World Journal of Gastroenterology

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DOI: 10.3748/wjg.v22.i22.5211

*World J Gastroenterol* 2016 June 14; 22(22): 5211-5227
ISSN 1007-9327 (print) ISSN 2219-2840 (online)


ORIGINAL ARTICLE

Basic Study

# Visceral hypersensitive rats share common dysbiosis features with irritable bowel syndrome patients

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Author contributions: Zhou XY, Li M and Li YQ designed the research; Zuo Xl and Li X performed the research; Li M analyzed the data; Li M and Zhou XY wrote the paper; Zhou XY, Hou XH, Cong YZ and Li YQ made critical revisions to this manuscript; Zhou XY and Li M contributed equally to this work.

Supported by National Natural Science Foundation of China, No. 81330012 and No. 81170352.

Institutional animal care and use committee statement: All experiments were reviewed and approved by the Ethical Committee and Institutional Animal Care and Use Committee of Qilu Hospital (KYLL-2013-005), and the methods were performed in strict accordance with the Animal Management Rules of the Chinese Ministry of Health.

Conflict-of-interest statement: The authors declare no conflict of interest.

Data sharing statement: Data will be available for scientific sharing. The sra number for the sequencing data is pending.



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Received: December 21, 2015
Peer-review started: December 22, 2015
First decision: January 13, 2016
Revised: March 1, 2016
Accepted: March 18, 2016
Article in press: March 1, 2016
Published online: June 14, 2016

## Abstract

**AIM:** To evaluate gut microbial dysbiosis in two visceral hypersensitive models in comparison with irritable bowel syndrome (IBS) patients and to explore the extent to which these models capture the dysbiosis of IBS patients.

**METHODS:** Visceral hypersensitivity was developed using the maternal separation (MS) rat model and post-inflammatory rat model. The visceral sensitivity of the model groups and control group was evaluated using the abdominal withdraw reflex score and electromyography in response to graded colorectal distention. The 16S ribosomal RNA gene from fecal samples was pyrosequenced and analyzed. The correlation between dysbiosis in the microbiota and visceral hypersensitivity was calculated. Positive findings were compared to sequencing data from a published human IBS cohort.

**RESULTS:** Dysbiosis triggered by neonatal maternal separation was lasting but not static. Both MS and post-inflammatory rat fecal microbiota deviated from that of

the control rats to an extent that was larger than the co-housing effect. Two short chain fatty acid producing genera, *Fusobacterium* and *Clostridium XI*, were shared by the human IBS cohort and by the maternal separation rats and post-inflammatory rats, respectively, to different extents. *Fusobacterium* was significantly increased in the MS group, and its abundance positively correlated with the degree of visceral hypersensitivity. *Porphyromonadaceae* was a protective biomarker for both the rat control group and healthy human controls.

**CONCLUSION:** The dysbiosis MS rat model and the post-inflammatory rat model captured some of the dysbiosis features of IBS patients. *Fusobacterium*, *Clostridium XI* and *Porphyromonadaceae* were identified as targets for future mechanistic research.

**Key words:** Animal model; Irritable bowel syndrome; Microbiota; Pyrosequencing; 16S rRNA gene



**Core tip:** Dysbiosis of the gastrointestinal microbiota and hypersensitivity to colonic distension are critical features of irritable bowel syndrome (IBS). For animal models, the correlation between dysbiosis in the microbiota and visceral hypersensitivity remains unknown. This study identified common biomarkers between the animal models and IBS patients, which may be targets for future mechanistic research.

Zhou XY, Li M, Li X, Long X, Zuo XL, Hou XH, Cong YZ, Li YQ. Visceral hypersensitive rats share common dysbiosis features with irritable bowel syndrome patients. *World J Gastroenterol* 2016; 22(22): 5211-5227 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5211.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5211

## INTRODUCTION

The human intestinal tract is home to trillions of bacteria that have co-evolved with their host over millennia[1]. Their combined genomes, called a metagenome, contain 150-fold more genes than do the human hosts, and they provide functions that humans otherwise do not have[2]. Complex interactions exist between the gut microbiota and the host[3]. Irritable bowel syndrome (IBS) is a common gastrointestinal disorder that is characterized by abdominal pain and alterations in bowel habits; statistically, IBS affects 7%-10% of people worldwide[4]. Accumulating evidence has indicated that the gut microbiota may participate in the pathogenesis of IBS[5]. Because collecting fecal samples both before and after a gastrointestinal infection from the same IBS patients is unfeasible for clinics, only gut dysbiosis in standing IBS patients has been evaluated to date[6,7]. However, how gut microbiota abnormalities arise and are maintained over time is unclear. These questions are critical for interventions targeting the microbiota, such as probiotic usage. In this work, we used visceral hypersensitive rat models to investigate the longitudinal changes of gut microbiota.

Currently, both post-infectious/inflammatory models and stress-related models have been frequently used to study the pathophysiology of IBS[8,9]. There are more than 12 major post-infectious/post-inflammatory models to mimic post-infectious IBS, which occurs after an initial episode of acute gastrointestinal infection. Chemicals such as trinitrobenzene sulfonic acid (TNBS)[10], mustard oil[11] and dextran sulfate sodium[12] were used to cause mucosal injury in the post-inflammatory models, and pathogens such as *Trichinella spiralis*[13] and *Campylobacter*[14] were used to infect the gut; both led to visceral hypersensitivity. Stress-related models[15] could also induce the modulation of visceral pain, and this may involve changes in the brain-gut axis[9]. However, one of the unsolved problems is the extent to which these models recapture the characteristics of gut dysbiosis in IBS patients. In this work, we used two visceral hypersensitive models, the TNBS post-inflammatory (pTNBS) model and the maternal separation (MS) model, to investigate: (1) whether and the extent to which these models reproduce the disturbance of gut microbiota in a similar way to that of the IBS patients; and (2) whether microbial dysbiosis, if it exists, is static or shifting in these visceral hypersensitive models. We also hoped to identify targets in the models' gut microbial communities that are suitable for use in developing probiotics to specifically modulate the microbiota.

## MATERIALS AND METHODS

### *Animal maintenance and modeling*

Sprague-Dawley rats were purchased from the animal center of Shandong University of Traditional Chinese Medicine. The rats were allowed to habituate for 7 d to the breeding facility prior to mating. They were kept under standardized specific pathogen-free conditions (21-22 ℃, 12:12-h light-dark cycle) with access to pellet food and water *ad libitum*. All experiments were approved by the Ethical Committee and Institutional Animal Care and Use Committee of Qilu Hospital (KYLL-2013-005), and the methods were performed in strict accordance with the Animal Management Rules of the Chinese Ministry of Health. The overall design and co-housing relationship of involved rats are indicated in Figure 1A.

The MS visceral hypersensitive models were developed as previously described[15]. Briefly, rat pups that were randomly assigned to the MS group were stressed by separating them from their mothers for 3 h daily between postnatal days 2-14. The control group (Ctl) received normal breeding during this session. All pups were weaned on postnatal day 22, and only the

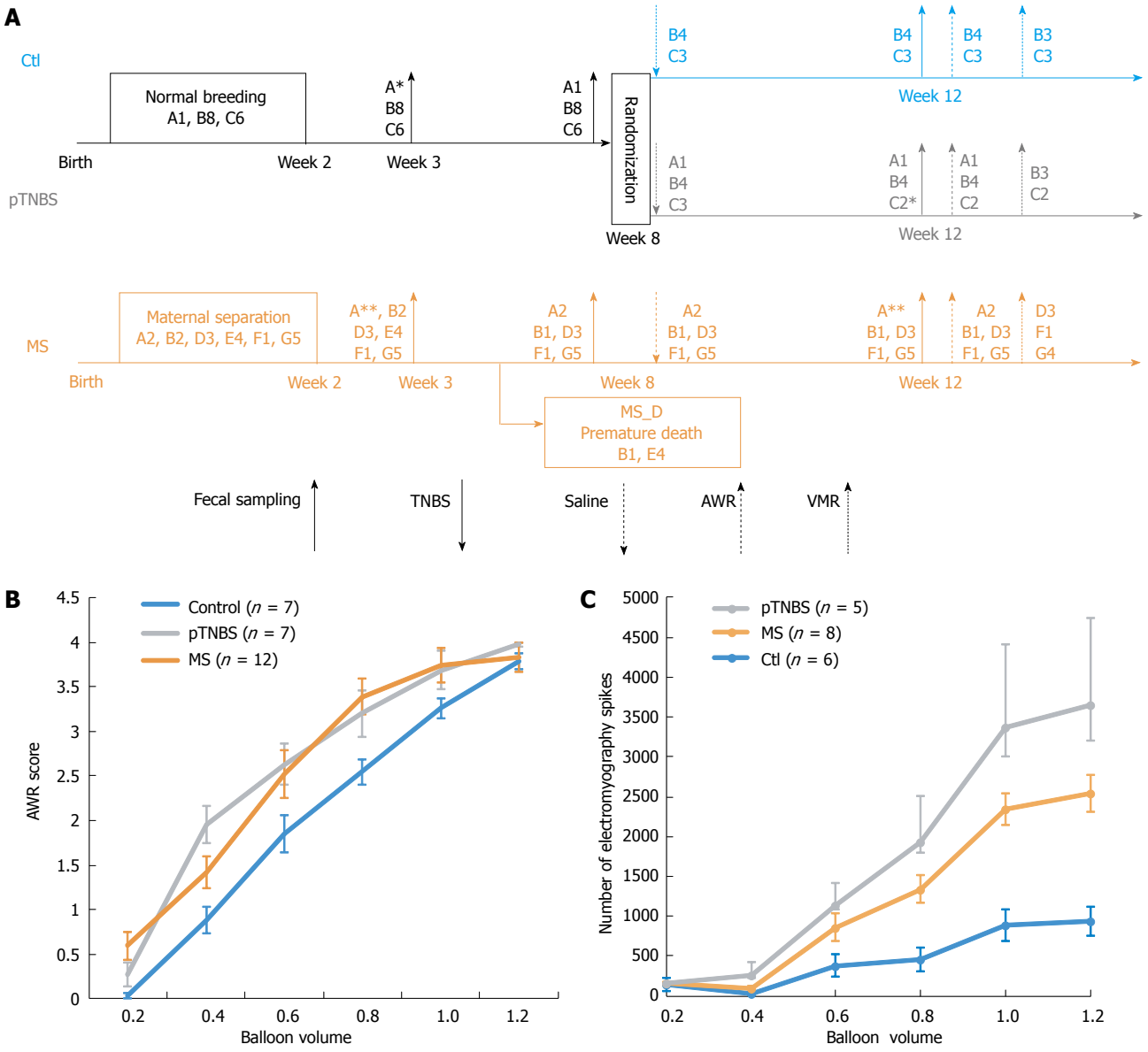



**Figure 1 Study design and visceral sensitivity evaluation.** A: Schematic flow chart showing the treatment and co-housing relationship of involved rats. For each code, such as "B8", the character "B" indicates the nest and number 8 indicates the number of rats. They were cohoused until "B4", which indicates that four of them were randomly chosen and cohoused together. The asterisk indicates fecal samples that failed to return the sequencing data; B: Abdominal withdrawal reaction (AWR) score in response to the graded colorectal distention (CRD); C: Visceromotor response (VMR) score in response to graded CRD. MS: Maternal separation; MS_D: MS early death; pTNBS: TNBS post-inflammatory.

male pups were used for the following study. Because some MS pups naturally died before they aged, 5 male rats were randomly chosen from those that were sampled at week 3 but did not survive to week 8. We indicated this group as the MS early death (MS_D) group. By including the MS_D group, we could test whether the dysbiosis caused by MS stress was more severe in the early dying pups.

