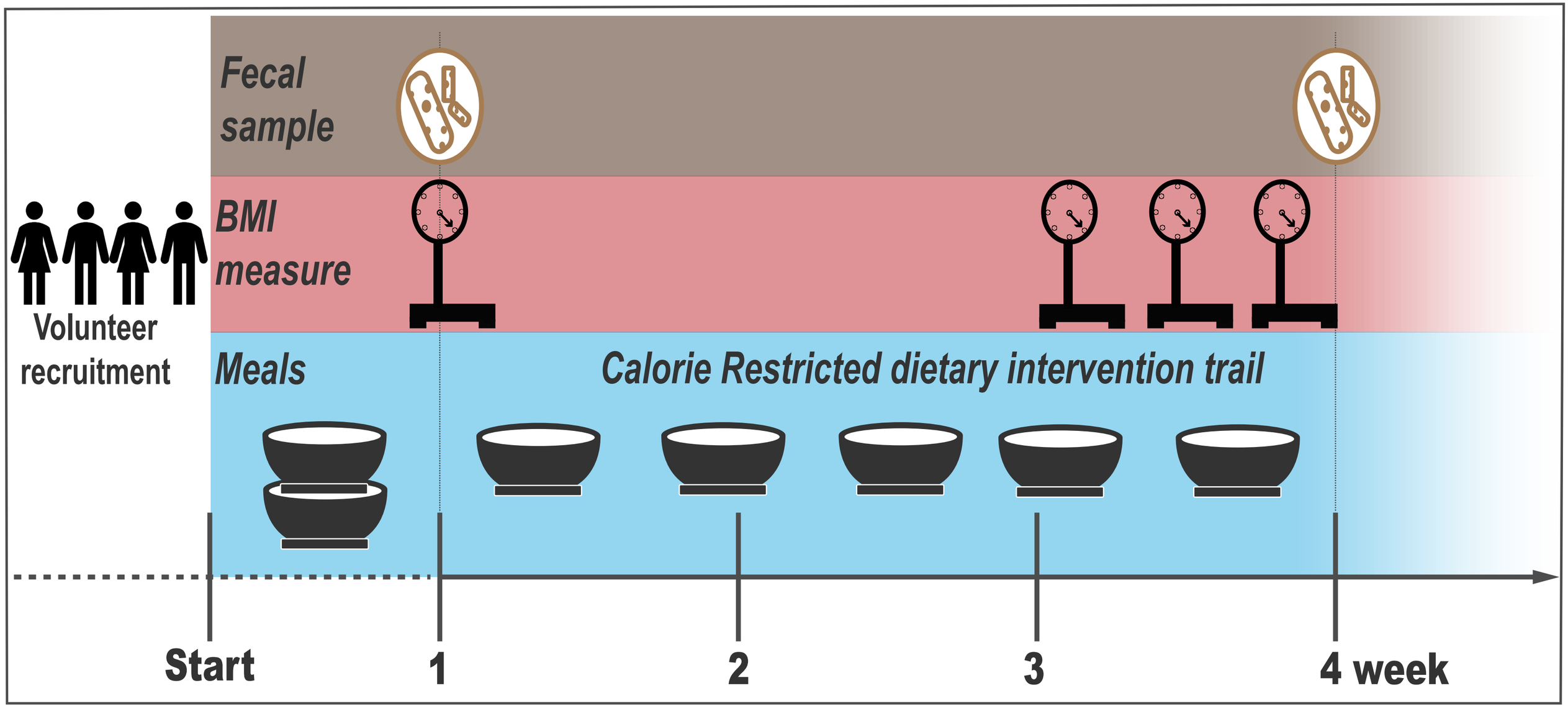
Supplemental Table 4. Common species

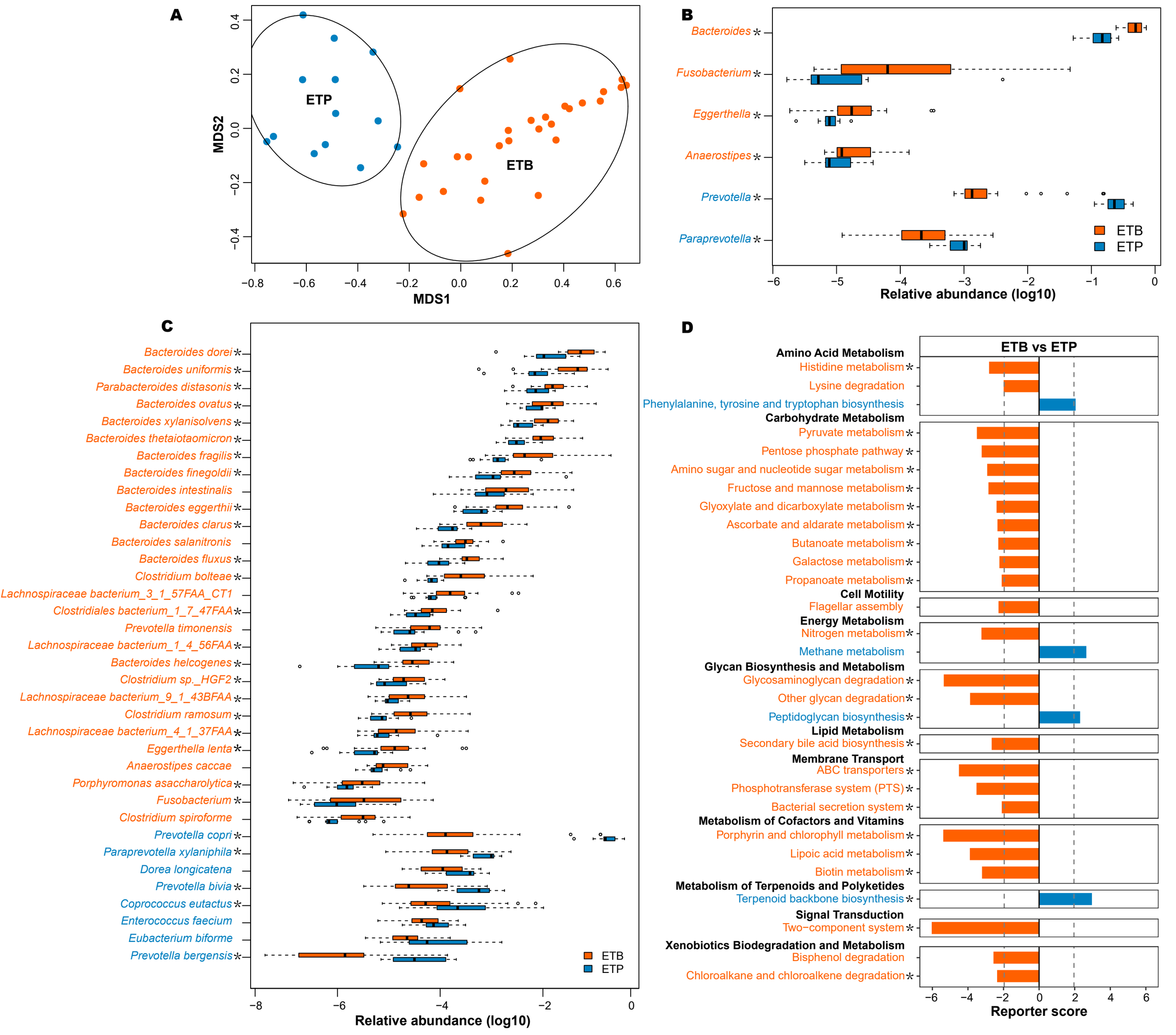
Table 1. Comparison of phenotypes of ETB and ETP subjects before CR intervention

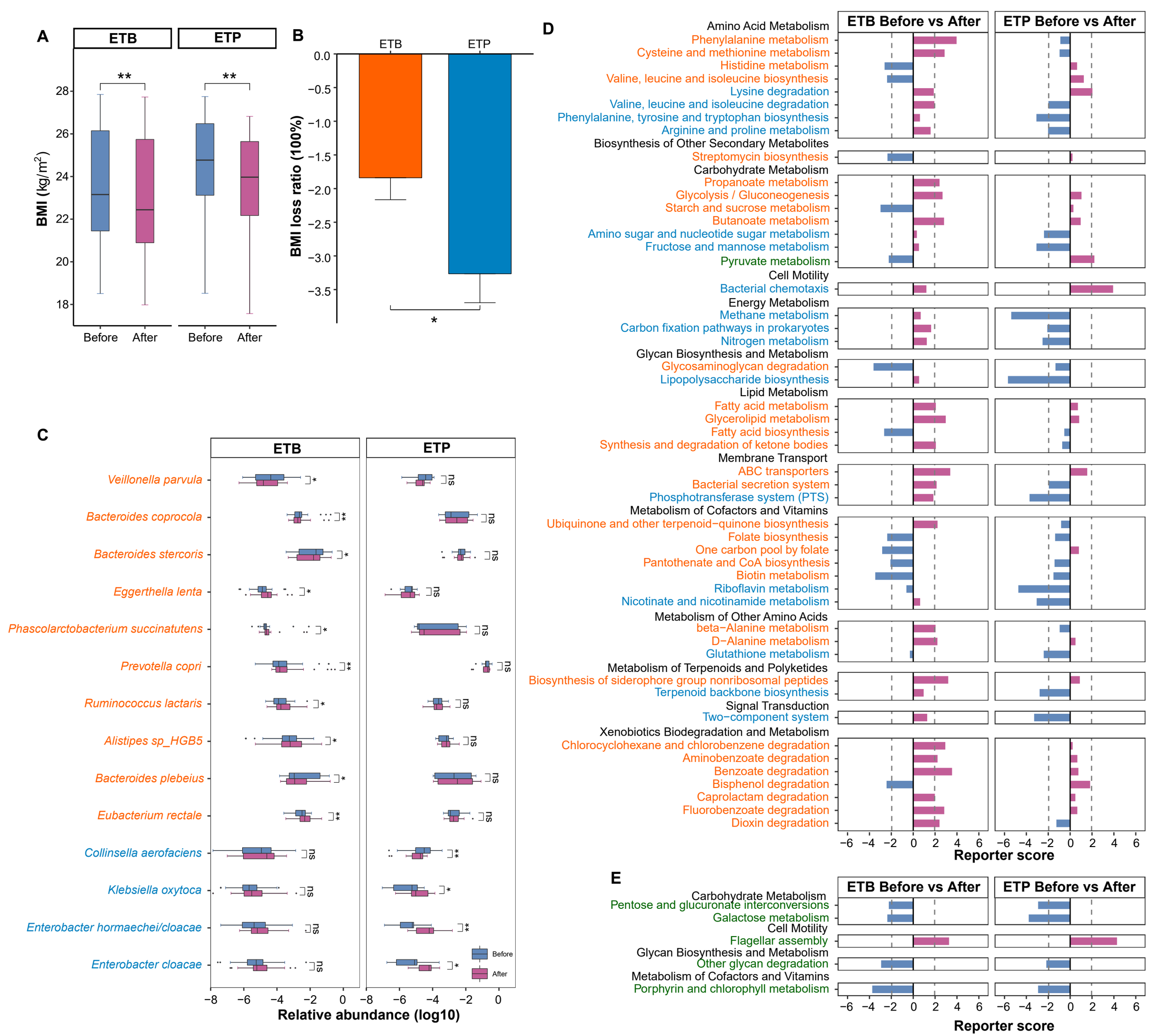
	ETB Group (Mean ± SD)	ETP Group (Mean ± SD)	P value ETB vs ETP
Number of subjects	28	13	
Sex (female/male)	16/12	8/5	1
Age	29 ± 6	30 ± 7	0.44
BMI (kg/m^2)	23.50 ± 2.81	24.21 ± 2.85	0.42
Weight (kg)	64.40 ± 11.37	65.81 ± 12.39	0.62

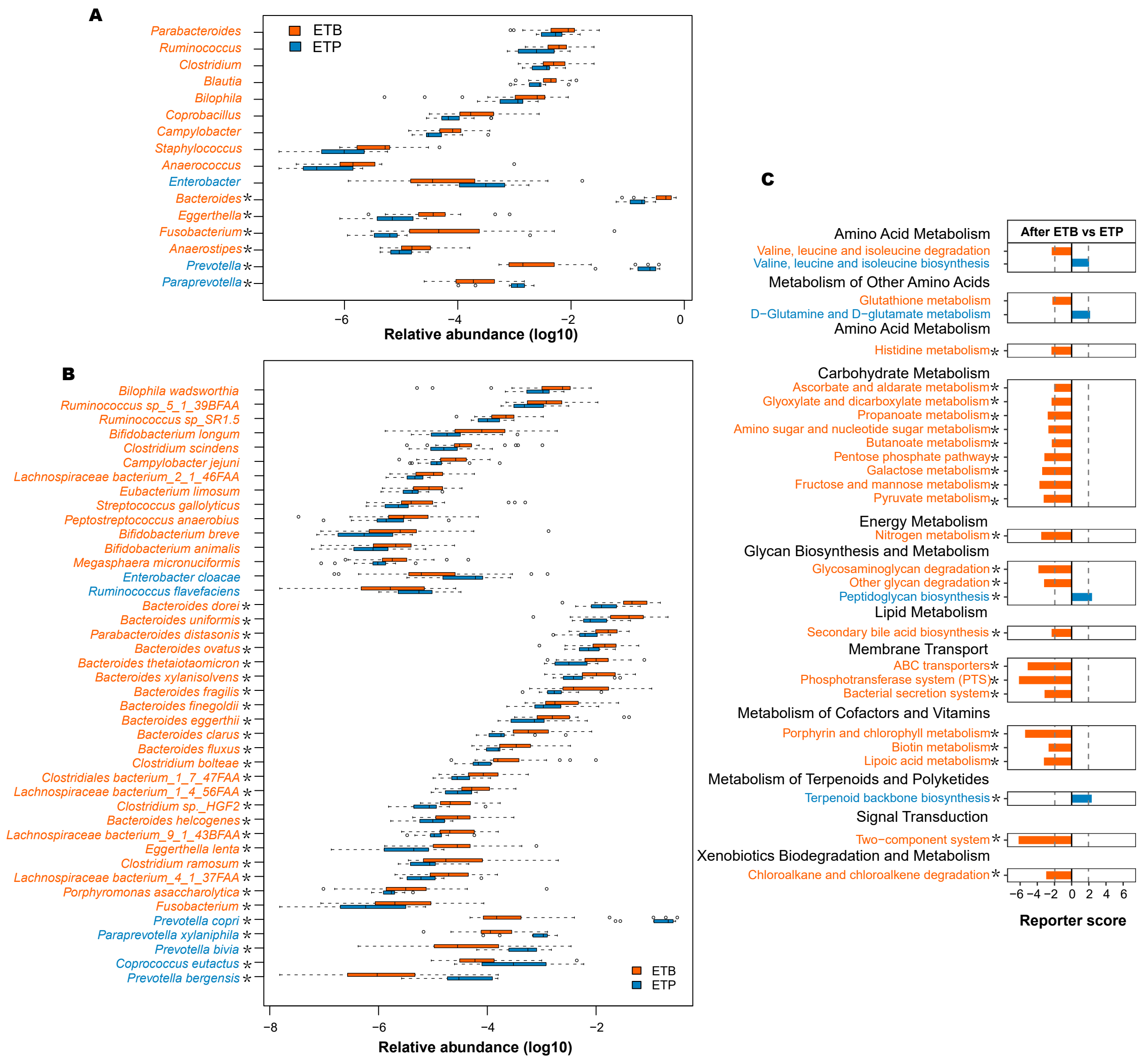
Table 2. Cohort Description

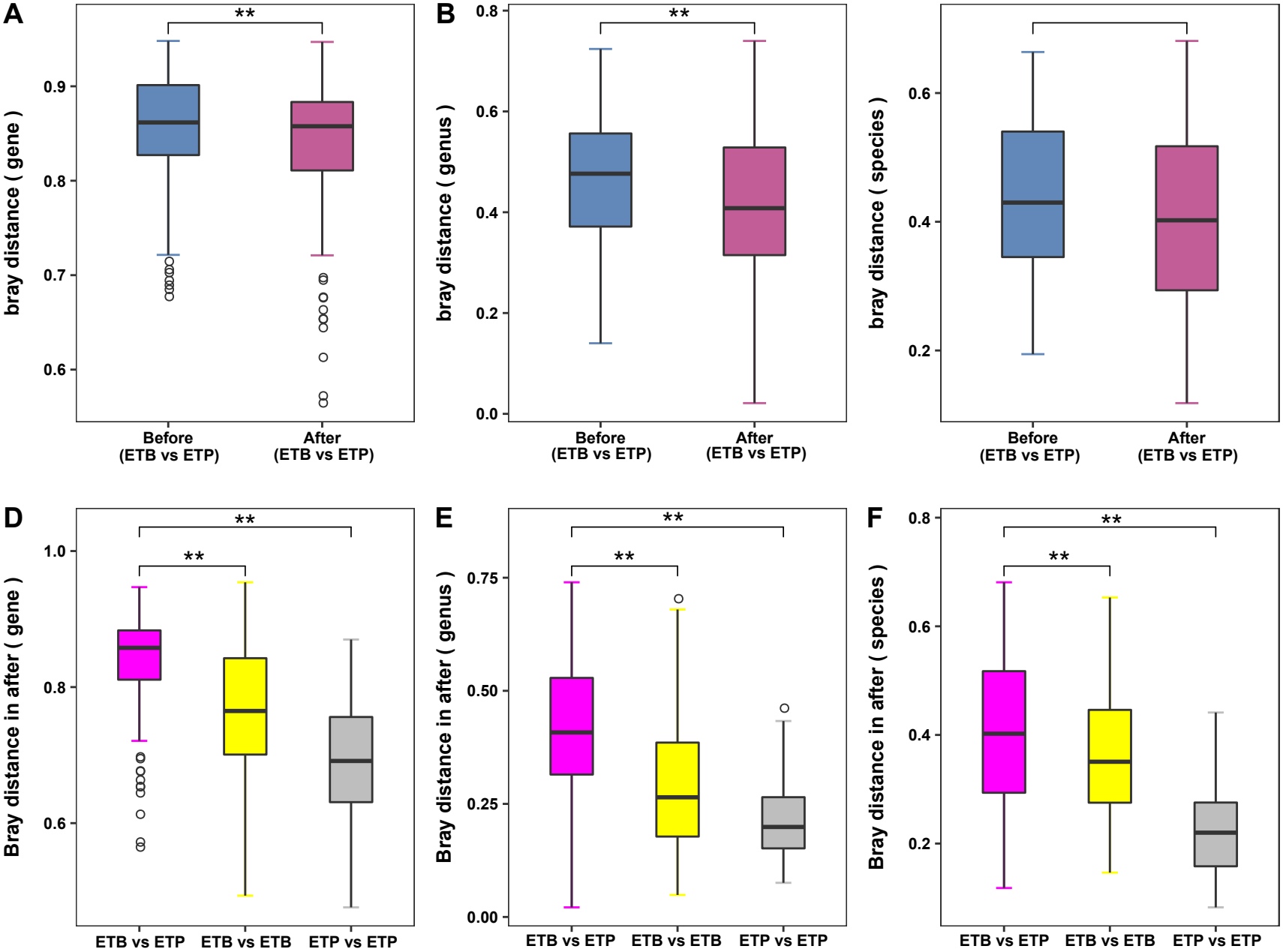
	Cohort (Mean ± SD)
Number of subjects	41
Sex (female/male)	24/17
Age	30 ± 6
BMI (kg/m^2)	23.72 ± 2.81
Weight(kg)	64.84 ± 11.57


Fecal sample
BMI measure
Meals
Calorie Restricted dietary intervention trail
Volunteer recruitment
Start
1
2
3
4 week


A
ETP
ETB
MDS2
MDS1
B
Bacteroides
Fusobacterium
Eggerthella
Anaerostipes
Prevotella
Paraprevotella
ETB
ETP
Relative abundance (log10)
C
Bacteroides dorei
Bacteroides uniformis
Parabacteroides distasonis
Bacteroides ovatus
Bacteroides xylanisolvens
Bacteroides thetaiotaomicron
Bacteroides fragilis
Bacteroides finegoldii
Bacteroides intestinalis
Bacteroides eggerthii
Bacteroides clarus
