

Comparison of the Gut Microbiota Disturbance in Rat Models of Irritable Bowel Syndrome Induced by Maternal Separation and Multiple Early-life Adversity

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Abstract

Background: The aims of this study was to identify the effect of modeling procedures on bacterial communities and investigate whether different modeling procedures lead to consistent patterns of gut microbiota compositions.

Methods: Two IBS rat models (MS alone and multiple-early-adversity modeling) were established and the gut microbiotas were analyzed using 16S-rRNA-based high-throughput sequencing methods.

Results: Rats from both models exhibited visceral hypersensitivity and the two model groups exhibited differences in the extent of visceral sensitivity and fecal water content. The microbial community structure of the two models exhibited significant differences compared to the controls, while the two model groups also exhibited significant differences between them. Furthermore, microbial community functional predictions suggested that the two models exhibited different abundances of metabolisms and pathways. Several common and distinct characteristic differences were also observed between the two model groups. *Alloprevotella* were more abundant in both model groups, while *Butyricoccus*, *Turicibacter*, *Ruminococcus*, and *Clostridium_sensu_stricto* along with the family it belongs to were less abundant relative to controls. In addition, the abundance of *Clostridium_IV*, *Corynebacterium*, *Rothia*, *Elusimicrobium*, *Romboutsia*, *Allobaculum*, *Parasutterella* and their related taxa were specifically associated with MS group, whereas *Butyricimonas* and *Vampirovibrio* along with its related taxa were specifically associated with MAM group. Among those, *Butyricimonas*, *Butyricoccus* and *Corynebacterium* were found partially mediate early adversity exposure-induced visceral hypersensitivity.

Conclusions: our results highlight the importance in evaluating gut microbiota characteristics in IBS research while also systematically considering potential modeling procedural differences. The microbial compositional/functional differences identified in this study were suggestive to further investigation of mechanism of early adversity induced IBS.

Background

Irritable bowel syndrome (IBS) is a chronic functional gastrointestinal disorder characterized by abdominal discomfort, bloating, and disturbed defecation in the absence of any identifiable abnormalities indicative of organic gastrointestinal disease [1]. The pathophysiology of IBS is likely heterogeneous and may involve visceral hypersensitivity, gut barrier dysfunction, altered brain–gut signaling, immune dysregulation, altered microbiota characteristics, and psychosocial factors [2, 3]

Various animal models have been developed to study the underlying mechanisms of IBS. Among these, the rodent maternal separation (MS) model is well-established and based on the premise that environmental changes during the early postnatal period can have long-lasting effects into adulthood, including visceral hyperalgesia and altered colonic motility [4, 5]. Combined animal models have the advantage of allowing the mimicking of multiple etiological factors of IBS and are developed by exposing animals to multiple stimuli, as has been used in several IBS studies [6–8].

Increasing data have demonstrated the relationship between alterations in gut microbiota characteristics and IBS. It was reported that IBS patients harbor altered gut microbiota compared to healthy controls, while fecal microbiota transplantation from healthy donors to IBS patients has shown promise in alleviating IBS symptoms [9, 10]. Among animal model studies, rats exposed to early-life adversity, including maternal separation, intestinal inflammation, or infection, restraint stress, and water avoidance stress have been shown to display visceral hypersensitivity and altered microbiota profiles in adulthood [11–13]. However, an understanding of the specific impacts of different modeling methodologies on gut microbiota is currently lacking, and the microbial signatures that are consistently associated with certain pathophysiological changes in IBS have not been identified.

In the present study, two common IBS rat models (MS alone and multiple early adversity modeling) were established. High-throughput microbial community analysis of their colon contents was then performed to identify the influence of modeling procedures on bacterial community and investigate whether different modeling procedures lead to consistent patterns of gut microbiota compositions. In addition, the possible mediating effects of gut microbial composition were explored between early adversity and altered pathophysiological variants.

Methods

Animals and ethics statement

Pregnant Sprague Dawley rats were obtained from SPF Biotechnology Co., Ltd. (Beijing, China), and arrived in the lab on day 15 of their pregnancies. Rats were maintained in an environment with controlled lighting (12: 12 h light–dark cycle) at 22–24 °C and allowed free access to water and a standard diet. Pregnant rats were checked daily for litter, and the day of birth was defined as the postnatal day (PND) 0 for the litter. After delivery, the dams and litters were housed together until weaning (PND22). After weaning on PND22, male and female rats were separated, and three animals were kept in a single cage. Only male rats were included in this study to avoid the effects of hormones (n = 7 for each group). The experimental animal protocol was approved by the Ethics Committee of Minzu University of China (ECMUC2019008CO).

Study design

Pregnant rats were randomly divided into three groups: the control (CTRL), MS, and multiple early adversity model (MAM) groups. Rats were left undisturbed in the CTRL group except for routine cage cleaning every two days. In the MS group, the maternal separation was performed once daily during PND1–PND21. Separations were conducted between 09:00 and 12:00 a.m. by placing the offspring in plastic cages placed on the top of heating pads set at 30–33 °C in a separate room from the main holding room. In the MAM group, the maternal separation was performed once daily during PND1–PND21, acetic acid instillation was performed once daily during PND15–PND28, and the rats were restrained for three hours every day during PND29–PND42. Rats are typically reared by PND43. Rats were anesthetized for acetic acid instillation, and 0.5% acetic acid was slowly instilled into the colon with a

plastic tube (external diameter of 1 mm) at a depth of 6 cm from the anus and then maintained for 3 min. The acetic acid dose on the first day was 0.2 ml and was increased by 0.1 ml every day. After increasing to 0.5 ml, enemas were administered at a dosage of 0.5 ml every day. To model restraint stress, rats were placed in a transparent plastic restraint cylinder, in which they could move forward and backward but could not turn around for 3 h. All rats were weighed once a week after PND22. Abdominal withdrawal reflex (AWR) scores in response to colorectal distension were measured at PND78, and fecal water content was measured at PND82, followed by euthanization and recovery of distal colon contents at PND84.