After the second fecal collection at week 8, half of the control group was randomly assigned to the post-TNBS inflammation group (pTNBS). The pTNBS group was fasted for 24 h with free access to tap water, and then 0.4 mL of 5% (v/v) TNBS (P2297, Sigma, Shanghai, diluted to 0.8 mL using 50% ethanol) was administered into the colorectum.

### *Visceral hypersensitivity evaluation*

After the last fecal collection at week 12, visceral hypersensitivity was evaluated using both the abdominal withdraw reflex (AWR) score and electromyography in response to graded colorectal distention (CRD). Graded CRD was induced by rapidly injecting (0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mL) saline into a urinary catheter balloon placed in the colon over 1 s and maintaining the distention for 20 s. AWR score was recorded according to a previously described method[16]. To represent the overall visceral sensitivity, a visceral hypersensitive index (VHI) for each rat was calculated by summing the rank of the AWR score at 0.4, 0.6, 0.8 and 1.0 of the total balloon volume.

The visceromotor responses (VMRs) to CRD were

quantified through electromyography of the rat obliqus external abdominis. Briefly, 5 d after embedding an electrode in the rat obliqus externus abdominis, the raw electromyography was recorded, rectified and quantified by counting the increased spike bursts during a 20 s window after graded CRD stimulation. The VMR index for each rat was calculated by summing the rank of electromyography spikes at 0.4, 0.6, 0.8 and 1.0 of the total balloon volume.

### *Fecal sample collection, DNA extraction, and pyrosequencing*

Fecal samples were collected 3, 8 and 12 wk after birth. The samples from the pTNBS group at weeks 3 and 8 were indicated as Ctl-pTNBS and were analyzed as the controls because their treatment was the same as that for the Ctl group. A chart illustrating the overall treatment, fecal collection and model evaluation time points is shown in Figure 1A.

The samples were snap-frozen in liquid nitrogen and stored at -80℃. Genomic DNA was extracted with a TIANamp Stool DNA Kit according to the manufacturer's instructions (Cat# DP328, Tiangen, Beijing). DNA purity and concentration were measured using a Nanodrop2000 (Thermo Fisher). The DNA samples were shipped to Majorbio (Shanghai), where the DNA integrity check, PCR amplification, DNA quantification, emPCR (using Roche GS FLX Titanium emPCR Kits) and pyrosequencing of the 16S rRNA gene V3 to V1 region (using Roche Genome Sequencer FLX+) were performed according to their optimized protocols. The sequencing results were archived in the Short Reads Achieve (number pending).

### *Taxonomy quantification using 16S rRNA gene sequences*

Raw sequencing data were prepared using Mothur v 1.33.0 according to their proposed 454 SOP (http://www.mothur.org/wiki/454_SOP)[17]. The raw sff files were decoded, denoised, trimmed and then aligned to Silva references (Release 102) using the default parameters. Chimeras were detected using the chimera.uchime command and were then removed. Distances between sequences were calculated with a cutoff value of 0.15. The sequences were clustered to the same operational taxonomic units (OTUs) if their distances were less than 0.03. The Shannon index and the inverse Simpson index (1/D) were calculated to indicate the diversity in each sample. Both indexes were calculated using Mothur, and the detailed formula can be accessed online (http://www.mothur.org/wiki/Shannon and http://www.mothur.org/wiki/Simpson). The OTU table was converted to biom files and the taxa abundance from domain to genus levels was generated using the summarize_taxa.py command in QIIME v1.8.0.

### *Statistical analysis*

The richness of each taxonomy and the Shannon index between groups were compared using the Kruskal-Wallis test (KW) or a student's *t*-test in SAS V.9.3 statistical software. The heatmap plot with dendrograms was drawn using the heatplot function in the made4 packages in R (version 3.1.1). For primary component analysis (PCA), the axis value of all 80 samples was calculated together using the prcomp function in the stats package in R, and then the samples were plotted by each time point (week 3, 8, and 12). Within each time point, samples were clustered based on the Euclidian distance using the vegdist and hclust in the vegan package. According to the cluster results, the PCA plot points were grouped and connected using the ordispider and ordiellipse functions, where the ellipse was estimated to cover 75% of the dots in this group. The distribution of each group in each cluster was checked using Fisher's exact test in SAS. The community dissimilarity was tested by the weighted and unweighted UniFrac test using Mothur. The specific taxa that were differentially present in each group were identified using the LEfSe [linear discriminant analysis (LDA) coupled with effect size measurements] method with an LDA cut-off value of 2.0[18]. The Spearman correlation between the VHI and the taxonomy richness was calculated using the cor.test function in the stats package in R.

### *Comparing rat models and human IBS cohort*

We downloaded the published 16S rRNA V4 region Miseq sequencing data by Jeffery *et al*[6]. This data set was analyzed by the same pipeline described above. We used LEfSe analysis on this dataset. Each positive finding from the rat experiment was checked against the human cohort. The relationship between human and rat biomarkers was indicated using a Venn plot.

## RESULTS

### *Modeling and visceral hypersensitivity evaluation*

The design and co-housing relationship of the rats involved in this study is shown in Figure 1A. Twenty-six of the 27 rats in this study (7 Ctl, 7 pTNBS, and 12 MS, see Figure 1B) were evaluated using AWR. The VHI score was calculated by summing the rank of the AWR score at 0.4, 0.6, 0.8 and 1.0 of the total balloon volume. A significant difference existed in the VHI among the three groups ($\chi^2$ = 9.98, *df* = 2, *P* = 0.0068, KW). The VHI difference in the pTNBS to Ctl comparison was 38.4 (95%CI: 15.7 to 61.0, *P* < 0.05), and the VHI difference in the MS to Ctl comparison was 32.9 (95%CI: 12.7 to 53.0, *P* < 0.05). There was no significant difference in the MS to pTNBS comparison, with a VHI difference of 5.48 (95%CI: -14.7 to 25.7, *P* > 0.05). Nineteen of the 27 rats were evaluated by VMRs (Figure 1C). The VMR index for each rat was calculated by summing the rank of electromyography spikes at 0.4, 0.6, 0.8 and 1.0 of the total balloon volume. The VMR index among groups was insignificant although the control group tended to

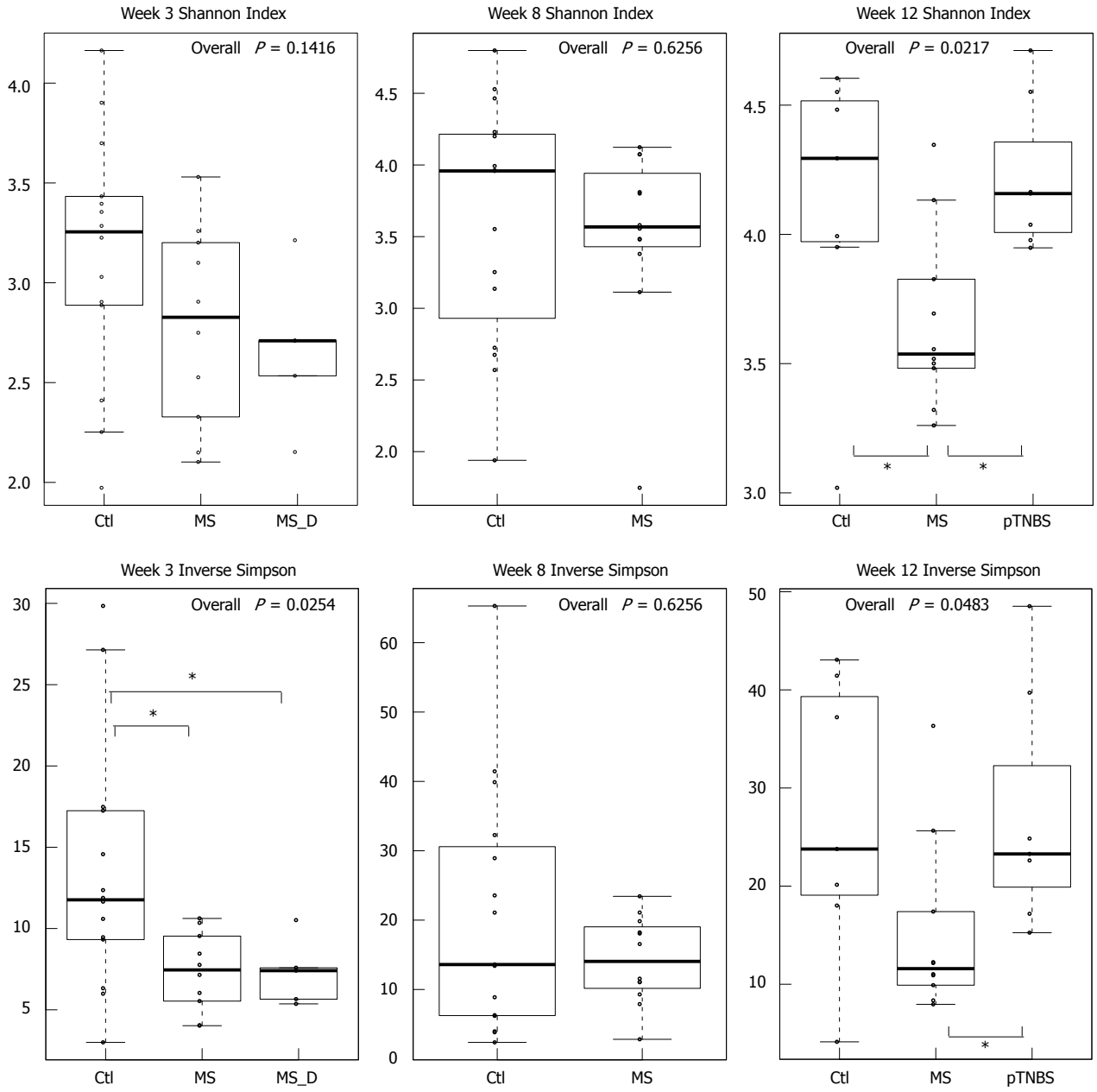



**Figure 2 Microbial diversity.** Shannon index (upper panel) and inverse Simpson (lower panel) of fecal microbiota at weeks 3, 8 and 12. Asterisk indicates $P < 0.05$ in pairwise comparison. MS: Maternal separation; MS_D: MS early death; pTNBS: TNBS post-inflammatory.

be lower than the MS and pTNBS groups (43.5 *vs* 86.5 and 60, respectively, $\chi^2 = 2.235$, $df = 2$, $P = 0.3271$, KW). Overall, these data indicate that both the MS and pTNBS groups developed visceral hypersensitivity at a comparable level.

