

Dynamic comparison of gut microbiota of mice infected with *Shigella flexneri* via two different infective routes

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Received May 29, 2019; Accepted December 6, 2019

DOI: 10.3892/etm.2020.8469

Abstract. *Shigella* is one of the main pathogens causing diarrheal disease, and is associated with high morbidity and mortality in developing countries. Previous clinical data and animal studies have shown that the outcomes of oral and peritoneal infections of *Shigella* differ, and that the latter is more serious. Furthermore, a variety of pathogenic bacteria are known to cause changes in intestinal flora after infection, and the influence of *Shigella* infection on intestinal flora remains poorly understood. In the present study, the 16S rRNA high-throughput sequencing method was used to compare the changes in gut microbiota profiles in feces of mice infected with *Shigella* via two routes. In addition, the present study investigated the association between the differences in infection performance and bacterial communities. The present results suggested that the intraperitoneal route induced a distinct decrease in α -diversity in the fecal microbiota when compared to the control at a later time, while the effect of the oral route on α -diversity was not obvious. Oral infection of *Shigella* had a rapid and significant effect on gut microbiota, mainly causing a decreased abundance of *Lactobacillus* and an increased abundance of *Prevotella* and *Escherichia/Shigella* in the early stage of infection. By contrast, the effect of intraperitoneal infection on the gut microbiota was relatively slow and small. The principal coordinate analysis results suggested that the dynamic profile of gut microbiota between the two infective routes was consistent with the infection process. Probiotics, such as *Lactobacillus reuteri* and *Faecalitalea* exhibited significantly reduced abundance after *Shigella* infection. Collectively, the present results suggested that gut microbiota

may play a pivotal role in the pathogenesis of *Shigella* infection. Future studies should investigate the effect of *Shigella* infection on the interaction between pathogenic bacteria and intestinal flora. The present results suggested that the use of probiotics may facilitate the prevention and treatment of shigellosis.

Introduction

Shigella is a common cause of bacterial dysentery, and can be classified into four serogroups: *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* (1). Among these groups, *S. flexneri* is the most dominant pathogen found in developing countries (2,3). The low-infectivity inoculum facilitates person-to-person spread by fecal-oral contact, which is the predominant mode of transmission (1). The clinical symptoms of shigellosis mainly include fever, diarrhea, abdomen convulsions and purulent bloody stools (4). The lesions are mainly confined to the colon, and result in mucosal congestion, edema and inflammatory cell infiltration between the ulcers (5). Bacteremia with *Shigella* is rare, especially in adults (6). In addition to the common route of oral infection, *Shigella* can also cause damage to multiple organs via the route of extra-intestinal infection, such as kidney and spleen abscesses induced by bacteremia (7,8), perihepatitis-salpingitis caused by unhygienic sexual practices, and vulvovaginitis induced by fecal contamination (9,10). In particular, when *Shigella* infection is combined with intestinal perforation, appendicitis and peritonitis, clinical manifestations of abdominal infection, such as high fever and septicemia, may occur with a high mortality rate (11). It was reported that 13 out of 57 children with surgical complications of Shigellosis died, despite having received antimicrobial therapy (12). In addition to the clinical reports, animal experiments have shown that diarrhea symptoms disappear within 24 h after oral infection with *Shigella*, while persistent diarrhea symptoms and mortality occur after intraperitoneal injection of *Shigella* (13). Therefore, the clinical manifestations of parenteral *Shigella* infection are more serious compared with those of intestinal infection, and the pathogenic mechanisms underlying these differences require further investigation.

Due to its long co-evolution with the host species, the gut microbiota influences numerous aspects of host physiology,

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Key words: *Shigella flexneri*, gut microbiota, intraperitoneal route, oral route

including digestion, metabolism, immunity and behavior (14). In addition, the gut microbiota plays crucial roles in resisting the invasion and clearance of pathogenic microorganisms (15). Previous studies have shown that infectious diarrhea, such as that caused by *Salmonella*, *Escherichia coli*, parasites and viruses, is usually accompanied by changes in the intestinal flora (16-19). However, it remains unclear whether the gut microbiota in mice can be influenced by *S. flexneri* infection. In order to investigate the changes in intestinal flora after *Shigella* infection, and to identify the different clinical manifestations after oral or peritoneal infection based on gut microbiota changes, mice were challenged through two infective routes in the present study. The diversity and abundance of intestinal flora between these two groups were compared at different infection stages using 16S ribosomal RNA (rRNA) high-throughput sequencing.

The results from the present study provided an insight into *Shigella* infection-induced gut microbiome changes, and identified potential biomarkers for *Shigella* infection, thereby facilitating the development of therapeutics for the prevention and treatment of shigellosis.

Materials and methods

Animals. Specific pathogen-free BALB/c mice (female; n=30; weight, 18-22 g; age 6-8 weeks) were purchased from the Shanghai Laboratory Animal Center CAS, and housed at 22±2°C with 12 h light/dark cycle under specific pathogen-free conditions in the experimental facility for ≥1 week prior to use in the experiments. The mice received a standard batch of food and water *ad libitum*. The present study was approved by The Institutional Animal Care and The Ethic Committee of Fujian Medical University.