Visceral sensitivity assessment

Visceral hypersensitivity was evaluated using the AWR score at PND78, as described previously [14, 15]. Briefly, a flexible balloon (6 cm) constructed from a surgical glove finger was attached to Tygon tubing and inserted 8 cm into the descending colon via the anus and held in place by taping the tubing to the tail. Rats were placed in transparent plastic cubicles (8 × 10 × 20 cm) and allowed to adapt for 10 min. colorectal distension was then established by rapidly inflating the balloon to constant pressure. Pressure was measured using a sphygmomanometer connected to a pressure transducer. The balloon was inflated to various pressures (20, 30, 40, 50, 60, 70, 80, 90, and 100 mmHg) for a 10 s stimulation period followed by a 1 min rest period. Behavioral responses to colorectal distension were measured by visual AWR observations by two blinded observers, and AWR score assignments were as follows: 0, normal behavior without response; 1, slight head movement without abdominal muscle contraction; 2, contraction of abdominal muscles; 3, lifting of abdominal wall; and 4, body arching and lifting of pelvic structures. The minimal distention pressures that evoked abdominal visceromotor responses corresponding to AWR scores of 2, 3, and 4 were recorded by both observers and defined as the distension threshold.

Fecal water content

Fresh feces expelled by each rat were collected on PND82 in 5 ml sterile tubes and immediately weighed, followed by drying them in an oven to calculate fecal water content with the following formula: water content% = [wet weight of the feces (g) - dried weight of the feces (g)]/wet weight of the feces (g) × 100%.

Collection of the samples

All of the rats were anesthetized using Isoflurane and were sacrificed by exsanguinations after blood collected from the posterior vena cava on PND84 as described in previously[16]. Colorectal tissue samples and fecal contents were collected just after sacrifice. Fresh fecal pellets were collected directly from the distal colon, placed in 5 ml sterile tubes, and preserved with 4 M guanidine thiocyanate solution for further microbial community characterization. All rats were then sacrificed by blood sampling. Fecal samples were immediately frozen at -20 °C, then transferred to the laboratory within 24 h and stored at -80 °C prior to DNA extraction and characterization of the microbiota using 16S rRNA gene sequencing.

Histological examination of colonic inflammation

To examine the extent of colonic inflammation, histological tissues were collected at PND84. The tissues were fixed in formalin for 12 h, and pathological sections were generated at a thickness of 5 μm and processed for hematoxylin-eosin staining. The slides were observed under an optical microscope, and inflammatory cells were observed by a pathologist blinded to the treatments.

Microbiota sequencing

Total bacterial DNA was extracted from samples using the Power Soil DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer's protocol. DNA quality and quantity were assessed by the ratios of 260 nm/280 nm and 260 nm/230 nm. Then DNA was stored at $-80\text{ }^{\circ}\text{C}$ until further processing.

The V3-V4 region of the bacterial 16S rRNA gene was amplified with the common primer pair (Forward primer, 5'- ACTCCTACGGGAGGCAGCA-3'; reverse primer, 5'- GGACTACHVGGGTWTCTAAT-3') combined with adapter sequences and barcode sequences. PCR amplification was performed in a total volume of 50 μl , which contained 10 μl Buffer, 0.2 μl Q5 High-Fidelity DNA Polymerase, 10 μl High GC Enhancer, 1 μl dNTP, 10 μM of each primer and 60 ng genome DNA. Thermal cycling conditions were as follows: an initial denaturation at $95\text{ }^{\circ}\text{C}$ for 5 min, followed by 15 cycles at $95\text{ }^{\circ}\text{C}$ for 1 min, $50\text{ }^{\circ}\text{C}$ for 1 min and $72\text{ }^{\circ}\text{C}$ for 1 min, with a final extension at $72\text{ }^{\circ}\text{C}$ for 7 min. The PCR products from the first step PCR were purified through VAHTSTM DNA Clean Beads. A second round PCR was then performed in a 40 μl reaction which contained 20 μl 2 \times Phusion HF MM, 8 μl ddH₂O, 10 μM of each primer and 10 μl PCR products from the first step. Thermal cycling conditions were as follows: an initial denaturation at $98\text{ }^{\circ}\text{C}$ for 30 s, followed by 10 cycles at $98\text{ }^{\circ}\text{C}$ for 10 s, $65\text{ }^{\circ}\text{C}$ for 30 s min and $72\text{ }^{\circ}\text{C}$ for 30 s, with a final extension at $72\text{ }^{\circ}\text{C}$ for 5 min. Finally, all PCR products were quantified by Quant-iT™ dsDNA HS Reagent and pooled together. High-throughput sequencing analysis of bacterial rRNA genes was performed on the purified, pooled sample using the Illumina HiSeq 2500 platform (2 \times 250 paired ends) at Biomarker Technologies Corporation, Beijing, China.

Bioinformatic analyses

Raw 16S rRNA gene sequence reads were demultiplexed and then trimmed, merged, and filtered using the Usearch v11.0.667linux32 program using the UNOISE pipeline. Paired-end sequence reads with an expected error (ER) value > 1.00 were removed during filtering. Sequences were dereplicated and classified into zero-radius operational taxonomic units (ZOTUs) after denoising (error-correcting) and chimera checking. The RDP classifier was then used to assign taxonomic classifications to 16S rRNA gene sequences with a confidence threshold of 80%. Representative sequences from the ZOTUs were aligned and further filtered to create a phylogenetic tree using the QIIME pipeline. The ZOTU table was subsampled randomly to the number of reads in the smallest sample (19,511) in order to obtain equal sequencing depths among samples. The functional profiles of the microbial communities were also predicted using the open-source R package Tax4Fun and the Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog dataset based on 16S rRNA gene profiles.

Statistical analyses

Categorical variables were evaluated as frequencies and percentages. Chi-squared tests and Fisher's exact tests were then used to assess statistical associations between variables. Numerical variables were expressed as means \pm standard deviations (SD). One-way analysis of variance (ANOVA) tests were used to compare differences in measured parameters among groups.

The Chao1, observed OTU numbers (obs_otus), phylogenetic diversity (PD_whole_tree), Shannon and Simpson indices were used to evaluate community richness and diversity of the gut microbial communities. Principal coordinates analysis (PCoA), adonis tests, and db-RDA analyses were used to evaluate differences among communities based on beta diversity metrics including the unweighted and weighted Unifrac distance metrics. Finally, the Linear discriminant analysis Effect Size (LEfSe) analysis with a threshold of ± 2 were used to explore significant differences among treatment groups using the relative abundance data of taxa and the KEGG-based ortholog and pathway abundances.