### *DNA sequence data and microbial diversity comparison*

A total of 489556 valid reads were assigned to 80 sequenced samples after barcode trimming. Sequence length varied between 230 and 327 bp per read. After removing chimeras and non-bacterial reads, 434594 reads remained. Each fecal sample included 3313 to 8161 reads. Based on a 97% species similarity, 2413 OTUs were identified from all of the fecal samples. Good's coverage for each sample varied from 95.57% to 99.72%. The rarefaction curve reached a plateau for most samples, suggesting that the present study captured the dominant phylotypes.

We first compared the microbial diversity among groups using the Shannon index and the inverse Simpson index (Figure 2). By week 3, the inverse Simpson index was significantly higher in the Ctl group ($\chi^2 = 7.34$, $df = 2$, $P = 0.0254$, KW). By week 8, the Shannon index and the inverse Simpson index were similar between the control group and the MS group. By week 12, the MS group has the lowest Shannon index ($\chi^2 = 7.67$, $df = 2$, $P = 0.217$, KW) and inverse Simpson index ($\chi^2 = 6.06$, $df = 2$, $P = 0.0483$, KW) compared with other groups. The pTNBS group had roughly same diversity indexes compared to the Ctl

group. These data indicate that the MS model, but not the pTNBS model, developed fecal microbiota with reduced diversity in a non-static manner.

### Dysbiosis of major phyla

We then investigated whether differences in the phylum abundance exist at different time points (Figure 3). *Bacteroidetes* was the dominant phylum across all samples, and *Firmicutes* and *Proteobacteria* were the second and third most abundant phyla. No significance was reached for these three phyla at any of the 3 time points. The *Firmicutes* to *Bacteroidetes* (F/B) ratio was not significantly different ($P > 0.05$, KW).

*Fusobacteria* was abundant by week 3 (up to 0.25) and dropped to zero in most control rats. No difference in *Fusobacteria* existed by week 3 and week 8; however, by week 12, the MS group had significantly more *Fusobacteria* ($\chi^2 = 6.83$, $df = 2$, $P = 0.0328$, KW, $P < 0.05$ in MS-Ctl comparison). The control group had significantly more *Actinobacteria* than the MS group at week 8 ($P = 0.0034$, $\chi^2 = 8.58$, $df = 1$, KW). However, by week 12, the pTNBS group had significantly more *Actinobacteria* than the Ctl and MS groups ($\chi^2 = 8.07$, $df = 2$, $P = 0.0176$, KW, $P < 0.05$ in the pTNBS-Ctl and pTNBS-MS comparison). These data suggest that the dysbiosis of the major phyla may be phase dependent and different among the visceral hypersensitive rat models.

### PCA and cluster analysis

We based the cluster analysis and PCA on the OTU data from the 16S rRNA gene pyrosequencing. The primary components for all 80 samples were calculated from the relative abundance of the 2413 OTUs. The relative importance of the first 20 primary components is plotted in Figure 4A. Primary component 1 and primary component 2 explained 27.7% and 14.6% of the total variance, respectively (Figure 4A). The differences in the primary components between the time points and experimental groups are listed in Table 1. Primary component 1 mainly reflected the effect of time points ($\chi^2 = 37.7$, $df = 2$, $P = 0.0000$, KW). Primary components 2 and 4 reflected the effect of experimental groups on fecal microbiota composition. The other 3$^{rd}$, 6$^{th}$, and 9$^{th}$ components were different both among time points and among groups.

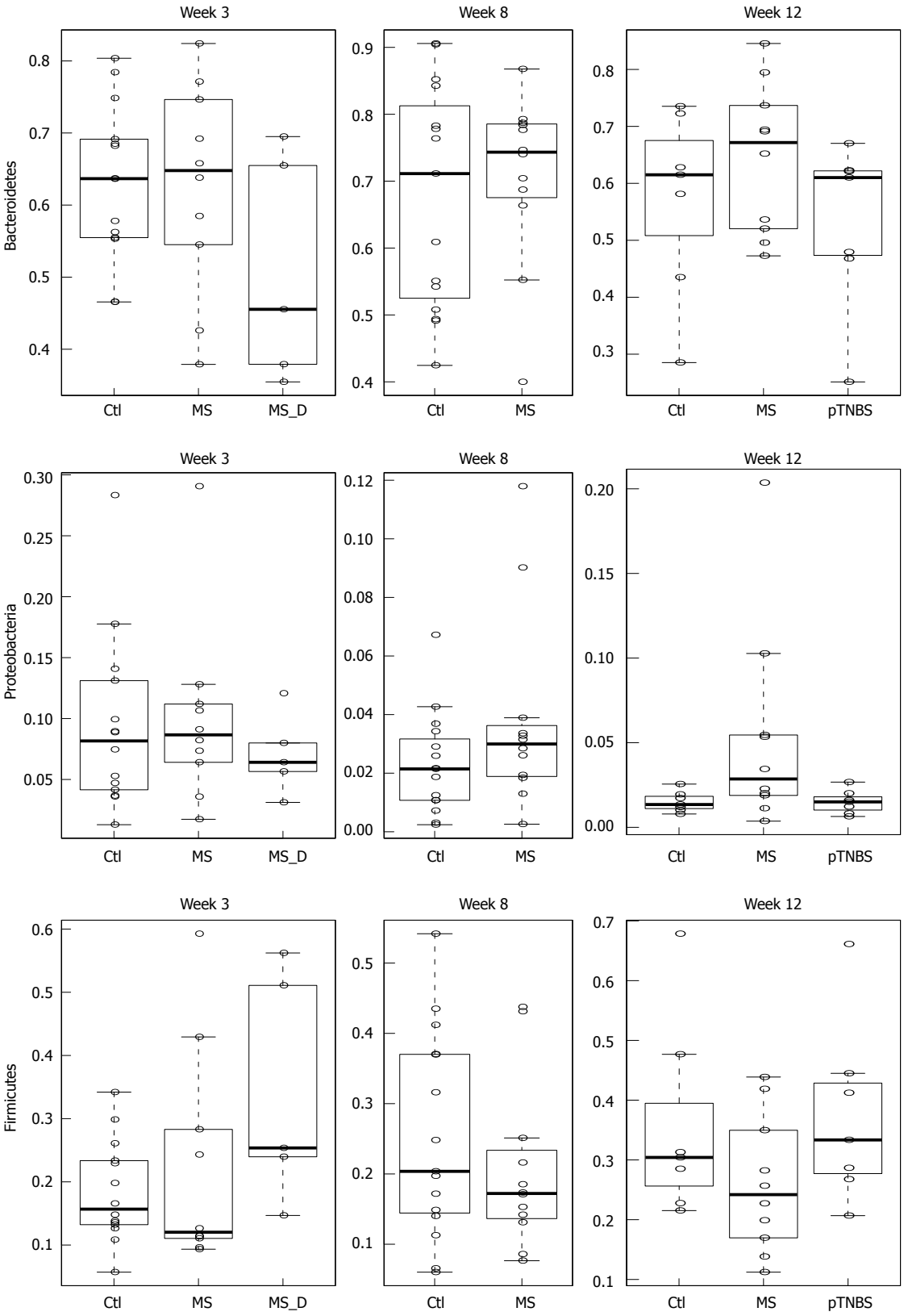
We then used cluster analysis to test whether the groups would fall into the same or different clusters. At each time point, the samples were fitted into the 3 top clusters based on the Euclidean distance. We designated the names of each cluster according to the samples it included. By week 3, the normal cluster included 8 Ctl, and the MS cluster included 4 MS and 4 MS_D samples. The mixed cluster included 6 Ctl, 6 MS and 1 MS_D samples (Figure 4B). The 3 groups' distribution in the clusters was significantly different ($P = 1.702 \times 10^{-4}$, Fisher's test). This result suggests that MS caused dysbiosis in rat models at early ages.

By week 8, the 27 fecal microbiota samples clustered into 3 mixed clusters (Figure 4C). The control group dominated mixed cluster 3 (12/16) while the MS group dominated mixed cluster 2 (7/8). The cluster distribution of the MS and control groups was significantly different ($P = 0.0114$, Fisher's test). By week 12, the 24 fecal microbiota samples formed 3 clusters (Figure 4D). The MS cluster included 5 MS samples, and the mixed cluster included roughly the same number of samples from the Ctl ($n = 6$), MS ($n = 5$), and pTNBS ($n = 7$) groups. Another "orphan" cluster included only one control sample. The difference among groups was significant ($P = 0.0150$, Fisher's test). These data suggest that the dysbiosis triggered by MS during childhood is still substantial in a fraction of adult rats. Four weeks after TNBS administration, the fecal microbiota of the post-inflammatory rat model was more similar to that of the control group as revealed by PCA and cluster analysis.