***S. flexneri* treatment.** The standard strain of *S. flexneri* (cat. no. 12022; American Type Culture Collection) was inoculated in Gram Negative Enrichment Broth (HuanKai Microbial Sci. and Tech. Co., Ltd.) overnight at 37°C with shaking at 180 rpm on an air table. After the culture reached the logarithmic growth stage (0.4-0.5 optical density at 600 nm), the suspension was centrifuged at 825 x g for 1 min at 4°C to collect the bacterial precipitate. Then, this was suspended in normal saline buffer. The mice were randomly divided into three groups (n=10 in each group): i) Control group with normal saline gavage; ii) intraperitoneal (IP) injection group, 5x10⁷ colony-forming units (CFU) of *Shigella* given by IP injection; and iii) oral gavage group, 5x10⁸ CFU of *Shigella* given orally. Each mouse was administered the treatment at a volume of 0.1 ml. The administered bacterial doses were confirmed by plating serial dilutions onto *Salmonella-Shigella* agar plates (HuanKai Microbial Sci. and Tech. Co., Ltd.). After treatment with *S. flexneri*, the mice received sterilized food and water *ad libitum* for 7 days, and were then sacrificed. During this period, body weight, general physical activity, fur ruffling, abdominal swelling and body temperature of the mice in each group were recorded every day. To calculate the survival rates of mice infected with *Shigella* in the groups, Olfert's guidelines on humane endpoints (20) were adopted as follows: When a mouse lost >20% of its body weight, showed signs of coma

and lethargy, and had a body temperature <34°C, the mouse was considered to have reached a humane endpoint and was euthanized. The remaining mice were euthanized 7 days after *Shigella* infection.

Fecal sample collection. To evaluate *S. flexneri* shedding in the feces, one piece of fecal pellet was collected and weighed (0.1-0.3 g) from each mouse at the time points of 0, 6, 12, 24 and 48 h after infection. Then, these were suspended in sterile PBS by extensive vortexing to prepare a uniform fecal suspension, which was cultivated on *Salmonella-Shigella* agar (HuanKai Microbial Sci. and Tech. Co., Ltd.) by incubation overnight at 37°C and the number of colonies was counted. For 16S high-throughput sequencing, five fresh feces samples per day were randomly collected from five mice in each group using aseptic technique on days 2, 3 and 4 after the administration of *Shigella*. Three pieces of feces from each mouse were combined to be a single sample to meet the requirement of sample size. In total, 45 samples of feces were collected which were immediately frozen in liquid nitrogen and stored at -80°C for further analysis.

DNA extraction, PCR amplification and 16S rRNA sequencing. Bacterial genomic DNA was extracted from the stool samples using a QIAamp Fast DNA Stool Mini kit (cat. no. 51604; Qiagen GmbH), according to manufacturer's protocol. The hypervariable (V)3-V4 region of the bacterial 16S rRNA was amplified by PCR (ABI GeneAmp® 9700, USA) with Phusion® High-Fidelity DNA Polymerase (New England BioLabs, Inc.) under the following conditions: Initial denaturation at 95°C for 5 min; followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec; and a final extension at 72°C for 4 min. The resulting PCR product was stored at 4°C. The universal primers used in the PCR reaction were 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'). The 5'-end of 341F carried a special sequence tag to encode each sample. The obtained PCR products were separated by electrophoresis using 2% agarose gel, and purified using the GeneJET Gel Extraction kit (Thermo Fisher Scientific, Inc.). Then, DNA libraries were constructed using an Ion Plus Fragment Library kit (Thermo Fisher Scientific, Inc.) and sequenced using an Ion S5 XL system (Thermo Fisher Scientific, Inc.), according to manufacturer's protocol. Raw FASTQ files were pretreated and quality-filtered using the Quantitative Insights Into Microbial Ecology (QIIME) bioinformatics pipeline (version 1.17; <http://qiime.org/>). Next, the sample data were separated from the reads obtained according to the barcodes, and the barcodes and primer sequences were removed to obtain raw reads. After data filtering, the remaining high-quality clean data were assembled based on tags and sequence overlaps.