Mediation analyses

To further assess the contribution of alteration in gut microbiota to the association between early adversity and the altered physiological factors in adulthood including body weight, visceral hypersensitivity, and fecal water content, mediation analyses were conducted using the "mediation" package in R. Bacterial taxa were considered as mediators in mediation analysis to evaluate the apparent causal effects of early adversity on the physiological factors in adulthood. The total effect (TE) and average causal mediation effect (ACME) values were determined between predictors and outcomes via the mediators, and the percent of mediation effect was estimated using a two-tailed P-value cutoff of < 0.05 to assess statistical significance.

Results

Model assessment

Visceral sensitivity is one of the most important pathophysiological characteristics of IBS. Here, we used colonic stretch stimulation AWR scores to evaluate the visceral sensitivity of treatment groups. Both MAM and MS groups exhibited increases in visceral hypersensitivity to colorectal distension compared to control group individuals (Fig. 1a, b). The MAM group showed even higher visceral hypersensitivity than did the MS group. The AWR scores were significantly higher in the MAM rats compared to those of the control group at pressures of 40, 50, 60, 70, and 80 mmHg and in MS rats at pressures of 50 and 60 mmHg. The threshold values for the MS group for AWR score 2 were significantly lower than those of the control group, while the threshold values of AWR scores 2, 3, and 4 were all significantly lower in the MAM group compared to the control group. These results thus indicate that visceral hypersensitivity was induced in both the MAM and MS groups.

No statistically significant differences were observed in body weights between different model groups, although the body weights of rats in the MAM group were generally higher than those of the other two groups (Fig. 1c). Fecal water content was measured to evaluate differences in general stool features, and a significant decrease in the fecal water content of MAM rats was observed compared to control rats

(11.6% \pm 4.3% vs 20.1% \pm 4.7%, $p = 0.0059$, $n = 7$). No such effect was observed between the control and MS rats (18.3% \pm 4.8%) (Fig. 1d). Histological examination did not indicate remarkable inflammatory features in all groups on PND84, suggested that the two modeling methods did not cause obvious intestinal organic lesions.

Overall assessment of intestinal microbiota variation

A total of 1,395,522 paired-end 16S rRNA gene sequence reads covering the V3–V4 hypervariable regions were generated from 21 samples, with an average of 66,453 (\pm 13,675.83 SD) reads per sample, and ranging from 45,296 to 80,200 per sample. After trimming and filtering, a total of 1,058,795 high-quality paired-end reads remained. Further denoising resulted in the removal of 1.091 chimeric sequences.

A total of 4,498 zero-radius operational taxonomic units (ZOTUs) were ultimately generated and further used for data analysis. All ZOTUs were annotated by comparison against the RDP database. Among these, 4,453 ZOTUs were annotated at the phylum levels, comprising 7 phyla. In addition, a total of 4,401, 4,335, 4,125 and 1,940 ZOTUs were annotated at the class, order, family, and genus level comprising 13 classes, 14 orders, 21 families, and 33 genera, respectively. Bacteroidetes (48.1% of all sequence reads) and Firmicutes (47.7%) were the most dominant taxa at the phylum level, with Bacteroidia (48.1%), Clostridia (32.1%), and Bacilli (12.1%) dominating at the class level. Bacteroidales (48.1%), Clostridiales (32.1%), and Lactobacillales (12.1%) were dominant at the order level, while Porphyromonadaceae (23.4%), Prevotellaceae (23.4%), Lachnospiraceae (16.8%), Lactobacillaceae (12.1%) and Ruminococcaceae (11.3%) were the most dominant taxa at the family level (considering those comprising > 10% of the reads). A total of 31 genera exhibited an abundance of greater than 0.1%. Among these, Prevotella (22.50%) and Lactobacillus (12.10%) were the most dominant genera among those comprising > 5% of the overall reads.

Comparison of microbial community structures among different model groups

Several different richness and diversity indices were calculated for the microbial communities of different treatment groups after randomly subsampling the ZOTU table to 18,429 reads per sample, which was the size of the smallest sample. No significant differences were observed for all five diversity indices among the different model groups (Fig. 2). To examine bacterial community structural differences among model groups, two distance matrices were created from the subsampled ZOTU table based on the unweighted Unifrac distance metric and the weighted Unifrac distance metric. Principal coordinate analysis (PCoA) of beta-diversity differences based on the unweighted UniFrac distance metric indicated that the microbial communities from the three model groups were completely differentiated into three different clusters. Furthermore, PCoA based on the weighted Unifrac distance metric also indicated the partial separation of the microbiota communities based on the different model groups (Fig. 3a, b).

Comparison of intra-group and inter-group distances for each model group based on the unweighted Unifrac distance metric suggested that the extent of variation within the MS and MAM groups was

significantly higher than that of the control group, with no significant difference between the two model groups, indicating that the modeling process not only altered the structure of the gut microbiota, but the extent of instability of the gut microbiota also increased significantly.. Pairwise comparisons of the distances between the groups indicated that the distance between the two model groups was significantly higher than the distances between either the MS or MAM communities to those of the control group, suggesting that the gut microbiota structure varies in different directions based on the modeling methodology (Fig. 3c).

In contrast to the comparisons among groups using the unweighted Unifrac metric, comparison of the intra-group and inter-group distances of each model group based on the weighted Unifrac distance metric did not yield statistically significant differences (Fig. 3d). In other words, significant differences generated from the distance matrix generated from the presence/absence data are masked after taking into account ZOTU abundances. Thus, the differences in low-abundance ZOTUs were minimal when species abundance weighting is considered, suggesting that the observed differences between different model groups are more influenced by less abundant species.

Adonis tests and db-RDA analyses of both distance matrices indicated the presence of significant associations between the different model groups and bacterial community structures (Table 1). In addition, several parameters associated with visceral hypersensitivity, body weight and fecal water content, were also significantly associated with bacterial community structural differences based on the unweighted Unifrac distance metric.

Table 1

Adonis and db-RDA analysis results investigating the association between gut microbiota and models inducing exposure and other variants regarding the visceral hypersensitivity, body weight, and fecal water content based on the unweighted and weighted Unifrac distance metrics.