We tracked the longitudinal dysbiosis of 23 rats whose fecal samples were collected at all 3 time points. We analyzed whether the 10 MS rats clustered to the different or same clusters at week 3 and week 12. Seven out of 10 rats shifted to different clusters (mixed-to-MS or MS-to-mixed) from week 3 to week 12. The agreement Kappa value for cluster classification at week 3 and week 12 was -0.4000 (95%CI: -0.9566 to 0.1566). This result indicates that although MS stress generated an isolated dysbiosis cluster in a fraction of rats, each rat's gut microbiota might shift between the less disturbed (mixed) cluster and the severely disturbed (MS) cluster.

### UniFrac test on animal models and co-housing effect

We further tested the effect of modeling and co-housing on fecal microbiota using the weighted and unweighted UniFrac test (Table 2). A phylogenetic tree was built for all samples at each time point, and weighted and unweighted UniFrac scores were calculated to evaluate the community similarity. According to the unweighted UniFrac test, we found that by week 3, the fecal community in Ctl and MS groups was significantly different ($P < 0.001$). The co-housing effect caused community dissimilarity in the 2 houses of control rats (B8 *vs* C6, $P < 0.001$) but not in the 2 houses of MS rats (D3 *vs* G5, $P = 0.507$). By week 8, the co-housing effect was non-significant within the MS and Ctl groups, but the difference was still significant between these two groups. By week 12, a significant community difference existed among the Ctl, MS, and pTNBS groups; the co-housing effect was not obvious within any of the groups. The weighted UniFrac test was significant in all of the above comparisons. Overall, these data suggest that both the MS model and the pTNBS model developed dysbiosis of the fecal microbiota, and the differences were not caused by the co-housing relationship.


Week 3
Week 8
Week 12
Bacteroidetes
Proteobacteria
Firmicutes
Ctl
MS
MS_D
pTNBS

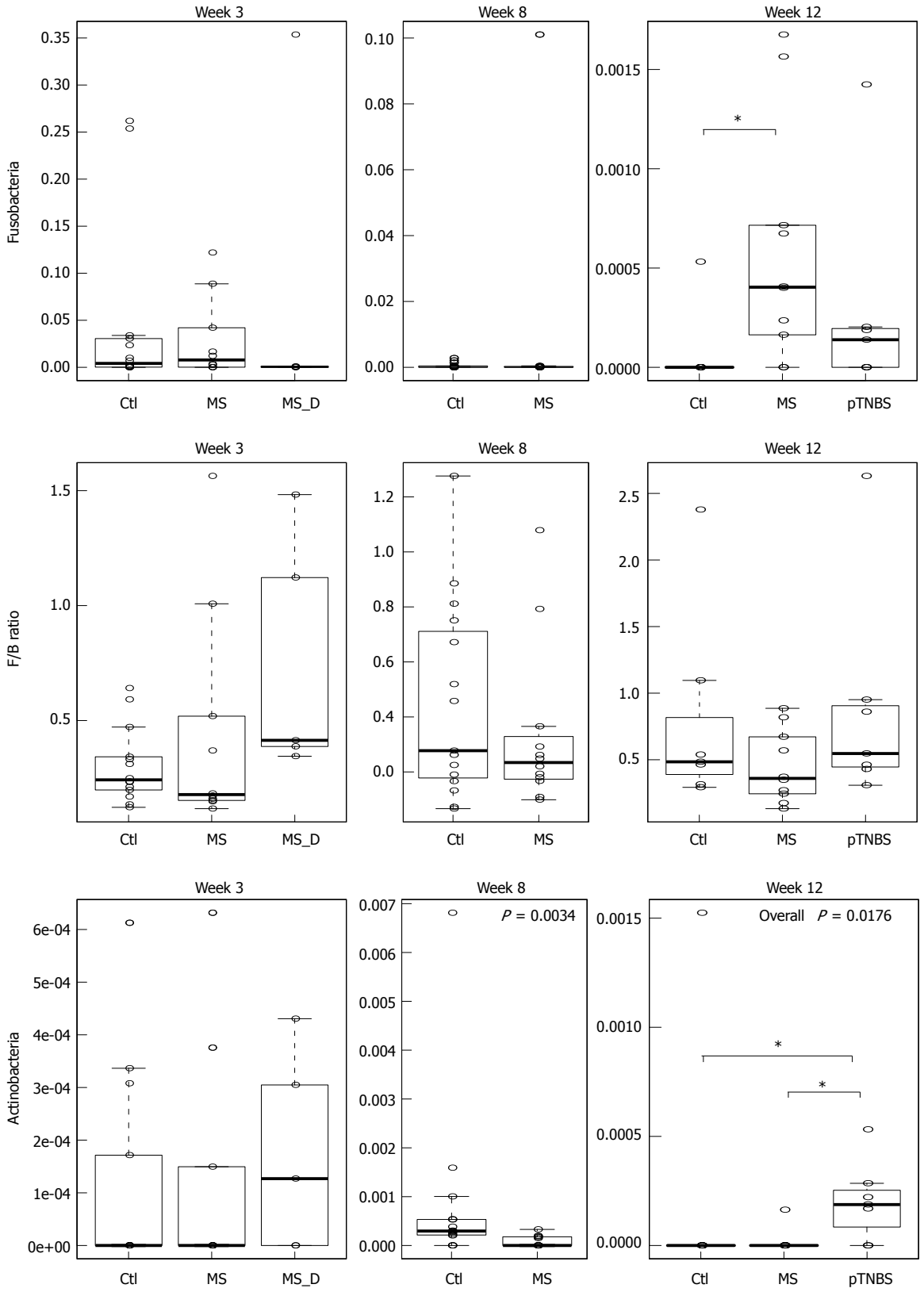



**Figure 3 Phyla abundance by weeks 3, 8 and 12**. Asterisk indicates $P < 0.05$ in pairwise comparison. $P > 0.05$ (no significant), unless the $P$-value is drawn in the plot box. MS: Maternal separation; MS_D: MS early death; pTNBS: TNBS post-inflammatory.

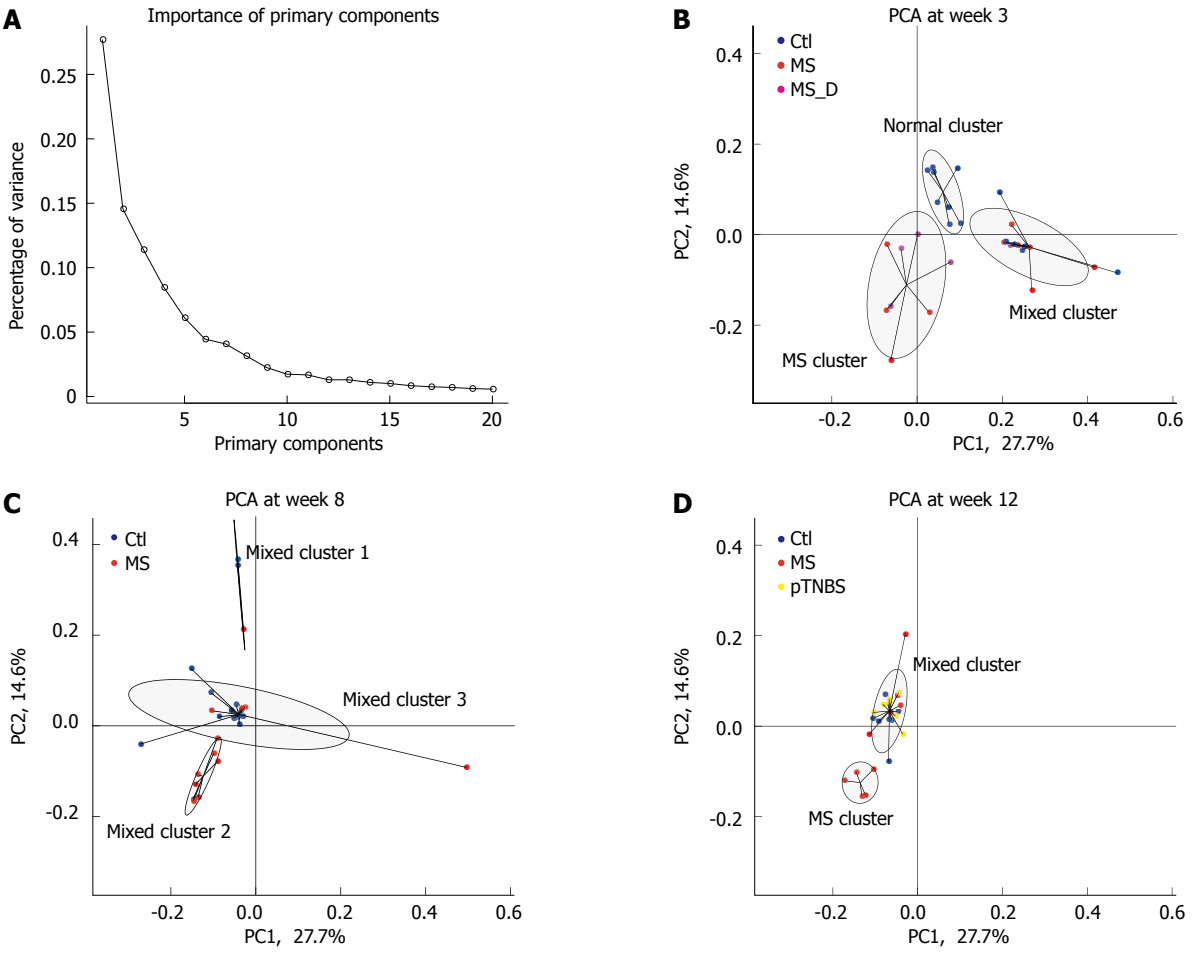



**Figure 4 Primary component analysis and cluster analysis by time points**. Primary components were calculated from the relative abundance of all 2413 OTUs. A: The percentage of variances explained by the first 20 primary components. The primary component was separately plotted at week 3 (B), week 8 (C), and week 12 (D). The cluster analysis divided the samples into three clusters, and the samples in the same cluster were connected together. The ellipse was estimated to cover 75% of dots in this cluster. Each cluster was named according to the samples involved in this cluster. The MS group formed isolated clusters, indicated as "MS cluster", at week 3 and week 12. MS: Maternal separation; PCA: Primary component analysis.