Bacteroides salanitronis
Bacteroides fluxus
Clostridium bolteae
Lachnospiraceae bacterium_3_1_57FAA_CT1
Clostridiales bacterium_1_7_47FAA
Prevotella timonensis
Lachnospiraceae bacterium_1_4_56FAA
Bacteroides helcogenes
Clostridium sp._HGF2
Lachnospiraceae bacterium_9_1_43BFAA
Clostridium ramosum
Lachnospiraceae bacterium_4_1_37FAA
Eggerthella lenta
Anaerostipes caccae
Porphyromonas asaccharolytica
Fusobacterium
Clostridium spiroforme
Prevotella copri
Paraprevotella xylaniphila
Dorea longicatena
Prevotella bivia
Coprococcus eutactus
Enterococcus faecium
Eubacterium biforme
Prevotella bergensis
ETB
ETP
Relative abundance (log10)
D
ETB vs ETP
Amino Acid Metabolism
Histidine metabolism
Lysine degradation
Phenylalanine, tyrosine and tryptophan biosynthesis
Carbohydrate Metabolism
Pyruvate metabolism
Pentose phosphate pathway
Amino sugar and nucleotide sugar metabolism
Fructose and mannose metabolism
Glyoxylate and dicarboxylate metabolism
Ascorbate and aldarate metabolism
Butanoate metabolism
Galactose metabolism
Propanoate metabolism
Cell Motility
Flagellar assembly
Energy Metabolism
Nitrogen metabolism
Methane metabolism
Glycan Biosynthesis and Metabolism
Glycosaminoglycan degradation
Other glycan degradation
Peptidoglycan biosynthesis