Data analysis. For the analysis of microbial diversity, operational taxonomic units (OTUs) were generated for all sequences based on different similarity levels (21). In the present study, the OTUs were clustered according to a similarity cutoff value of 97% using UPARSE software (version 7.1; <http://drive5.com/uparse/>), while USEARCH (version 10.0.240, <http://www.drive5.com/usearch/>) was used to identify and remove chimeric sequences. SSUrRNA database of SILVA132 (

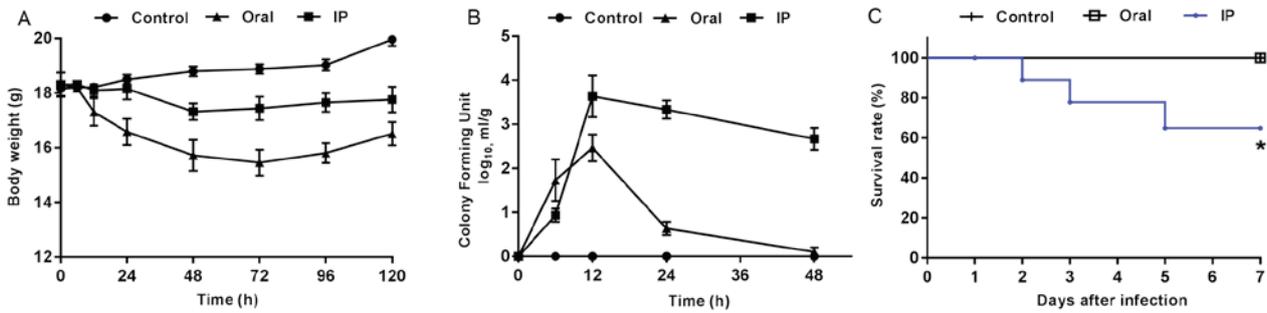


Figure 1. General status of mice after infection in the different groups. (A) Body weight changes were monitored at different post-infection time points. Data are presented as the mean \pm SEM. n=10 mice/group. (B) Colony-forming units in fecal homogenates are expressed as the mean \pm SEM from six mice/group at different time points. (C) Survival curves indicate the survival rates of mice in different groups. *P<0.05 vs. control group. IP, intraperitoneal injection group.

arb-silva.de) was selected for species annotation analysis, where the threshold was set as 0.8-1). The taxonomy of 16S rRNA gene sequences was analyzed using the Ribosomal Database Project (RDP) classifier (version 2.11; <http://sourceforge.net/projects/rdp-classifier/>), and the sequences were classified to different levels, including phylum, class, order, family, genus and species. Subsequently, the composition and relative abundance of each sample at each classification level were calculated. The OTU profiling table and α -diversity indices, including Chao1, Shannon and Simpson (22), were calculated using QIIME. The β -diversity among different groups was analyzed by principal coordinate analysis (PCoA) based on weighted UniFrac distances (23). Bray-Curtis dissimilarities were used to construct a hierarchical cluster, while non-metric multidimensional scaling (NMDS) was plotted based on the weighted UniFrac dissimilarity (24). Linear discriminant analysis Effect Size (LEfSe) was used to find the biomarkers with statistical differences between groups, which was presented by Histogram of Linear Discriminant Analysis distribution and cladogram (25). Data are presented as the mean \pm SEM. The statistical analyses were performed with a one-way ANOVA followed by Tukey's post-hoc test, while Kruskal-Wallis analysis followed by Dunn's multiple comparisons test was used for data with variance inhomogeneity using SPSS 19.0 (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference. GraphPad Prism (version 7.0; GraphPad Software, Inc.) was used to compare mouse survival rates between the groups using a Mantel-Cox test, and to generate a graphical representation of the results.

Results

General condition of mice after infection. The present results suggested that the animals challenged with *S. flexneri* via IP and oral routes exhibited marked changes in physical activity, food intake, fur ruffling and abdominal swelling. At 2 h after the oral administration of *S. flexneri*, mice began to show diarrhea symptoms, which were evident at 6 h and gradually disappeared after 24 h. At 12 h after IP injection, mice began to present with diarrhea, some of which lasted until day 7. Furthermore, pronounced abdominal edema was observed, and mice presented with high irritability upon touching of the abdomen. Compared with the control group, all mice in the infection groups presented with varying degrees of weight loss within 3 days after *S. flexneri* infection (Fig. 1A). The

mice infected orally with *S. flexneri* had greater weight loss compared with both the controls and the IP group. In addition, the CFU value of *S. flexneri* detected in the feces of the oral group peaked at 12 h, and then exhibited a rapid decline (Fig. 1B). In the IP group, the maximum CFU value also appeared at 12 h, but was followed by a slower rate of decline compared with that in the oral group. During the experiment, three mice in the IP group reached the humane endpoint which were euthanized and excluded from the further experiments, while none of the mice in the control or oral groups died. The present results suggested that the differences between the survival rates in the three groups was statistically significant (Fig. 1C; P=0.019).

α -diversity. In the present study, 3,517,768 high-quality reads were generated from the 45 samples by filtering the chimera. In total, ~78,172 reads were obtained from each sample for further analysis, and the average read length was 415 bp. Then, OTU clustering was performed with 97% identity and 1,468 OTUs were obtained by removing those with low abundance (threshold, 0.005%). These were then annotated with the species from the Silva132 database. The present results suggested that a total of 738 (50.27%) OTU annotations were made at the genus level. Furthermore, there were significant differences in α -diversity between the IP and control groups on day 4 (P<0.05 and P<0.01; Table I). The Shannon index and Chao1 were lower in the IP group on day 4 compared with the control group, while there were no significant differences between the oral group and control group.