**Table 1 Importance and meaning of the first 10 primary components of fecal microbiota**

| Primary component | % of variance | Difference among time points ($n$ = 80, $df$ = 2) | | Difference among groups ($n$ = 80, $df$ = 3) | |
|---|---|---|---|---|---|
| | | $\chi^2$ | *P*-value | $\chi^2$ | *P*-value |
| 1 | 27.7% | 37.67931 | 0.00000[1] | 4.6999109 | 0.19514 |
| 2 | 14.6% | 1.352165 | 0.50861 | 20.4278998 | 0.00014[1] |
| 3 | 11.4% | 6.165561 | 0.04583[1] | 8.6830899 | 0.03382[1] |
| 4 | 8.5% | 0.719395 | 0.69789 | 21.7793862 | 0.00007[1] |
| 5 | 6.1% | 0.623716 | 0.73209 | 4.6400976 | 0.20013 |
| 6 | 4.5% | 8.388254 | 0.01508[1] | 9.3688439 | 0.02477[1] |
| 7 | 4.1% | 0.844768 | 0.65548 | 0.7871023 | 0.85255 |
| 8 | 3.2% | 0.412918 | 0.81346 | 6.6021743 | 0.08572 |
| 9 | 2.2% | 8.880049 | 0.01180[1] | 9.9866961 | 0.01868[1] |
| 10 | 1.7% | 1.762893 | 0.41418 | 2.6274074 | 0.45270 |

[1]The *P*-value is less than 0.05.

### *Biomarkers and correlation to visceral hypersensitivity*

We performed linear discriminant analysis coupled with effect size measurement (LEfSe) analysis at different time points to screen biomarkers for each group (Figure 5, Figure S1-3). By week 3, 29 samples were analyzed using LEfSe. The control group was strongly associated with higher abundances of unclassified *Bacteroidales*, *Veillonella*, *Treponema*, and unclassified *Clostridiales*. The MS group was associated with higher abundances of unclassified *Burkholderiales*, *Coprobacillus*, and *Clostridium_XIVa*. By week 8, 27 samples were analyzed. The MS group was associated with higher abundances of *Helicobacter*, unclassified *Burkholderiales*, and unclassified *Desulfovibrionaceae*,

**Table 2 Weighted and unweighted UniFrac test on experiment groups and co-housing**

| Time point | Comparison | Number of samples to build phylogenetic tree | Weighted UniFrac test | | Unweighted UniFrac test | |
|---|---|---|---|---|---|---|
| | | | Weighted UniFrac score | *P*-value | Unweighted UniFrac score | *P*-value |
| Week 3 | Ctl-MS | 29 | 0.863902 | < 0.0010 | 0.957176 | < 0.0010[1] |
| | Ctl-MS_D | 29 | 0.926521 | < 0.0010 | 0.963861 | 0.0130[1] |
| | MS-MS_D | 29 | 0.833168 | < 0.0010 | 0.909242 | 0.2790 |
| | Ctl(B8)-Ctl(C6) | 29 | 1.000000 | < 0.0010 | 1.000000 | < 0.0010[1] |
| | MS(D3)-MS(G5) | 29 | 1.000000 | 0.0070 | 1.000000 | 0.5070 |
| Week 8 | Ctl-MS | 27 | 0.719683 | < 0.0010 | 0.951668 | 0.0190[1] |
| | Ctl(B8)-Ctl(C6) | 27 | 0.83063 | < 0.0010 | 0.955082 | 0.0870 |
| | MS(D3)-MS(G5) | 27 | 0.617715 | < 0.0010 | 0.874196 | 0.5220 |
| Week 12 | Ctl-MS | 24 | 0.754046 | < 0.0010 | 0.944729 | 0.0390[1] |
| | Ctl-pTNBS | 24 | 0.942882 | < 0.0010 | 0.978698 | 0.0160[1] |
| | MS-pTNBS | 24 | 0.828407 | < 0.0010 | 0.973938 | 0.0130[1] |
| | Ctl(B4)-Ctl(C3) | 24 | 0.928051 | < 0.0010 | 0.976209 | 0.3200 |
| | MS(D3)-MS(G5) | 24 | 0.771421 | < 0.0010 | 0.912219 | 0.4550 |
| | pTNBS(B4)-pTNBS(C2) | 24 | 1.000000 | < 0.0010 | 1.000000 | 0.6390 |

[1]The *P*-value is less than 0.05. MS: Maternal separation; pTNBS: TNBS post-inflammatory.

which all belong to *Proteobacteria*. The control group was associated with higher abundances of *Barnesiella*, *Actinobacteria*, *Clostridium_XI*, *Allobaculum*, and *Odoribacter*.

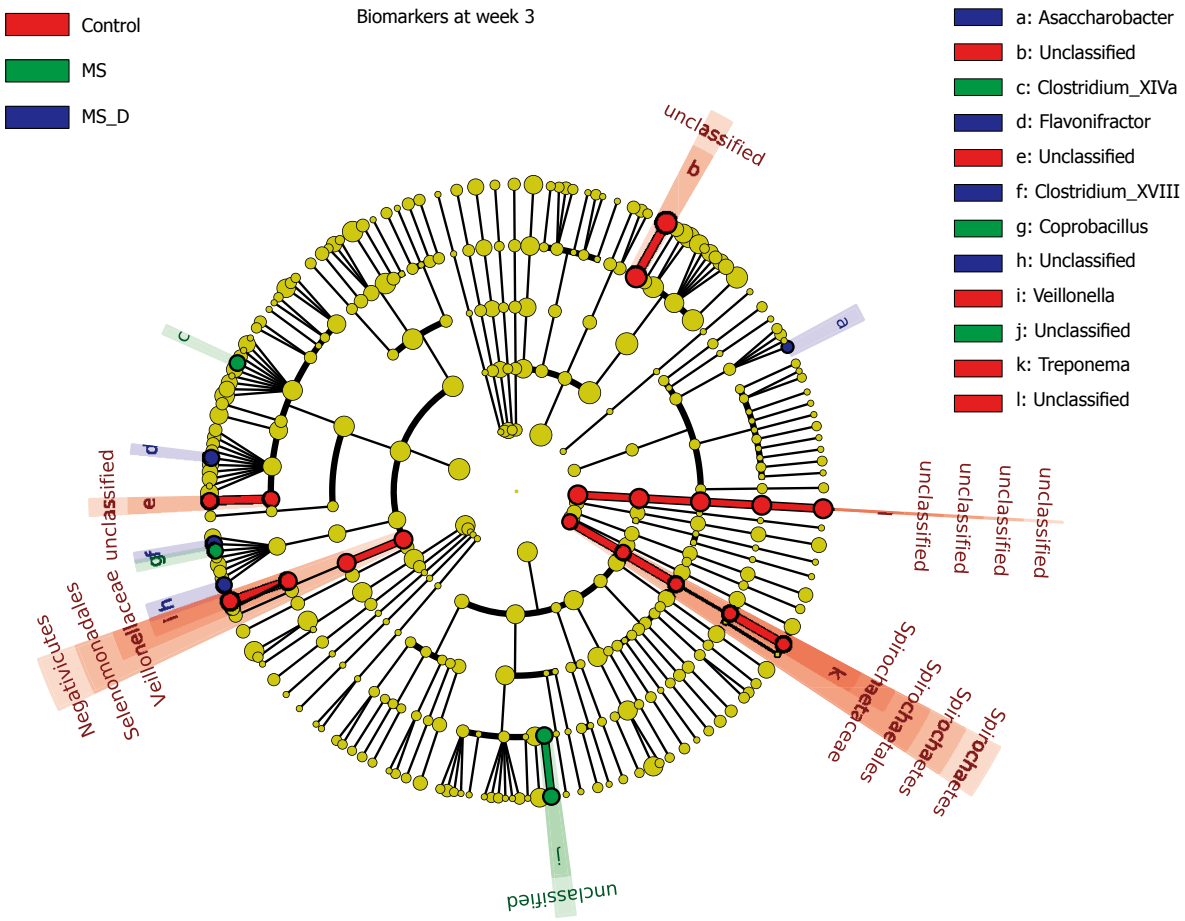
To investigate whether the differentially abundant taxa correlated with the visceral hypersensitivity level, we listed the biomarkers in each group by week 12, and calculated their Spearman correlation to the VHI both within the respective group and across groups (Table 3). By week 12, *Fusobacterium* was associated with the MS group (LDA Score = 2.766). The *Fusobacterium* abundance was also significantly and positively correlated with the VHI across the all 24 samples by week 12 ($r$ = 0.4564, $P$ = 0.0250). Unclassified *Erysipelotrichaceae* was associated with the control group (LDA Score = 3.097) and significantly and negatively correlated with the VHI across groups ($r$ = -0.4944, $P$ = 0.0140). These data suggest that *Fusobacterium* may participate in the pathogenesis of visceral hypersensitivity and that *Erysipelotrichaceae* might protect against the hypersensitivity.