Lipid Metabolism
Secondary bile acid biosynthesis
Membrane Transport
ABC transporters
Phosphotransferase system (PTS)
Bacterial secretion system
Metabolism of Cofactors and Vitamins
Porphyrin and chlorophyll metabolism
Lipoic acid metabolism
Biotin metabolism
Metabolism of Terpenoids and Polyketides
Terpenoid backbone biosynthesis
Signal Transduction
Two−component system
Xenobiotics Biodegradation and Metabolism
Bisphenol degradation
Chloroalkane and chloroalkene degradation
Reporter score


A
ETB
ETP
BMI (kg/m2)
Before
After
B
ETB
ETP
BMI loss ratio (100%)
C
ETB
ETP
Veillonella parvula
Bacteroides coprocola
Bacteroides stercoris
Eggerthella lenta
Phascolarctobacterium succinatutens
Prevotella copri
Ruminococcus lactaris
Alistipes sp_HGB5
Bacteroides plebeius
Eubacterium rectale
Collinsella aerofaciens
Klebsiella oxytoca
Enterobacter hormaechei/cloacae
Enterobacter cloacae
Before
After
Relative abundance (log10)
D
ETB Before vs After
ETP Before vs After
Amino Acid Metabolism
Phenylalanine metabolism
Cysteine and methionine metabolism
Histidine metabolism
Valine, leucine and isoleucine biosynthesis
Lysine degradation
Valine, leucine and isoleucine degradation
Phenylalanine, tyrosine and tryptophan biosynthesis