Taxonomic differences between groups at different time points. The relative abundances of OTUs from different groups at different time points were compared, and the relative abundances of the top ten bacterial species at the level of phylum and family are presented in Fig. 2. The present results suggested that almost all OTUs belonged to the following five phyla: Bacteroidetes, Firmicutes, Proteobacteria, Deferribacteres and unidentified_Bacteria, accounting for >99% of the total OTUs. The main taxa at the phylum level, family level and genus level at different time points (days 2, 3 and 4) in the three groups were compared (Fig. 3). At the phylum level, the oral group showed a higher relative abundance of Bacteroidetes and Proteobacteria on day 2 compared with the control group and IP group (P<0.05), but had lower abundance of Firmicutes compared with the control group

Table I. α -diversity indices of the gut microbiota from the three groups at different time points.

Time	Group	Average length of sequence, bp	Effective reads	Shannon index	Simpson index	Chao1
Day 2	C	415	80140	6.74±0.58	0.97±0.02	575.21±36.30
	IP	413	80133	6.51±0.35	0.98±0.01	552.17±20.87
	Oral	415	80146	6.71±0.20	0.98±0.01	545.29±35.02
Day 3	C	417	82278	6.64±0.21	0.97±0.01	574.90±116.48
	IP	418	80872	5.96±0.41	0.98±0.01	517.84±127.05
	Oral	418	72138	6.65±0.51	0.97±0.01	532.22±155.22
Day 4	C	415	80225	6.73±0.31	0.97±0.01	600.88±96.70
	IP	415	80170	6.02±0.21 ^a	0.97±0.01	424.79±29.74 ^b
	Oral	413	80151	6.49±0.33	0.97±0.01	634.16±82.14

^aP<0.05, ^bP<0.01 vs. control group. IP, intraperitoneal; C, control.

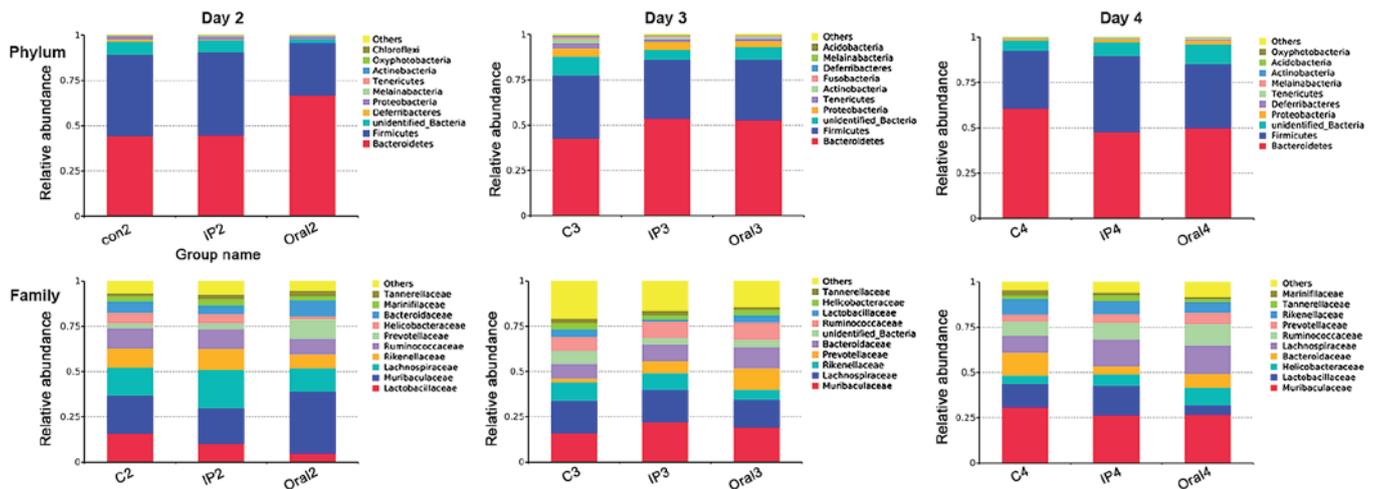


Figure 2. Relative percentage abundance of the main taxa at the phylum level and family level in the three groups. Relative percentage abundance was assessed using 16S high-throughput sequencing at days 2, 3 and 4. The sequences that could not be classified into any known group were designated as 'unclassified_Bacteria'. C, control; IP intraperitoneal.