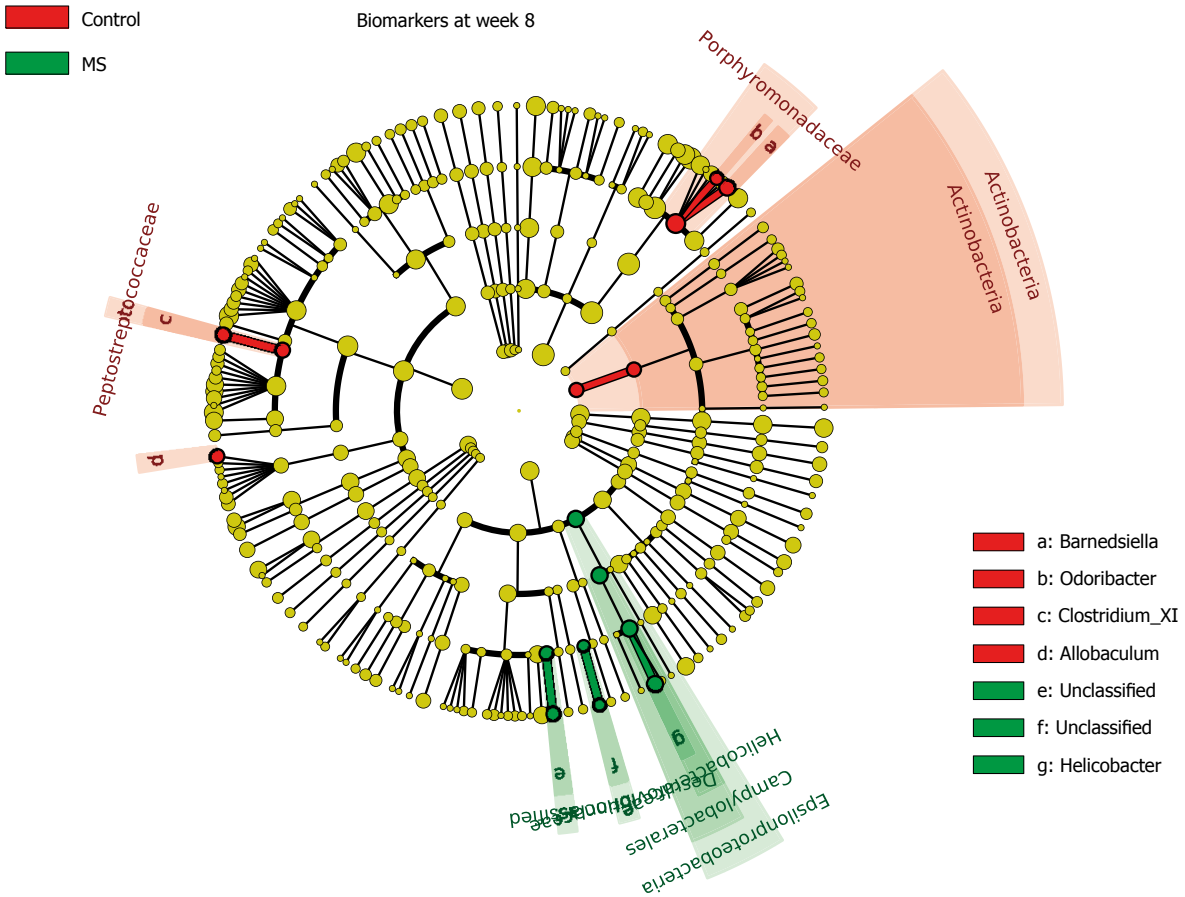
### *Comparing visceral hypersensitive rat models to IBS patients*

Next, we asked to what extent visceral hypersensitive rats' dysbiosis resembles that of IBS patients. We downloaded the pyrosequencing data published by Jeffery *et al*[6], which included 37 IBS patients and 20 controls. We used the LEfSe method to analyze disease and healthy biomarkers in human fecal samples (Figure 6A); this data was further compared to the data from our animal models (Figure 6B). We identified 36 biomarkers of IBS patients and 15 biomarkers of human controls[6] (Figure 6A). The biomarkers of disease were largely different in human IBS and visceral hypersensitive rats, and only a few disease or control biomarkers were shared between the human study[6] and our rat model study. *Fusobacteria* marginally increased in IBS patients compared with human controls[6] ($P$ = 0.063, KW, Figure 6C). The MS rat model did not share increased common biomarkers with human patients. Both control rats and human health controls have fecal microbial markers of *Porphyromonadaceae* and unclassified *Porphyromonadaceae* (Figure 6B). In the IBS cohort published by Jeffery *et al*[6], *Porphyromonadaceae* was significantly lower in the IBS groups ($P$ = 0.0006, KW, Figure 6D), and the MS rats also had lower *Porphyromonadaceae* concentrations ($P$ = 0.0097, KW, Figure 6D). The genus *Clostridium_XI* (belonging to family *Peptostreptococcaceae*, order *Clostridiales*) was a biomarker of both IBS patients and pTNBS rats (Figure 6E). *Clostridium_XI* accounted for up to 6% of the fecal microbiota in the IBS group and was significantly higher than the level in the healthy control group ($P$ = 0.0484, KW). Additionally, *Clostridium XI* also colonized at a higher level in the pTNBS rats ($P$ = 0.0422, KW).

## DISCUSSION

In this study, we found that (1) both the MS and the pTNBS rat models developed dysbiosis of fecal microbiota; (2) the fecal microbiota of the MS model was characterized by a lower diversity and a higher level of *Fusobacteria* at week 3 and week 12. A fraction of the MS rats formed an isolated MS cluster that indicated clear-cut dysbiosis in comparison to the controls but the rats in this cluster tended to alternate; (3) the pTNBS model was characterized by higher *Actinobacteria* but did not develop any isolated clusters; (4) among the biomarkers observed by week 12, the *Fusobacterium* positively and unclassified *Erysipelotrichaceae* negatively correlated to visceral hypersensitivity; and (5) in comparison to a previously published fecal microbial profile in human IBS patients[6], *Porphyromonadaceae* was a protective biomarker for both healthy humans and rat controls;


Control
MS
MS_D
Biomarkers at week 3
a: Asaccharobacter
b: Unclassified
c: Clostridium_XIVa
d: Flavonifractor
e: Unclassified
f: Clostridium_XVIII
g: Coprobacillus
h: Unclassified
i: Veillonella
j: Unclassified
k: Treponema
l: Unclassified
unclassified
Veillonellaceae
Selenomonadales
Negativicutes
Spirochaetaceae
Spirochaetales
Spirochaetes
Spirochaetae
unclassified


Control
MS
Biomarkers at week 8
a: Barnedsiella
b: Odoribacter
c: Clostridium_XI
d: Allobaculum
e: Unclassified
f: Unclassified
g: Helicobacter
Porphyromonadaceae
Actinobacteria
Peptostreptococcaceae
Helicobacteraceae
Campylobacterales
Epsilonproteobacteria

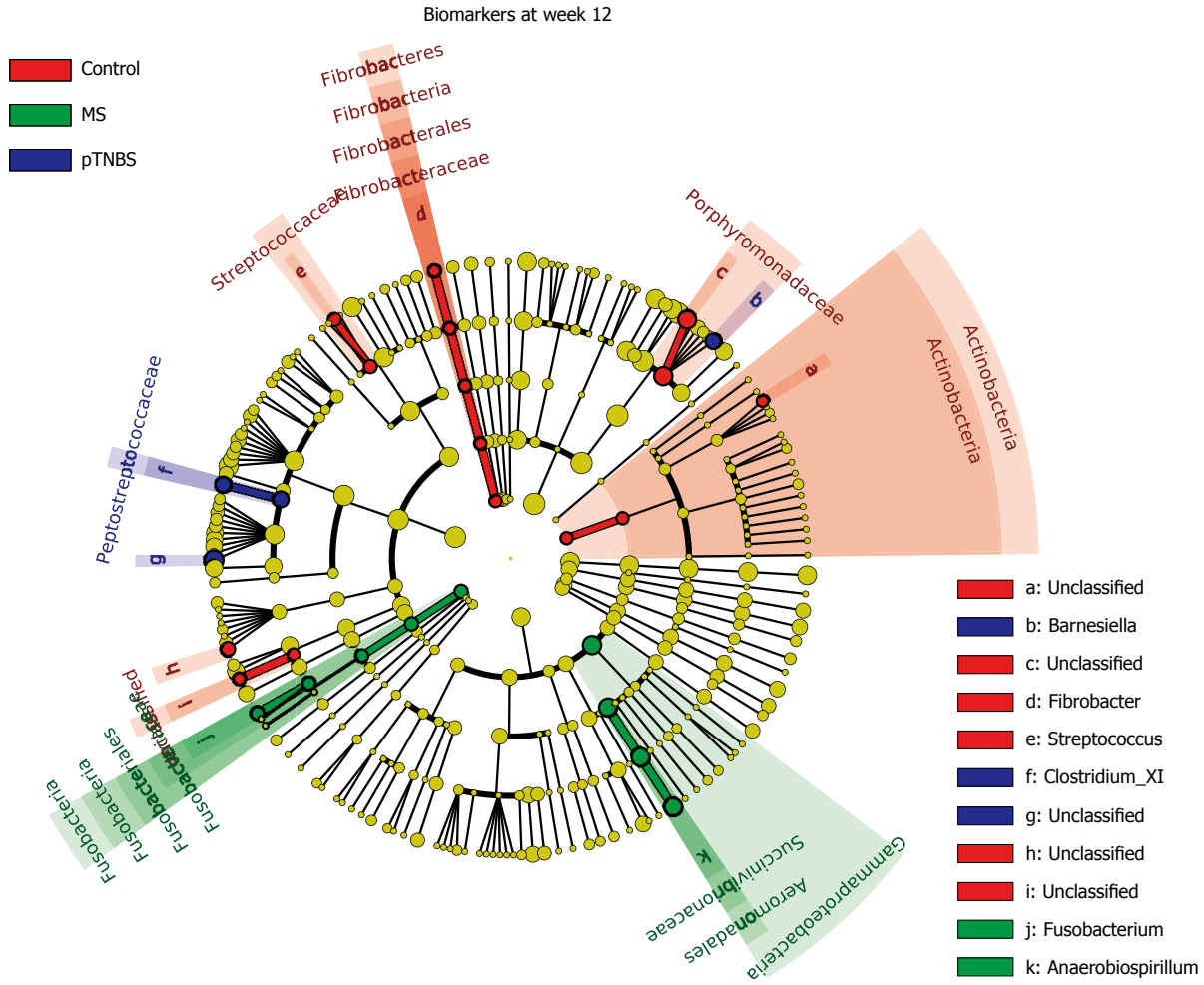



**Figure 5 Microbial markers for different groups at weeks 3, 8 and 12.** Biomarkers for each time point were calculated using the LEfSe Method. The abundances of taxa at the phylum, class, order, family, and genus levels were compared between the groups. Taxa with different abundances between groups and with an LDA score larger than 2.0 were considered to be a biomarker; biomarkers were indicated with corresponding colors on the cladogram. See also Figure S1, S2, and S3. MS: Maternal separation; MS_D: MS early death; pTNBS: TNBS post-inflammatory.

*Clostridium_XI* was a shared biomarker for human IBS patients and pTNBS rats.

Rodent models have been frequently used to study the pathogenesis and treatment of IBS, where the intestinal microbiota plays an important role. Before this study, dysbiosis in IBS rat models had not been specifically profiled, and whether any members in microbial community were correlated to visceral hypersensitivity was not known. We evaluated the fecal microbiota of MS and control rats at three time points and found that dysbiosis happened shortly after weaning (third week) and at the adult phase (12 wk). The pTNBS rat model did not develop an isolated microbial cluster but had biomarkers of *Barnesiella*, *Clostridium_XI*, and unclassified *Ruminococcaceae*. Through the unweighted UniFrac test, we found that the co-housing effect was no more significant at week 8 or week 12. Thus, both models developed significant dysbiosis of the fecal microbiota.