Arginine and proline metabolism
Biosynthesis of Other Secondary Metabolites
Streptomycin biosynthesis
Carbohydrate Metabolism
Propanoate metabolism
Glycolysis / Gluconeogenesis
Starch and sucrose metabolism
Butanoate metabolism
Amino sugar and nucleotide sugar metabolism
Fructose and mannose metabolism
Pyruvate metabolism
Cell Motility
Bacterial chemotaxis
Energy Metabolism
Methane metabolism
Carbon fixation pathways in prokaryotes
Nitrogen metabolism
Glycan Biosynthesis and Metabolism
Glycosaminoglycan degradation
Lipopolysaccharide biosynthesis
Lipid Metabolism
Fatty acid metabolism
Glycerolipid metabolism
Fatty acid biosynthesis
Synthesis and degradation of ketone bodies
Membrane Transport
ABC transporters
Bacterial secretion system
Phosphotransferase system (PTS)
Metabolism of Cofactors and Vitamins
Ubiquinone and other terpenoid−quinone biosynthesis
Folate biosynthesis
One carbon pool by folate
Pantothenate and CoA biosynthesis
Biotin metabolism
Riboflavin metabolism
Nicotinate and nicotinamide metabolism
Metabolism of Other Amino Acids
beta−Alanine metabolism
D−Alanine metabolism
Glutathione metabolism
Metabolism of Terpenoids and Polyketides
Biosynthesis of siderophore group nonribosomal peptides