Adonis test				
Variants	Unweighted Unifrac		Weighted Unifrac	
	p	r ²	p	r ²
Model group (Ctrl, MAM and MS)	0.001	0.21127	0.047	0.1743
db-RDA analysis				
Variants	Unweighted Unifrac		Weighted Unifrac	
	p	Adjusted r ²	p	Adjusted r ²
Model group (control, MAM and MS)	0.001	0.123634	0.048	0.069004
AWR score at 20 mmHg	0.762	-0.00996	0.249	0.539272
AWR score at 30 mmHg	0.995	-0.01763	0.743	0.848851
AWR score at 40 mmHg	0.15	0.009689	0.113	0.406235
AWR score at 50 mmHg	0.01	0.029906	0.138	0.433366
AWR score at 60 mmHg	0.003	0.034062	0.381	0.642073
AWR score at 70 mmHg	0.008	0.02841	0.509	0.721735
AWR score at 80 mmHg	0.004	0.031782	0.676	0.81294
AWR score at 90 mmHg	0.03	0.019883	0.594	0.766233
Distention threshold for AWR score 2	0.005	0.032491	0.099	0.029877
Distention threshold for AWR score 3	0.013	0.025983	0.454	-0.00282
Distention threshold for AWR score 4	0.045	0.018325	0.572	-0.00857
Body weight at Day 29	0.464	-0.00095	0.802	-0.01954
Body weight at Day 36	0.351	0.002996	0.429	-0.0019
Body weight at Day 43	0.06	0.014273	0.293	0.008548
Body weight at Day 50	0.016	0.022061	0.262	0.008548
Body weight at Day 57	0.067	0.015648	0.446	-0.00092
Bold values indicate P < 0.05				

Adonis test				
Body weight at Day 71	0.024	0.023843	0.283	0.009416
Body weight at Day 84	0.054	0.016281	0.591	-0.00916
Fecal Water content at Day 82	0.045	0.019433	0.282	0.009067
Bold values indicate P < 0.05				

Differences in microbiota composition and functionalities among model groups

Linear discriminant analysis effect size (LEfSe) analysis was performed to identify differences in the taxonomic composition of microbial communities that were associated with different model groups (Fig. 4a, b). Comparison of the control and MAM groups indicated the presence of 14 taxa with significant differences. Specifically, the genus *Vampirovibrio* and the family, order, class, and phylum to which it belongs as well as *Alloprevotella* were more abundant in the MAM group. In contrast, *Clostridium sensu stricto* and the family, order, and class to which it belongs along with the *Butyricimonas*, *Turicibacter*, *Ruminococcus*, and *Butyricoccus* genera were more abundant in the control group samples.

A total of 26 taxa exhibited significantly different abundances in the comparison between the control and MS groups. *Corynebacterium* and the family to which it belongs as well as *Alloprevotella* were more abundant in the MS group. The genus *Rothia* and the family to which it belongs along with *Elusimicrobium* and the family, order, class, and phylum to which it belongs were more abundant in the control group. Other taxa that were more abundant in the control group communities included *Clostridium sensu stricto* and the family to which it belongs, *Ruminococcus*, *Romboutsia* and the family to which it belongs, *Butyricoccus*, *Clostridium IV*, *Allobaculum* and the family, order, and class to which it belongs, *Turicibacter*, and lastly, *Parasutterella* and the family, order, and class to which it belongs.

The abundance of six taxa was significantly higher in both model groups compared to the control group and included *Alloprevotella*, *Butyricoccus*, *Turicibacter*, *Ruminococcus*, and *Clostridium sensu stricto* in addition to the family it belongs to.

The functional capacities of the bacterial communities were predicted with the Tax4Fun R package using the SILVA database v.123 as a reference. A total of 5,971 KEGG orthologs (KO) and 261 metabolic pathways were predicted from these inferences. LEfSe analysis indicated that 10 KOs had significantly different inferred abundances between the control and MS groups (Fig. 4c). Eight KOs also exhibited significant differences when comparing the control and MAM groups (Fig. 4d). At the functional pathway level, eight pathways had significantly different inferred abundances among the control and MS groups and between the control and MAM groups, respectively (Fig. 4e, f).

Mediation analyses

Mediation analyses were performed to investigate the possibility that gut microbiota composition mediates the association between early adversity and altered physiological variants (Table 1). Among the 14 taxa identified at different taxonomic levels described above that correlated with MAM exposure, variable abundances of *Butyricimonas* and *Butyricicoccus* were found to potentially mediate MAM exposure-induced abnormalities in visceral hypersensitivity (Table 2). Among the 26 taxa correlated with MS exposure, *Corynebacterium* variation was found to potentially mediate the association of MS exposure and the visceral hypersensitivity variable corresponding to the distention threshold score of 2 for AWR.

Table 2

Gut microbiota composition mediates the association between early adversity and altered physiological variants.

MAM exposure correlated alteration							
Taxa	Physiological variants	ACME		Total		Prop. Mediated	
		beta	p	beta	p	%	p
<i>Butyricimonas</i>	AWR score at 50 mmHg	1.3	0.150	2.46	< 0.001	52.44	0.030
<i>Butyricicoccus</i>	AWR score at 80 mmHg	0.5	0.170	0.97	< 0.001	51.41	0.026
MS exposure correlated alteration							
Taxa	Physiological variants	ACME		Total		Prop. Mediated	
		beta	p	beta	p	%	p
<i>Corynebacterium</i>	Distention Threshold for AWR score 2	-8.27	0.026	-10	0.018	80.79	0.044
Note: ACME; average causal mediation effect. Prop. Mediated: proportion of mediation effect							

Discussion

In the present study, we established two IBS rat models using maternal separation (MS) and multiple early adversities (MAM) modeling and investigated their intestinal microbiota. Rats from both IBS models exhibited visceral hypersensitivity compared with the control group rats. However, the two model groups exhibited differences in the extent of visceral pain sensitivity and fecal water content. The microbial community structure from rats with the two visceral hypersensitivity models exhibited significant differences compared to the controls, while communities from the two model groups also exhibited

significant differences between them. Furthermore, community functional predictions suggested that rats from the two models exhibited different abundances of metabolisms and pathways associated with structural changes. Several common and distinct characteristic differences were also observed between rats from the two model groups. Some of these bacterial taxa may play a mediating role in the association between early adversity and specific pathological changes of IBS.

Several studies have evaluated early adversity events and alterations in intestinal flora diversity. Some of these have suggested that exposure to early adversity can affect the gut microbiota, manifesting as a reduction in microbial diversity and richness [17, 18]. However, other studies have reported no changes in diversity in these scenarios [19]. Comparison of five alpha-diversity indices between different model groups did not yield any significant differences in this study. The apparent discrepancies in associations between microbial community alpha-diversity and early adversity could be explained by differences in study designs and environments, or even by factors of the same study including the type of early adverse events that were used and the timing of material collection, which can lead to variable results. For example, Zhou et al. [20] evaluated the composition and diversity of the gut microbiota of MS, PI-IBS, and control rats at three different time points. No differences in alpha diversity were observed among the three groups in the third week and the eighth week, only the MS group exhibited a significant decrease in the Simpson and Shannon indices at week 12, while the PI-IBS group did not exhibit significant differences.