Approximately 10% of IBS patients believe that their symptoms began with an infectious illness, and prospective studies have shown that 3% to 36% of enteric infections lead to IBS symptoms[19]. Understanding underlying gut microbial dysbiosis associated with PI-IBS is critical for the prevention and management of this disease. *Campylobacter jejuni*, *Campylobacter rodentium*, and *Salmonella enterica* are available bacterial infectious murine models that mimic aspects of the pathogenesis of post-infectious IBS[8]. In this study, two rat models, MS and pTNBS, were not given any specific infector but were colonized more frequently by *Fusobacterium* and *Clostridium XI,* respectively. The latter includes the *Clostridium difficile*, *Clostridium litorale*, and *Clostridium lituseburense*. These two genera were also found to increase or tend to increase in the downloaded Miseq 16S rRNA gene sequencing data from Jeffery's IBS cohort[6]. Thus, these two bacteria may be common dysbiosis features across human and rat models. In a chip-based study by Jalanka-Tuovinen *et al*[7], *C. cellulosi* and its relatives (members from *Clostridium* cluster IV) significantly decreased in IBS-D patients. Thus, whether *Clostridium* plays a mechanistic role in the pathogenesis of IBS warrants further study.

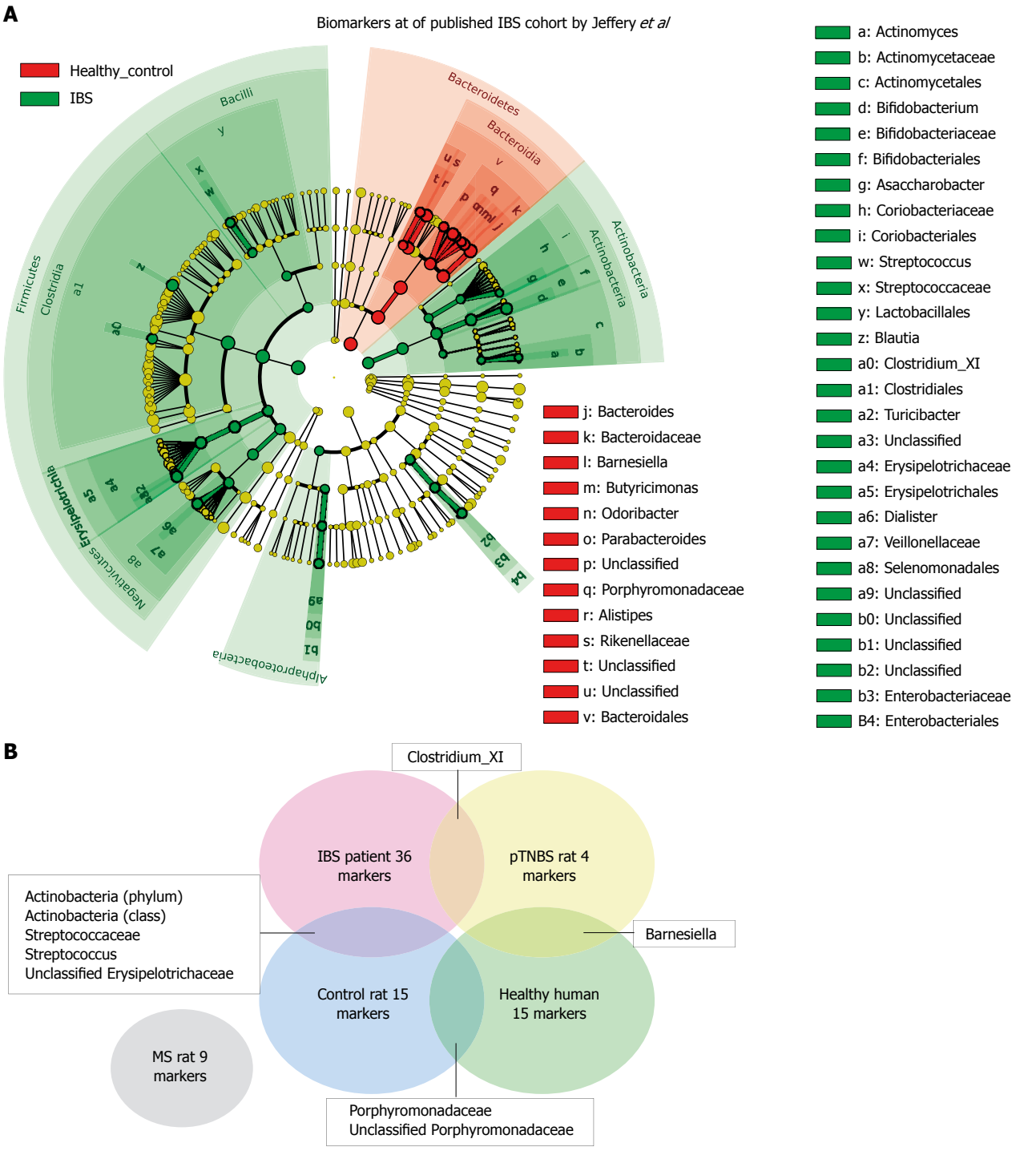
**Table 3 Abundance of different taxa among groups by week 12 and correlations to visceral hypersensitivity**

| Taxonomy | LDA score (Log10) | Group | Across groups ($n$ = 24) | | Within group | |
|---|---|---|---|---|---|---|
| | | | r_all_sample | p_all_sample | r_ingroup | p_ingroup |
| Actinobacteria | 2.338 | Control $n$ = 7 | 0.1835 | 0.3906 | 0.2041 | 0.6606 |
| Actinobacteria \| Actinobacteria | 2.338 | | 0.1835 | 0.3906 | 0.2041 | 0.6606 |
| Actinobacteria \| Actinobacteria \| Coriobacteriales \| Coriobacteriaceae \| Unclassified | 2.192 | | 0.2639 | 0.2127 | 0.2041 | 0.6606 |
| Bacteroidetes \| Bacteroidia \| Bacteroidales \| Porphyromonadaceae | 4.610 | | -0.1005 | 0.6405 | 0.1429 | 0.7825 |
| Bacteroidetes \| Bacteroidia \| Bacteroidales \| Porphyromonadaceae \| Unclassified | 4.543 | | -0.1344 | 0.5313 | 0.1429 | 0.7825 |
| Fibrobacteres | 2.435 | | -0.3601 | 0.0839 | -0.0741 | 0.8745 |
| Fibrobacteres \| Fibrobacteria | 2.435 | | -0.3601 | 0.0839 | -0.0741 | 0.8745 |
| Fibrobacteres \| Fibrobacteria \| Fibrobacterales | 2.435 | | -0.3601 | 0.0839 | -0.0741 | 0.8745 |
| Fibrobacteres \| Fibrobacteria \| Fibrobacterales \| Fibrobacteraceae | 2.435 | | -0.3601 | 0.0839 | -0.0741 | 0.8745 |
| Fibrobacteres \| Fibrobacteria \| Fibrobacterales \| Fibrobacteraceae \| Fibrobacter | 2.435 | | -0.3601 | 0.0839 | -0.0741 | 0.8745 |
| Firmicutes \| Bacilli \| Lactobacillales \| Streptococcaceae | 2.710 | | 0.1215 | 0.5717 | 0.4447 | 0.3174 |
| Firmicutes \| Bacilli \| Lactobacillales \| Streptococcaceae \| Streptococcus | 2.710 | | 0.1215 | 0.5717 | 0.4447 | 0.3174 |
| Firmicutes \| Erysipelotrichia \| Erysipelotrichales \| Erysipelotrichaceae \| Unclassified | 3.097 | | -0.4944 | 0.0140[1] | -0.1429 | 0.7825 |
| Firmicutes \| Negativicutes \| Selenomonadales \| Unclassified | 2.396 | | -0.2047 | 0.3374 | 0.7027 | 0.0782 |
| Firmicutes \| Negativicutes \| Selenomonadales \| Unclassified \| Unclassified | 2.396 | | -0.2047 | 0.3374 | 0.7027 | 0.0782 |
| Fusobacteria | 2.766 | | 0.4564 | 0.0250[1] | 0.2067 | 0.5667 |
| Fusobacteria \| Fusobacteria | 2.766 | | 0.4564 | 0.0250[1] | 0.2067 | 0.5667 |
| Fusobacteria \| Fusobacteria \| Fusobacteriales | 2.766 | | 0.4564 | 0.0250[1] | 0.2067 | 0.5667 |
| Fusobacteria \| Fusobacteria \| Fusobacteriales \| Fusobacteriaceae | 2.766 | | 0.4564 | 0.0250[1] | 0.2067 | 0.5667 |
| Fusobacteria \| Fusobacteria \| Fusobacteriales \| Fusobacteriaceae \| Fusobacterium | 2.766 | MS $n$ = 10 | 0.4564 | 0.0250[1] | 0.2067 | 0.5667 |
| Proteobacteria \| Gammaproteobacteria | 4.622 | | 0.1809 | 0.3976 | 0.0667 | 0.8648 |
| Proteobacteria \| Gammaproteobacteria \| Aeromonadales | 4.622 | | 0.1757 | 0.4115 | 0.0667 | 0.8648 |
| Proteobacteria \| Gammaproteobacteria \| Aeromonadales \| Succinivibrionaceae | 4.622 | | 0.1757 | 0.4115 | 0.0667 | 0.8648 |
| Proteobacteria \| Gammaproteobacteria \| Aeromonadales \| Succinivibrionaceae \| Anaerobiospirillum | 4.622 | | 0.1757 | 0.4115 | 0.0667 | 0.8648 |
| Bacteroidetes \| Bacteroidia \| Bacteroidales \| Porphyromonadaceae \| Barnesiella | 3.607 | | -0.1501 | 0.4840 | -0.0180 | 0.9694 |
| Firmicutes \| Clostridia \| Clostridiales \| Peptostreptococcaceae | 3.776 | pTNBS $n$ = 7 | 0.1946 | 0.3623 | 0.0180 | 0.9694 |
| Firmicutes \| Clostridia \| Clostridiales \| Peptostreptococcaceae \| Clostridium_XI | 3.776 | | 0.1946 | 0.3623 | 0.0180 | 0.9694 |
| Firmicutes \| Clostridia \| Clostridiales \| Ruminococcaceae \| Unclassified | 5.014 | | -0.0322 | 0.8813 | -0.1982 | 0.6701 |

[1]The $P$-value is less than 0.05. LDA: Linear discriminant analysis; MS: Maternal separation; pTNBS: TNBS post-inflammatory.