($P < 0.05$). The present results suggested that on day 4, the relative abundance of Melainabacteria in the oral group was significantly higher compared with the control group and IP group ($P < 0.05$). At the family level, on days 2 and 3 the relative abundance of Lactobacillaceae in the oral group was significantly lower compared with the control group ($P < 0.01$ and $P < 0.05$, respectively); while it was lower in the IP group compared with the control group, the difference was not statistically significant. On days 2 and 3, the relative abundance of Prevotellaceae bacteria in the oral group was higher compared with the control group ($P < 0.05$). The relative abundance of Muribaculaceae, belonging to the phylum Bacteroidetes, on day 2 in the oral group was significantly higher compared with the control group and IP group ($P < 0.05$). In addition, the present results suggested that the relative abundance of Lachnospiraceae on day 4 in the oral group was significantly higher compared with the control group. At the genus level, the relative abundances of *Lactobacillus* and *Alistipes* in the oral group on days 2 and 3 were significantly lower compared with

the control group ($P < 0.05$ and $P < 0.01$). By contrast, the relative abundance of *Alloprevotella* was significantly higher in the oral group compared with the control group and IP group ($P < 0.05$ and $P < 0.01$). The proportion of *Escherichia-Shigella* was significantly higher in the IP group and oral group on days 2, 3 and 4 compared with the control group ($P < 0.05$ and $P < 0.01$).

β -diversity. β -diversity describes the microbial community composition and can be used to evaluate the differences among microbial communities (22). In the present study, PCoA analysis was performed based on the weighted UniFrac distance, and then the main coordinate combination with the highest contribution rate was selected for plotting. The present results suggested that there were different distribution styles between the IP and oral groups at each sampling point (days 2, 3 and 4). On day 2, PC1 represented 67.69% of the variation between all the groups, and the main coordinate analysis could distinguish the oral group from the other two groups (Fig. 4A).

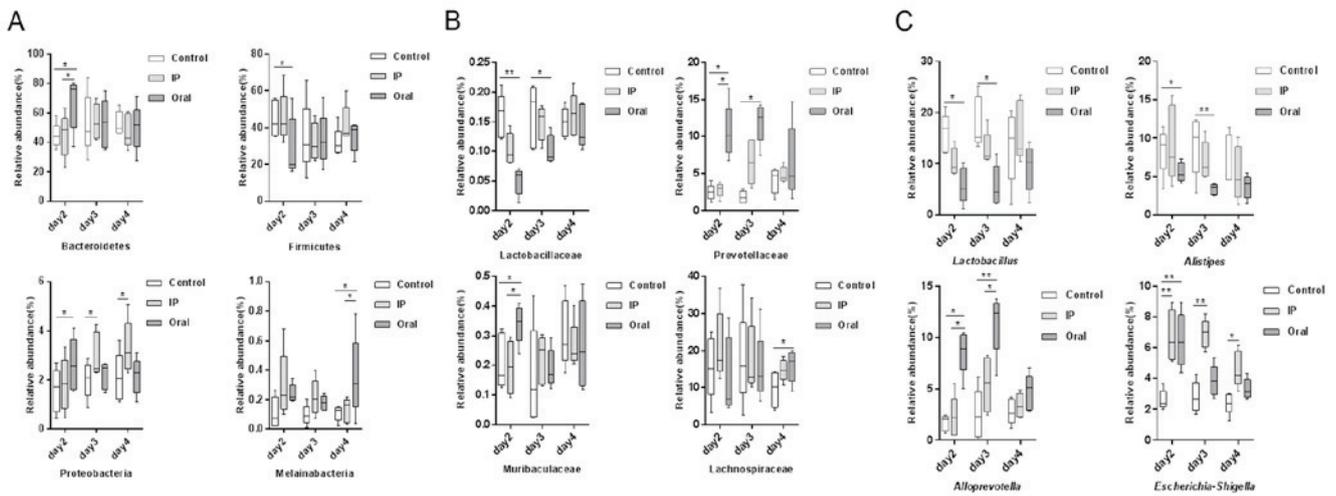


Figure 3. Comparison of the relative abundance in the main taxa among the three groups at different time points. Relative abundance at (A) the phylum level, (B) family level and (C) genus level. *P<0.05, **P<0.01. IP, intraperitoneal.