Inter-individual variation within host cohorts can also reflect the stability of gut microbiota. Healthy and homeostatic gut microbiota share higher similarities in the composition and richness of microbial taxa, while those with imbalanced microbiota exhibit drastic changes and tend to have higher inter-individual variation. Inter-group distances in the two model cohorts of this study exhibited significantly higher variation than those of the control group (Fig. 3c). Thus, induced early negative events resulted in a significant increase in the instability of gut flora. This result supports the hypothesis that exposure to early adversity can induce significant dysbiosis of fecal microbiota [13]. The PCoA analyses based on the unweighted UniFrac distance metric indicated that the microbiota from the three model groups could be completely divided into three disparate clusters. Further comparison of intra-group distances indicated that the distances between the two model groups were significantly higher than the distances between those groups and control rat communities. These results indicated that the flora structure of the two visceral hypersensitive model rats significantly changed relative to the controls, and that obvious differences were present between the two model groups.

To further evaluate the effects of different modeling processes on gut floral compositions, the composition and functionality differences of the communities among the model and control groups were further investigated. The two model groups exhibited several significantly altered taxonomic group abundances compared to the control group. The bacterial taxonomic differences unique to the MAM group comprised five taxa, where the genus *Butyricimonas* decreased significantly and *Vampirovibrio* along with the family, order, class, and the phylum to which it belongs all increased significantly compared to the control group. Bacterial taxonomic differences unique to the MS group involved 20 taxa,

where *Corynebacterium* and the family to which it belongs increased significantly, while many taxa decreased significantly compared to the control group including *Rothia* and the family to which it belongs; *Elusimicrobium* and the family, order, class, and phylum to which it belongs; *Romboutsia* and the family to which it belongs; *Clostridium IV*; *Allobaculum* and the family, order, and class to which it belongs; in addition to *Parasutterella* and the family, order, and class to which it belongs.

Some studies have suggested an association and role for *Butyricimonas* and *Vampirovibrio* with IBS, consistent with our results [21, 22]. *Butyricimonas* have been reported to produce butyrate that reduces inflammation and helps maintain healthy gut functioning. Therefore, decreased abundances of *Butyricimonas* in gut systems may play an inflammation-based role in the pathogenesis of IBS [21]. *Vampirovibrio* has been reported as significantly more abundant in trinitrobenzene sulfonic acid-induced PI-IBS rat model individuals, and its lowered abundance has been associated with the alleviation of symptoms for visceral hypersensitivity after treatment [22].

The abundances of *Parasutterella* species and their related taxonomic groups were significantly lower in the MS group compared to the control group. These results contrast with those of Chen et al. [23]. The latter group found that *Parasutterella* was abundant in the stool of IBS patients. However, our results are consistent with other studies indicating that *Parasutterella* abundances are associated with beneficial outcomes. Zhang et al. [24] reported that *Parasutterella* abundances were significantly lower in CDI patients and asymptomatic carriers than in healthy controls, while Kreutzer et al. [25] also reported a negative correlation between *Parasutterella* abundances and high-fat diet-induced hypothalamic inflammation. Although the association of *Parasutterella* and different health outcomes is clearly controversial, these observations nevertheless indicate that *Parasutterella* has a role in modulating the microbial activities and host responses in certain disease states and, thus, necessitate further investigation.

The associations identified here between *Corynebacterium*, *Rothia*, *Elusimicrobium*, *Romboutsia*, *Clostridium IV*, and *Allobaculum* with maternal separation stress have not been reported elsewhere. Consequently, their associations with IBS require additional confirmation in further studies.

Predictive analysis of microbiota functionalities also suggested that changes in the microbial composition of the two model groups corresponded to functional differences associated with diverse metabolisms and pathways. Specifically, we observed higher abundances of the pathways in the MS group compared to the control group involving the citrate (TCA) cycle (ko00020), protein export (ko03060), carbohydrate digestion and absorption (ko04973), and NF-kappa B signaling (ko04064), along with lower abundances of alanine, aspartate, and glutamate metabolism (ko00250), non-ribosomal peptide structures (ko01054), tyrosine metabolism (ko00350), and porphyrin and chlorophyll metabolism (ko00860) (Fig. 4e). In the MAM group, pathways that were significantly more abundant than in the control group included epithelial cell signaling in *Helicobacter pylori* infection (ko05120), galactose metabolism (ko00052), glutathione metabolism (ko004800), and the Fanconi anemia pathway (ko03460), while significantly lower pathways included morphine addiction (ko05032), thiamine

metabolism (ko00730), methane metabolism (ko00680), and bacterial chemotaxis (ko02030) (Fig. 4f). These results together support the hypothesis that the complex metabolic interactions between microbes and host tissues are critical to the development of IBS symptoms [26]. Furthermore, they suggest that different modeling strategies may result in IBS symptoms by inducing changes in bacterial flora via different metabolic pathways.

In the present study, the microbial composition of MS group gut communities was associated with the enhanced functional capacity for the NF-kappa B signaling pathway. This result is consistent with previous studies indicating that neonatal MS induces VH and visceral pain in rats that is mediated by activation of TLR4 and the NF- κ B signaling pathway [27, 28] and further suggests that changes in the gut microbiomes caused by MS are involved in NF- κ B activation in a rat model of IBS.

The microbial composition of the MAM group was also associated with functions related to morphine addiction. Several opioid, cannabinoid, and 5-HT receptors play critical roles in visceral hypersensitivity and abnormal gut motility associated with IBS and also contribute to the maladaptive effects of chronic morphine analgesia including hyperalgesia, tolerance, and dependence [29–31]. Therefore, we speculate that the visceral hyperalgesia induced by MAM modeling may be mediated by interactions between microorganisms and their host receptors. Further investigations incorporating metagenomic analyses at the gene level could help us understand how gut microorganisms affect visceral pain sensation in IBS.