Terpenoid backbone biosynthesis
Signal Transduction
Two−component system
Xenobiotics Biodegradation and Metabolism
Chlorocyclohexane and chlorobenzene degradation
Aminobenzoate degradation
Benzoate degradation
Bisphenol degradation
Caprolactam degradation
Fluorobenzoate degradation
Dioxin degradation
Reporter score
E
ETB Before vs After
ETP Before vs After
Carbohydrate Metabolism
Pentose and glucuronate interconversions
Galactose metabolism
Cell Motility
Flagellar assembly
Glycan Biosynthesis and Metabolism
Other glycan degradation
Metabolism of Cofactors and Vitamins
Porphyrin and chlorophyll metabolism
Reporter score


A
ETB
ETP
Parabacteroides
Ruminococcus
Clostridium
Blautia
Bilophila
Coprobacillus
Campylobacter
Staphylococcus
Anaerococcus
Enterobacter
Bacteroides*
Eggerthella*
Fusobacterium*
Anaerostipes*
Prevotella*
Paraprevotella*
−6
−4
−2
0
Relative abundance (log10)
B
Bilophila wadsworthia
Ruminococcus sp_5_1_39BFAA
Ruminococcus sp_SR1.5
Bifidobacterium longum
Clostridium scindens
Campylobacter jejuni
Lachnospiraceae bacterium_2_1_46FAA
Eubacterium limosum
Streptococcus gallolyticus
Peptostreptococcus anaerobius
Bifidobacterium breve
Bifidobacterium animalis
Megasphaera micronuciformis
Enterobacter cloacae