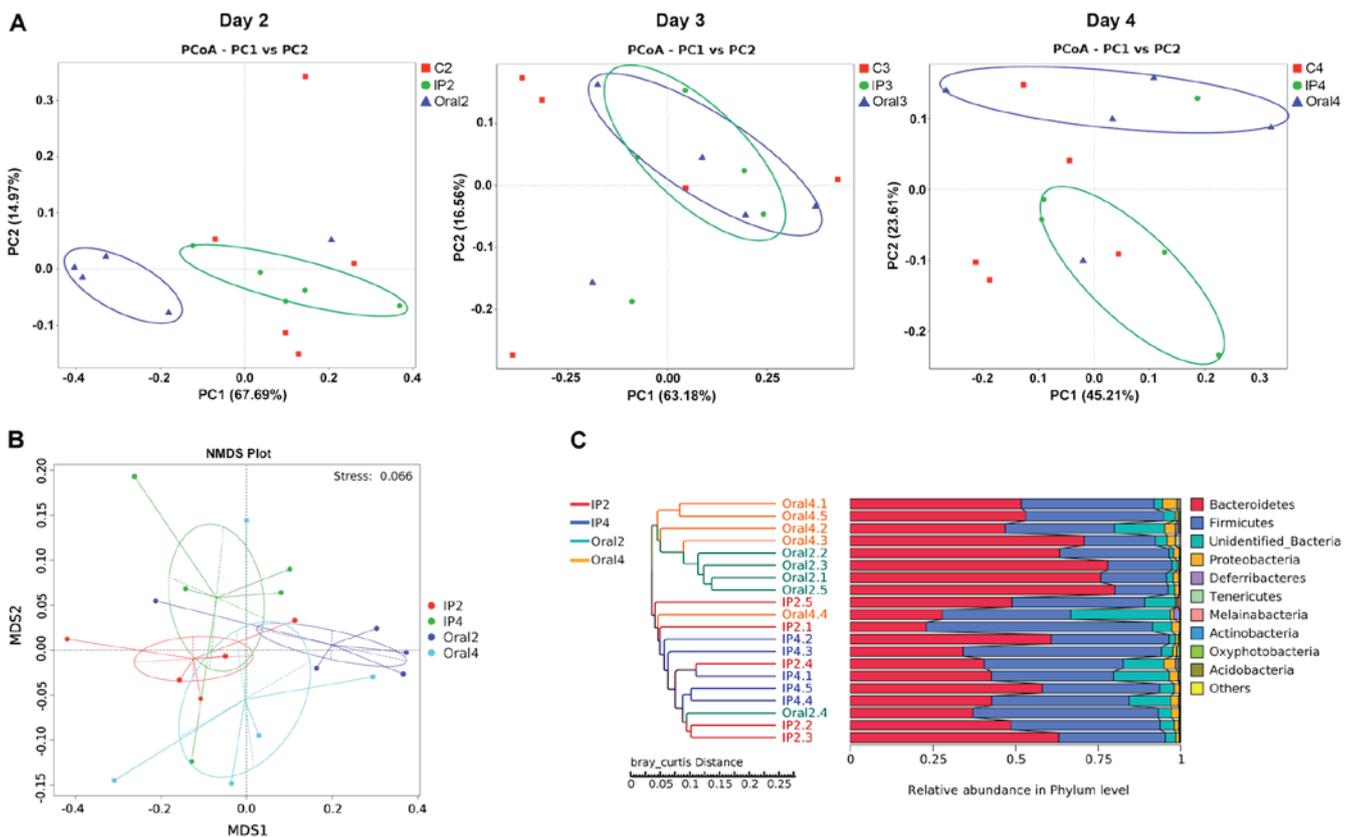


Figure 4. Analysis of the time-course of *S. flexneri* infection in mice. (A) PCoA of fecal microbiota on days 2, 3 and 4, based on the weighted UniFrac dissimilarity index. (B) Non-metric multidimensional scaling plot for the gut microbiota of the IP group and oral group on days 2 and 4, based on the weighted UniFrac dissimilarity index. (C) Hierarchical cluster analysis of operational taxonomic units in the IP group and oral group on day 2 and 4, based on the Bray-Curtis dissimilarity. IP, intraperitoneal; PCoA, principal coordinate analysis; NMDS, non-metric multidimensional scaling; MDS, metric multidimensional scaling.

Furthermore, the coordinates of the samples in the IP group and control group were similar. On day 3, the coordinates for the IP group overlapped with those of the oral group (Fig. 4A). On day 4, the positions of the IP group and the oral group were separated, and their coordinates were different compared with the control group, indicating a PC1 value of 45.17% between the three groups (Fig. 4A). NMD analysis showed

the degree of isolation of intestinal flora between the groups at different time points. The present results identified varying degrees of location differences between the IP group and the oral group on days 2 and 4 (Fig. 4B). The hierarchical cluster analysis of OTUs indicated that the IP and oral groups were well-separated. The present results suggested that the samples in the oral group (Oral2 and Oral4) were clustered according