Despite the significant differences in microbial composition and functionalities among the two model groups, six taxa were common in both the model groups relative to the control group communities. Specifically, *Alloprevotella* were more abundant in both the MS and MAM groups, while *Butyricoccus*, *Turicibacter*, *Ruminococcus*, and *Clostridium sensu stricto* along with the family it belongs to were less abundant in both the MS and MAM groups relative to controls. These results were consistent with those from previous studies. For example, *Alloprevotella* were significantly more abundant in model rats with early adversity stimuli compared to controls in one study [13]. In addition, *Turicibacter* were significantly less abundant in IBS-D patients [32]. *Ruminococcus*, *Butyricoccus*, and *Clostridium sensu stricto* are all short-chain fatty acid-producing genera that are considered beneficial for gastrointestinal tract functioning [33–35]. Furthermore, *Ruminococcus* spp. were less abundant in IBS patients relative to controls in another study [36]. Moreover, several studies have shown that the abundances of *Butyricoccus* were lower in ulcerative colitis patients and patients with inflammatory disease in general [35, 37, 38]. These microbial signatures exhibited similar abundance patterns in both early adversity modeling procedures, indicating that the responsive species may be involved in common characteristics of the complex pathophysiological changes that cause IBS. Thus, the variation in these taxa could provide clues to the etiology of IBS and potentially lead to novel therapies.

The changes in the gut microbiota and pathophysiological indicators described in this study were both induced by early adversity events. To further assess the contribution of altered gut microbial compositions to the association between early adversity and altered physiological outcomes, mediation analysis was performed to investigate the potential mediating effects of microbial populations on

pathophysiological changes induced by early adversity events. *Butyrivimonas* and *Butyrivibrio* populations were predicted to partially mediate MAM exposure-induced visceral hypersensitivity, while *Corynebacterium* was predicted to partially mediate MS exposure-induced visceral hypersensitivity. Thus, these genera might play critical roles in the development of early adversity-induced visceral hypersensitivity and may also represent targets for understanding the mechanisms underlying microbe-mediated visceral hypersensitivity.

A limitation of the present study is the lack of proven causal relationships between alterations in microbiota characteristics and visceral pain behaviors, despite the abundance of evidence suggesting that microbial compositions play roles in the development of visceral hypersensitivity. Future studies could deconvolute these effects by focusing on manipulating the gut microbiota through prebiotics, probiotics, or synbiotics to reverse the deleterious effects of early adversarial events on visceral pain or other IBS-related behaviors.

Conclusions

To conclude, the present study demonstrated that MS stress and multiple early adversity stress procedures could induce distinct alterations of the gut microbiota that also differ in stability, composition, and functionality in rat models. The large differences in gut microbiota between the two model groups coincide with the heterogeneity and complexity of IBS pathogenesis and highlight the importance in evaluating gut microbiota characteristics in IBS research while also systematically considering potential modeling procedural differences. The microbial compositional and functional differences identified in this study prompt further investigation of mechanisms of early adversity induced IBS related pathophysiological changes.

Abbreviations

Abdominal withdrawal reflex (AWR)

Average causal mediation effect (ACME)

Control (CTRL)

Colorectal distension (CRD)

Expected error (ER)

Kyoto Encyclopedia of Genes and Genomes (KEGG)

KEGG orthologs (KO)

Linear discriminant analysis Effect Size (LEfSe)

Maternal separation (MS)

Multiple early adversity model (MAM)

One-way analysis of variance (ANOVA)

Principal coordinates analysis (PCoA)

Postnatal day (PND)

Standard deviations (SD)

Total effect (TE)

Zero-radius operational taxonomic units (ZOTUs)

Declarations

Ethics approval and consent to participate

This study was conducted with approval from the Ethics Committee of Minzu University of China (ECMUC2019008CO).

Consent for publication

Not applicable.

Availability of data and materials:

The datasets generated and/or analysed during the current study are available in the SRA database repository, Accession No: PRJNA609270.

Competing interests

The authors declare no potential conflicts of interest.

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Authors' contributions

WE, SJ and LY performed experiments; WE and SJ analysed data; PL and LY obtained funding for the project and planned experiments ; WE, PL and LY and prepared the manuscript draft for submission; All authors read and approved the manuscript.