By week 12, *Fusobacterium* colonized in significantly greater abundance in MS rats, and its abundance was positively correlated with higher VHI scores. The *Fusobacteria* phylum also tends to be higher in published IBS cohorts[6]. *Fusobacterium* was invasive to the gut epithelial cells and has already been documented as being involved in the pathogenesis of colorectal adenoma[20-22] and inflammatory bowel disease[23,24]. This represents the first study documenting that *Fusobacterium* was involved in visceral hypersensitivity. Whether the increased colonization of *Fusobacterium* caused low grade inflammation and thus contributed to visceral hypersensitivity is not currently known. Moreover, both *Fusobacterium* and members in *Clostridium* are known short chain fatty acid (SCFA) producers[24-26]. The low fermentable oligo-, di-, and monosaccharides and polyol (FODMAP) diet has been shown to be an efficacious therapy for the reduction of IBS symptoms in randomized controlled trials[27-29]. Supplementing food containing FODMAP to IBS patients and healthy people would trigger gastrointestinal symptoms to a larger extent in the patient group[30]. However, the mechanism of treatment with a low FODMAP diet and why IBS patients are more sensitive to FODMAP remains unknown[31]. In this study, we identified two butyric producing taxa, *Fusobacterium* and *Clostridium*, which significantly correlate to visceral hypersensitivity or are disease biomarkers. Their colonization may render patients more ready to produce SCFA and gas in the presence of FODMAP. It was reported that the butyrate-producing *Clostridium* cluster XIVa significantly increased in IBS patients consuming a typical high FODMAP diet compared with those on a low FODMAP diet[32]. Farmer *et al*[33] showed that caecal intraluminal pH was significantly lower in IBS patients compared to controls. Thus, the detailed mechanistic role of *Fusobacterium* and *Clostridium* in IBS warrants further study.

*Porphyromonadaceae* is a family belonging to the order of *Bacteroidales* and the class *Bacteroidetes*. Among others, *Barnesiella* and *Butyricimonas* are genera under *Porphyromonadaceae*. Unfortunately, the biological function of *Porphyromonadaceae* has not been characterized in detail, and little attention has been paid to their role in gastrointestinal diseases. A previous study documented that *Barnesiella* was enriched in dextran sulfate sodium-induced colitis[34]. In our study, *Barnesiella* was also enriched 4 weeks after TNBS instillation. Whether *Barnesiella* promoted the inflammation or was passively enriched under inflammatory conditions was not determined in the current study. A recent review paper[35] summarized 29 relevant original research articles concerning microbiota analysis and IBS. Durbán's pyrosequencing


A
Biomarkers at of published IBS cohort by Jeffery et al
Healthy_control
IBS
Bacilli
Bacteroidetes
Bacteroidia
Actinobacteria
Actinobacteria
Firmicutes
Clostridia
Negativicutes
Erysipelotrichia
Alphaproteobacteria
j: Bacteroides
k: Bacteroidaceae
l: Barnesiella
m: Butyricimonas
n: Odoribacter
o: Parabacteroides
p: Unclassified
q: Porphyromonadaceae
r: Alistipes
s: Rikenellaceae
t: Unclassified
u: Unclassified
v: Bacteroidales
a: Actinomyces
b: Actinomycetaceae
c: Actinomycetales
d: Bifidobacterium
e: Bifidobacteriaceae
f: Bifidobacteriales
g: Asaccharobacter
h: Coriobacteriaceae
i: Coriobacteriales
w: Streptococcus
x: Streptococcaceae
y: Lactobacillales
z: Blautia
a0: Clostridium_XI
a1: Clostridiales
a2: Turicibacter
a3: Unclassified
a4: Erysipelotrichaceae
a5: Erysipelotrichales
a6: Dialister
a7: Veillonellaceae
a8: Selenomonadales
a9: Unclassified
b0: Unclassified
b1: Unclassified
b2: Unclassified
b3: Enterobacteriaceae
B4: Enterobacteriales
B
Clostridium_XI
IBS patient 36 markers
pTNBS rat 4 markers
Actinobacteria (phylum)
Actinobacteria (class)
Streptococcaceae
Streptococcus
Unclassified Erysipelotrichaceae
Barnesiella
Control rat 15 markers
Healthy human 15 markers
MS rat 9 markers
Porphyromonadaceae
Unclassified Porphyromonadaceae
C
Fusobacteria
P = 0.063
0.00030
0.00025
0.00020
0.00015
0.00010
0.0005
0.0000
Healthy control
IBS

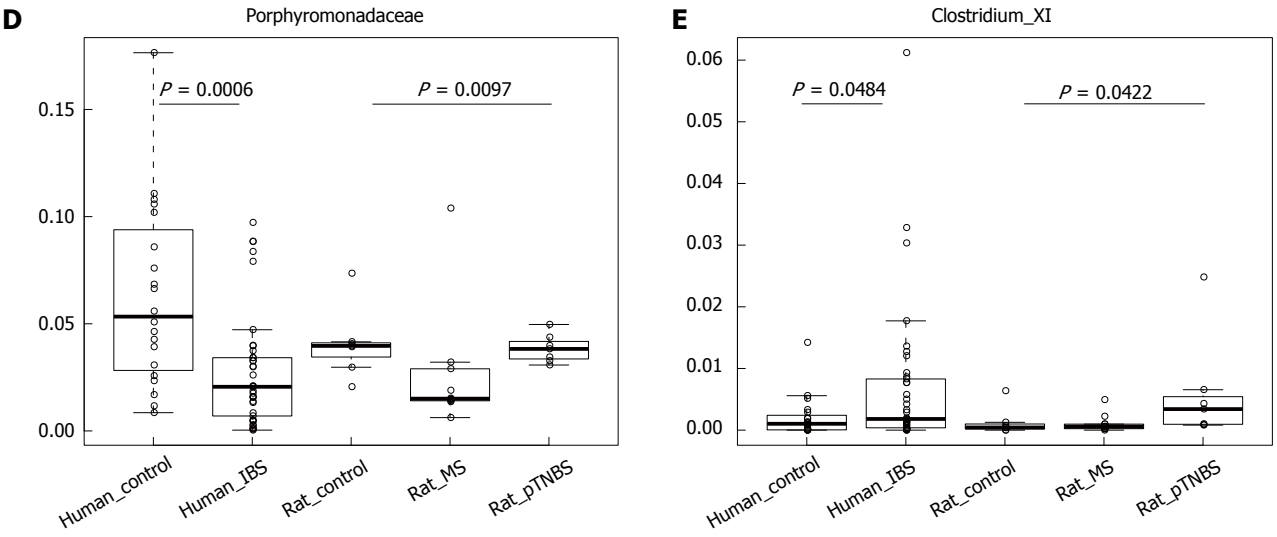



**Figure 6 Comparing rat model dysbiosis to that of Jeffery's human irritable bowel syndrome cohort.** A: Cladogram indicates the biomarkers of different abundances between groups; B: Venn plot of the positive (increased) biomarkers shared by rat models and Jeffery's human IBS cohort. The overlapped area indicates that the two groups have common biomarkers. The MS rat does not share biomarkers from the human cohort; C: Abundance of the *Fusobacteria* phylum marginally increased in the human IBS cohort; D: Abundance of *Porphyromonadaceae* in fecal samples of healthy human controls, human IBS patients, control rats, MS rats, and pTNBS rats. *Porphyromonadaceae* was depleted in both the human IBS group and the MS rats; E: Abundance of *Clostridium XI* in fecal samples of healthy human controls, healthy IBS patients, control rats, MS rats, and pTNBS rats. *Clostridium XI* increased in both human IBS patients and pTNBS rats. IBS: Irritable bowel syndrome; MS: Maternal separation; pTNBS: TNBS post-inflammatory.

study[36] found that the family *Porphyromonadaceae* was increased in the fecal samples of IBS subjects. In our study, the *Porphyromonadaceae* was highest in the control group by week 12. The discrepancy may be explained by the different nature between human patients and rat models.

IBS is a human disease with multifactorial pathophysiology[37], and the prevalence of IBS is associated with social-economic factors[38]. To date no available model could ideally model the IBS pathogenesis. IBS is heterogeneous and thus unlikely to be modeled in any single model. Although common biomarkers were found between human IBS patients and rat models, the limitations of rat models should also be taken into consideration. The pTNBS model was triggered by a pro-inflammatory molecule (TNBS). Therefore, this model resembles the human inflammatory bowel disease to some extent and can only mimic the post-infectious IBS, which is associated only to a percentage of patients. Furthermore, the causal relationship between visceral hypersensitivity, dysbiosis, and the symptoms of IBS is not clear and remains to be untangled in the future.

In summary, both the MS and the post-inflammation rat models developed dysbiosis in the fecal microbiota, and the models captured parts of the dysbiosis features of human IBS patients. The potential pathogenic role of *Fusobacterium* and *Clostridium XI*, as well as the protective role of *Porphyromonadaceae* warrants further mechanistic study.

## ACKNOWLEDGMENTS

We acknowledge Dr. Ian Jeffery and Dr. Paul W. O'Toole for sharing their sequencing data from human IBS patients and healthy controls.

## COMMENTS

### Background

Previous studies have indicated that the gut microbiota participated in the pathogenesis of irritable bowel syndrome (IBS).

### Research frontiers

Dysbiosis of the gastrointestinal microbiota and hypersensitivity to colonic distension are critical features of IBS. For animal models, the correlation between dysbiosis in the microbiota and visceral hypersensitivity remains unknown.

### Innovations and breakthroughs

Dysbiosis triggered by neonatal maternal separation (MS) was lasting but not static. Both MS and post-inflammatory rat fecal microbiota deviated from that of the control rats to an extent that was larger than the co-housing effect. *Fusobacterium*, *Clostridium XI* and *Porphyromonadaceae* were identified as targets for future mechanistic research.

### Applications

This study indicated that the two animal models could capture part of the dysbiosis features of IBS. Further mechanistic study on the biomarkers' role in the pathogenesis is warranted.

### Peer-review

The manuscript is excellent and addresses adequately the relationship between dysbiosis and visceral hypersensitivity in experimental animals. The quality of the study design and experimental investigations are very high.

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**P- Reviewer**: Guglielmetti S, Ierardi E, Soares RLS **S- Editor**: Ma YJ
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ISSN 1007-9327
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