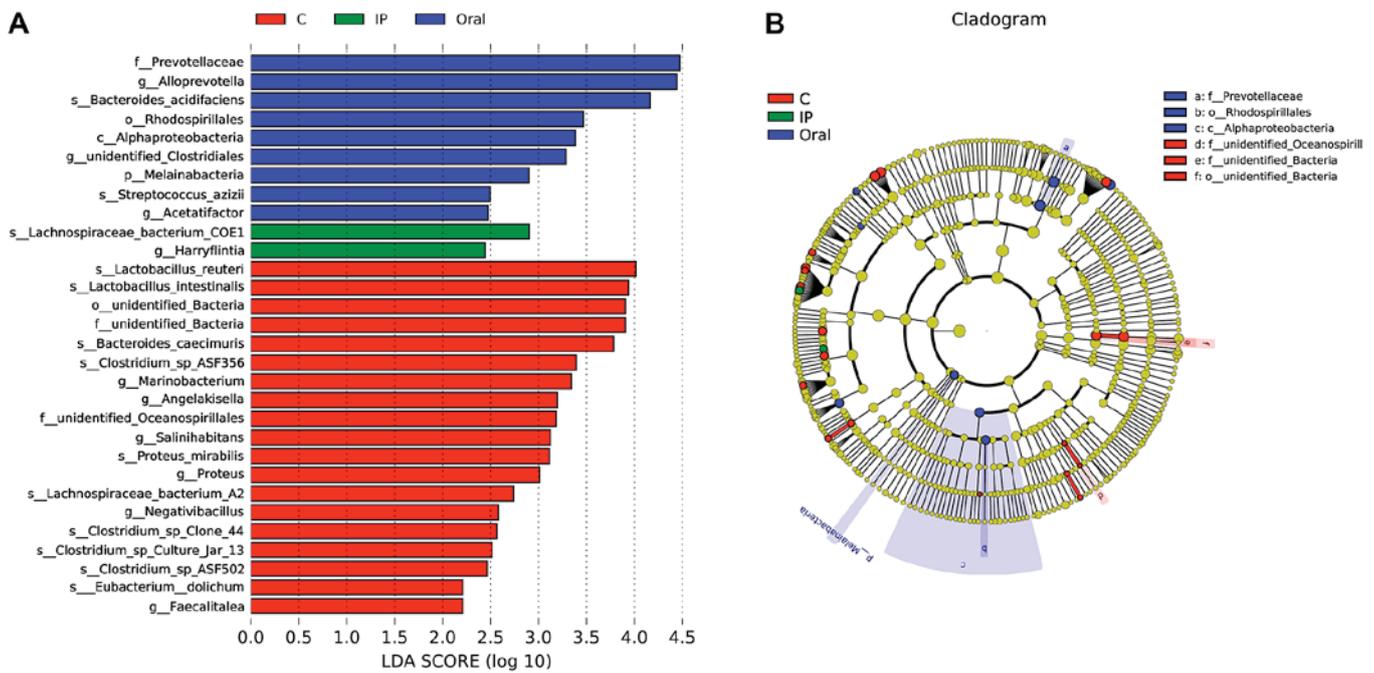


Figure 5. Microbial biomarkers among the different groups. (A) Linear discriminant analysis Effect Size analysis indicated the differentially abundant taxa as biomarkers. Kruskal-Wallis test produced $P < 0.05$ and an LDA score of > 2.0 . (B) The cladogram of taxa abundances in different groups. Taxa without significant differences are labeled in yellow, while significantly different taxa are labeled using the color of each group, red represents control, green represents the IP group and blue represented the oral group. C, control; IP, intraperitoneal; LDA, linear discriminant analysis.

to the time points, while samples in the IP group (IP2 and IP4) intersected the cluster tree to a certain extent (Fig. 4C).

LefSe analysis. LefSe, the computational approach to biomarker class comparisons contributes to the understanding of microbial communities (25). In order to assess the gut microbial responses associated with *S. flexneri* infection at the taxonomic level, microbial clade differences were determined using LefSe analysis. The present results suggested that a total of 19 bacterial taxa, such as *Lactobacillus reuteri*, *Lactobacillus intestinalis*, *Bacteroides caecimuris* and *Faecalitalea*, had a higher abundance in the control group compared with the IP group and oral group (Fig. 5A). However, the relative abundance of nine taxa of bacteria, such as Prevotellaceae, *Alloprevotella* and *Bacteroides acidifaciens*, in the oral group was higher compared with the control group and IP group (Fig. 5A). In addition, only two taxa of bacteria in the IP group were in higher abundance compared with the other groups. The present results suggested that three dominant bacteria were found in the control group, while Prevotellaceae, Rhodospirillales and Alphaproteobacteria were dominant in the oral group (Fig. 5B).

Discussion

Infections with *Shigella* spp. are usually self-limiting and confined to the mucosa of the distal ileum and the colon (26). However, in rare cases, parenteral infection can lead to bacteremia, multiple organ infections and abscesses (7). In order to investigate the differences in the effects of *Shigella* intestinal infection and parenteral infection on gut microbiota, and the possible role of gut microbiota in the different infection routes, a *Shigella* infection mouse model was established

by IP injection and gavage in the present study. The present results suggested that adult mice infected by oral infection and intraperitoneal injection exhibited different disease outcomes. Oral infection gradually self-cured within 3 days, while the IP infection developed into persistent diarrhea and other symptoms of dysentery. In the IP group, *S. flexneri* was constantly detected in the feces and a certain proportion of mice died, which is consistent with previous results from Yang *et al* (13) and Sharma *et al* (27). Furthermore, 4 day old mice (28) and young mice treated with antibiotics (29) are frequently used to construct animal models of shigellosis via oral administration. However, both these models neglect the role of the underdeveloped gut microbiome in the pathogenesis of shigellosis. By contrast, the adult mice included in the present study had a developed gut microbiome, which may better reflect the *in vivo* effects of *Shigella* on the gut microbiome.