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Not applicable

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Figures

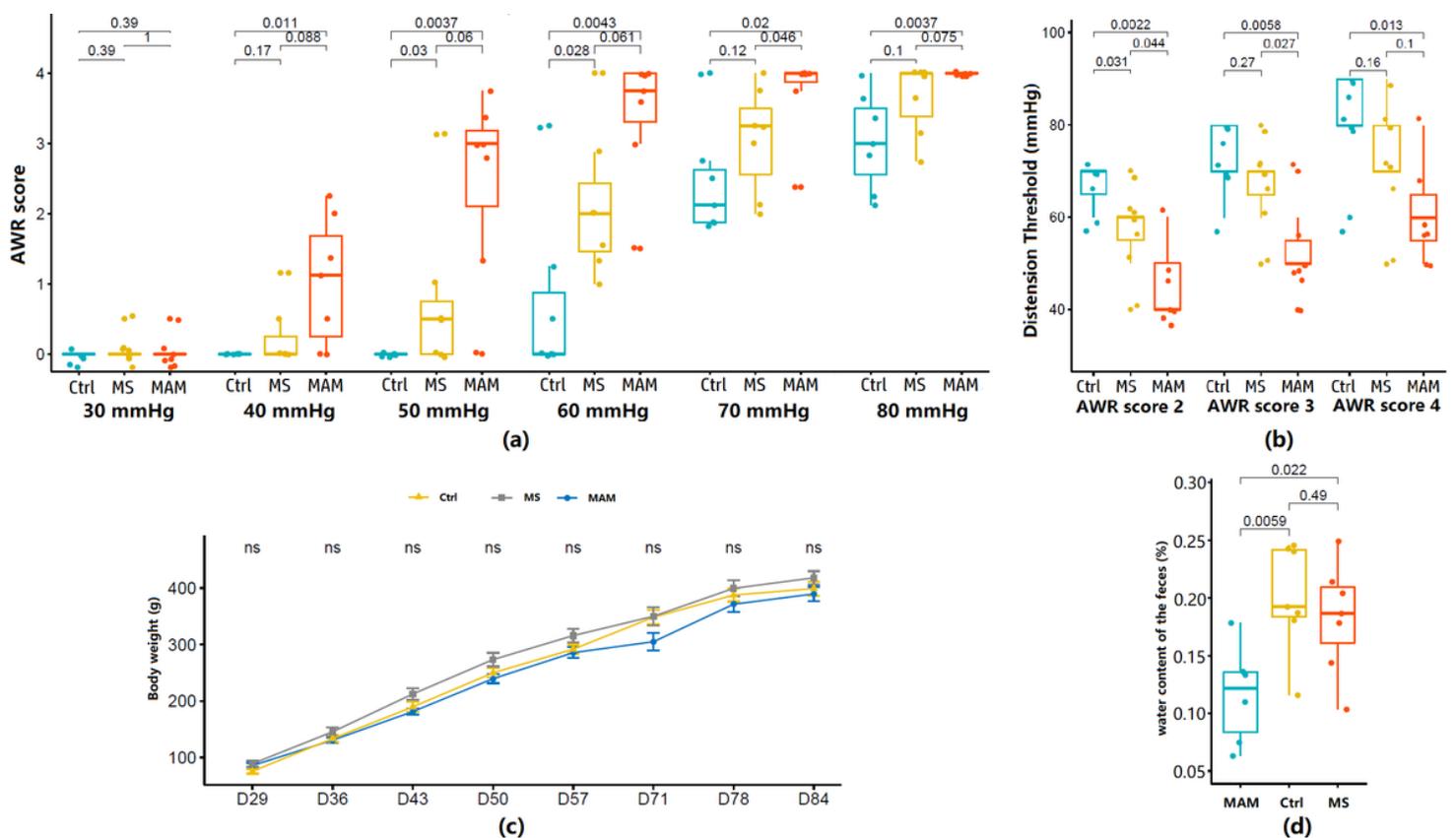


Figure 1

Comparison of visceral hypersensitivity, body weight, and fecal water content in rats of different model groups (n=7 for each group). a) Abdominal withdrawal reaction (AWR) scores in response to graded

colorectal distension (CRD). b) Distension threshold values for AWR scores of 2, 3, and 4. c) Body weight variation of rats. d) Fecal water content for rats in the control, MS, and MAM groups.

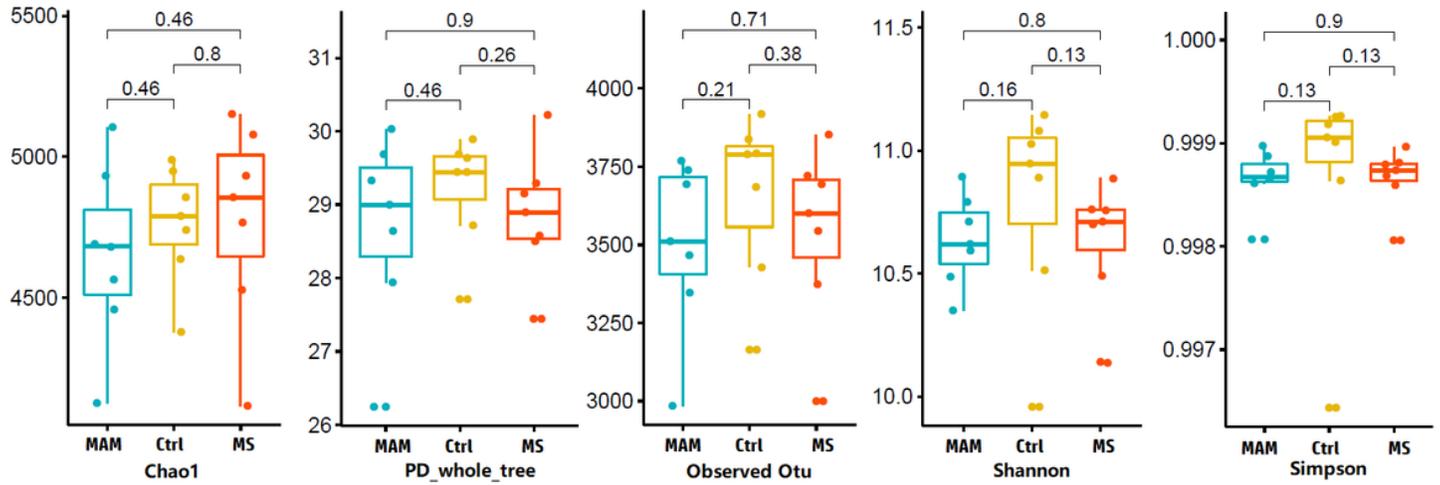


Figure 2

Alpha diversity values for gut microbial communities among different model group rats.