Ruminococcus flavefaciens
Bacteroides dorei*
Bacteroides uniformis*
Parabacteroides distasonis*
Bacteroides ovatus*
Bacteroides thetaiotaomicron*
Bacteroides xylanisolvens*
Bacteroides fragilis*
Bacteroides finegoldii*
Bacteroides eggerthii*
Bacteroides clarus*
Bacteroides fluxus*
Clostridium bolteae*
Clostridiales bacterium_1_7_47FAA*
Lachnospiraceae bacterium_1_4_56FAA*
Clostridium sp._HGF2*
Bacteroides helcogenes*
Lachnospiraceae bacterium_9_1_43BFAA*
Eggerthella lenta*
Clostridium ramosum*
Lachnospiraceae bacterium_4_1_37FAA*
Porphyromonas asaccharolytica*
Fusobacterium*
Prevotella copri*
Paraprevotella xylaniphila*
Prevotella bivia*
Coprococcus eutactus*
Prevotella bergensis*
ETB
ETP
−8
−6
−4
−2
Relative abundance (log10)
C
After ETB vs ETP
Amino Acid Metabolism
Valine, leucine and isoleucine degradation
Valine, leucine and isoleucine biosynthesis
Metabolism of Other Amino Acids
Glutathione metabolism
D−Glutamine and D−glutamate metabolism
Amino Acid Metabolism
Histidine metabolism*
Carbohydrate Metabolism
Ascorbate and aldarate metabolism*
Glyoxylate and dicarboxylate metabolism*
Propanoate metabolism*
Amino sugar and nucleotide sugar metabolism*
Butanoate metabolism*
Pentose phosphate pathway*