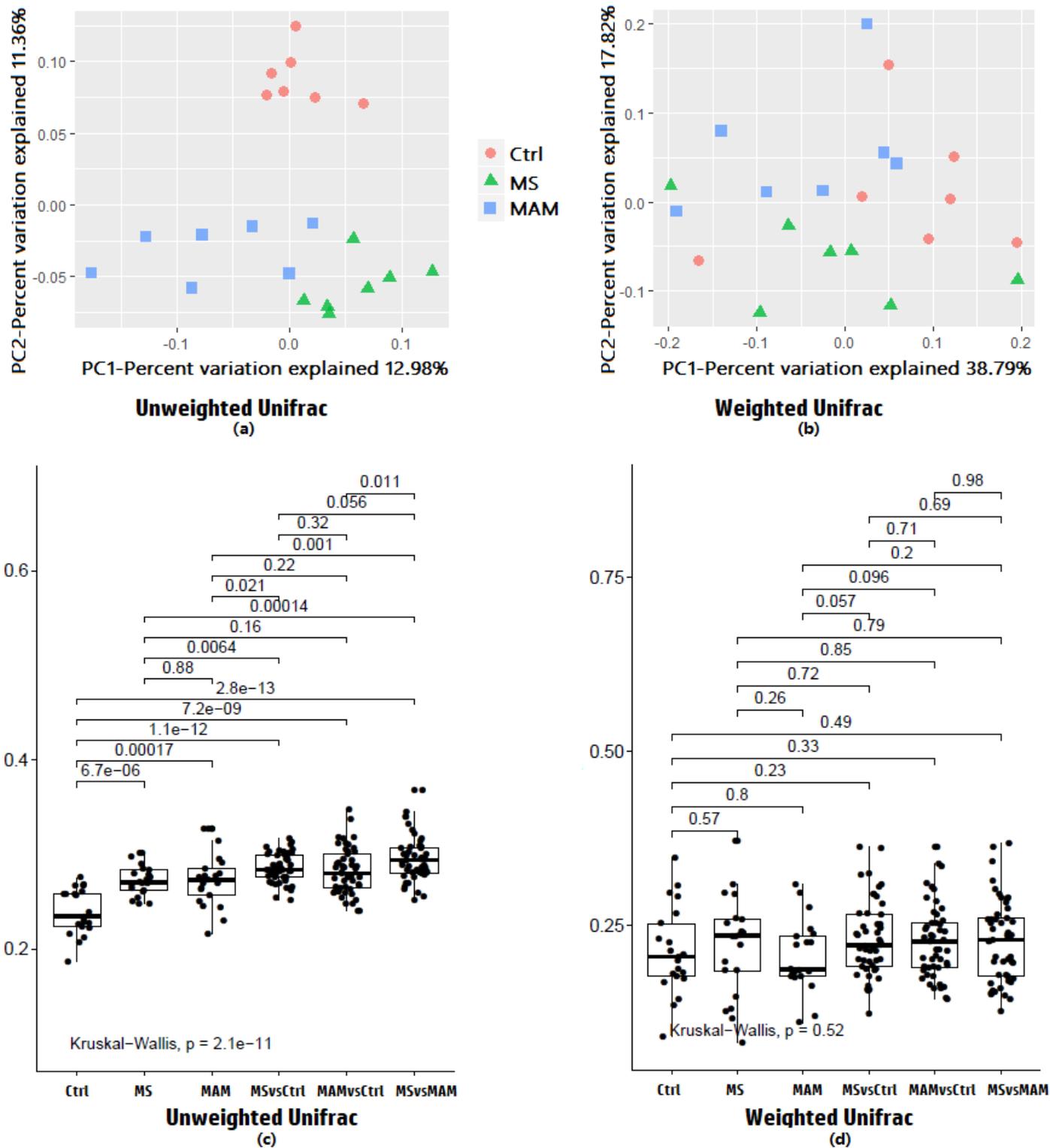


Figure 3

Beta diversity analysis among and within different model groups. a) 2D PCoA plots showing differences in the gut microbiota of rats from different model groups based on the unweighted Unifrac distance metric. b) 2D PCoA plots showing differences in the gut microbiota of rats from different model groups based on the weighted Unifrac distance metric. c) Comparison of inter-individual variation between and within different model groups based on the unweighted Unifrac distance metric. d) Comparison of inter-

individual variation between and within different model groups based on the weighted Unifrac distance metric.

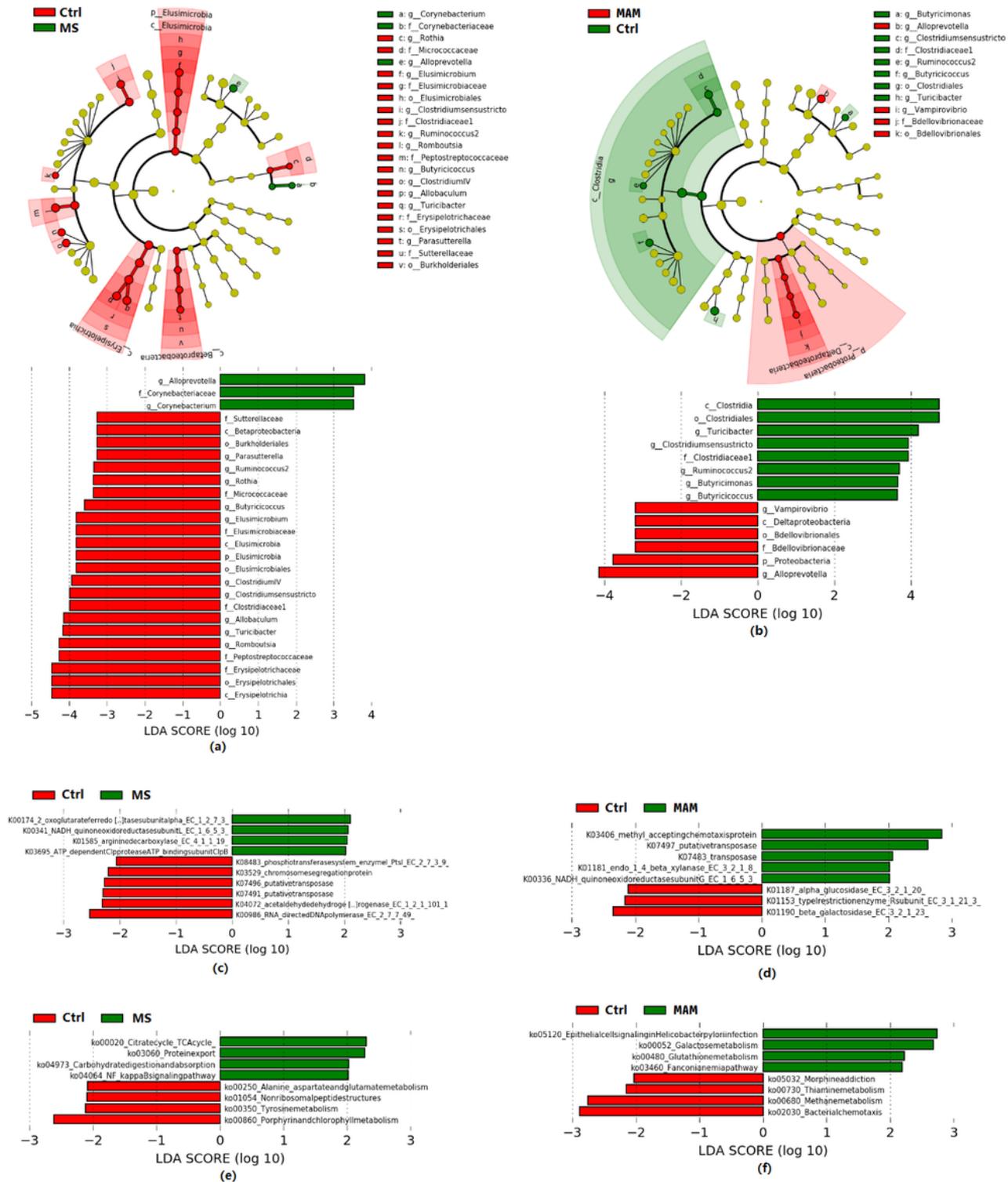


Figure 4

Linear discriminant effect size (LEfSe) analyses comparing differentially abundant taxa and the KEGG-based ortholog functions and pathways between control and MS group communities and control and MAM group communities. a), b) Differences in gut microbiota taxa between different model groups. c), d)

Differences of KEGG-based ortholog (KO) inferred functions in gut microbiota between different model groups. e), f) Differences in KO pathways predicted from gut microbiota compositions between different model groups.

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