Galactose metabolism*
Fructose and mannose metabolism*
Pyruvate metabolism*
Energy Metabolism
Nitrogen metabolism*
Glycan Biosynthesis and Metabolism
Glycosaminoglycan degradation*
Other glycan degradation*
Peptidoglycan biosynthesis*
Lipid Metabolism
Secondary bile acid biosynthesis*
Membrane Transport
ABC transporters*
Phosphotransferase system (PTS)*
Bacterial secretion system*
Metabolism of Cofactors and Vitamins
Porphyrin and chlorophyll metabolism*
Biotin metabolism*
Lipoic acid metabolism*
Metabolism of Terpenoids and Polyketides
Terpenoid backbone biosynthesis*
Signal Transduction
Two−component system*
Xenobiotics Biodegradation and Metabolism
Chloroalkane and chloroalkene degradation*
−6 −4 −2 0 2 4 6
Reporter score


A
bray distance (gene)
0.9
0.8
0.7
0.6
**
Before
(ETB vs ETP)
After
(ETB vs ETP)
B
bray distance (genus)
0.8
0.6
0.4
0.2
0.0
**
Before
(ETB vs ETP)
After
(ETB vs ETP)
bray distance (species)
0.6
0.4
0.2
Before
(ETB vs ETP)
After
(ETB vs ETP)
D
Bray distance in after (gene)
1.0
0.8
0.6
**
**
ETB vs ETP
ETB vs ETB
ETP vs ETP
E
Bray distance in after (genus)
0.75
0.50
0.25
0.00
**
**
ETB vs ETP
ETB vs ETB
ETP vs ETP
F
Bray distance in after (species)
0.8
0.6
0.4
0.2
**
**
ETB vs ETP
ETB vs ETB
ETP vs ETP