α -diversity indexes, including Chao1, Shannon, Simpson, Good's coverage and the Abundance-based Coverage Estimator, can be used to analyze the abundance and diversity of microbial communities (30). In the present study, no significant change in α -diversity was identified after oral *Shigella* infection in mice. A possible explanation may be that *Shigella* was cleared in a relatively short time under the action of the intestinal immune system of mice (31); hence, the effect of *Shigella* on the intestinal flora was short-lived and limited. Only Bacteroidetes, Firmicutes and Proteobacteria with high relative abundance were significantly affected, while other bacteria with low abundance were less affected. Therefore, the present results suggested that there was no significant change in the α -diversity of the gut microbiota after oral infection. In addition, the α -diversity in the IP group was unchanged until day 4, which was 3 days after *Shigella* infection induction. In the present study the dose challenge in the IP group was set as 5×10^7 CFU in order to reduce the

incidence of mortality; this dosage was lower than that used by both Yang *et al* (13) and Sharma *et al* (27), at 5×10^8 CFU and 10^8 CFU, respectively. In these previous studies, all mice in the IP group died within 7 days (13,27). Although in the present study, the symptoms of dysentery were pronounced and persistent, a dose-dependent relationship may exist between *S. flexneri* and the diversity of the gut microbiota.

To investigate the effect of *Shigella* infection on the relative abundance of specific microbiome taxa, relative abundances of the species in each group on days 2, 3 and 4 were analyzed in the present study. The present results suggested that the bacteria taxa of the oral group significantly varied compared with the control and IP groups. On days 2 and 3, the relative abundances of Prevotellaceae and *Alloprevotella* were higher compared with the IP group and control group, while Firmicutes, Lactobacillaceae and *Lactobacillus* had lower abundances compared with the IP group and control group. On day 4, there was no significant difference between the oral group and the other two groups. Therefore, the present results suggested that the effect of oral infection on microbiome taxa in mice was relatively rapid, since this route of infection directly exposes the gut microbiome to *S. flexneri*. Probiotics such as *Lactobacillus* may inhibit the proliferation of *Shigella*, and are constantly consumed when fighting against *Shigella*, which results in significant decreases in their abundance (32). Several mechanisms have been suggested for the inhibitory activity of lactic acid bacteria against pathogenic bacteria, especially Gram-negative pathogens (33). These mechanisms include the production of organic acids, hydrogen peroxide and bacteriocin, as well as the competition for colonization sites with pathogenic bacteria (32,33). The protective effects of *Lactobacilli*, probiotic bacteria, are greater against invasive bacteria such as *S. sonnei*, when compared to non-invasive bacteria such as *Vibrio cholerae* (34). However, the mechanism of the inhibitory effect of lactic acid bacteria on *Shigella* has been mostly investigated *in vitro* (35,36), and it is not fully understood how these inhibitory effects are exerted on pathogenic bacteria in the complex intestinal microecological environment. These inhibitory effects may be associated with decreases in host gene expression, microRNA regulation and a substantial reshaping of the *Listeria-monocytogenes* transcriptome (37). In the present study, on days 2 and 3, the abundance of Prevotellaceae significantly increased in the oral group compared with the control group. A similar trend was also observed in the IP group, indicating that the abundance of Prevotellaceae was affected by *Shigella* infection. Certain *Prevotella* strains have been reported to serve as clinically pivotal pathobionts that participate in human diseases by promoting chronic inflammation, such as in intestinal disorders with HIV infection (38), irritable bowel syndrome (39), rheumatic arthritis (40) and periodontitis (41). Therefore, the increased abundance of *Prevotella* after *Shigella* oral infection observed in the present study likely co-induces inflammatory responses via pathogenic bacteria and recruitment of inflammatory cells. However, the specific mechanism needs to be further investigated.

The present PCoA analysis results suggested that at the initial stage of infection (day 2), the coordinates of the samples in the IP group and the oral group were divided into two distinct groups. At day 3, the distribution differences between the two groups reduced, but the gap between the two groups

broadened at day 4, indicating that *Shigella* infection could affect the structure and composition of the intestinal flora at different time points. In addition, this effect correlated with the different symptoms and disease outcomes. For example, on the second day following oral infection of *Shigella* appeared to be the initial stage of shigellosis, but the symptoms had completely disappeared at day 4, while days 2-4 after IP infection indicated the progression of symptoms. The present results suggested that there were differences in shigellosis outcomes between the two infective pathways, based on the examination of intestinal flora. In addition, it has been shown that the invasion of *Shigella* into the large intestine via the abdominal cavity is by migration in the serosa and muscular layer, rather than via the blood circulation, suggesting that the early invasion of the pathogen from the abdominal cavity into the intestinal tract can escape the endogenous defense system of the host (13). The present results suggested that the influence of *Shigella* infection on intestinal flora is weak and slow at the early stage, but gradually becomes more pronounced with the development of bacteremia.

The present LEfSe analysis results suggested that *Lactobacillus reuteri* and *Faecalitalea* may be biomarkers of the control group. Previous studies have demonstrated that these bacterial groups are probiotics with certain protective effects, such as eliminating infections, attenuating both GI diseases and diseases in remote tissues, producing lactate and butyrate (42,43). The direct supplementation and prebiotic modulation of *Lactobacillus reuteri* may be an attractive preventive and therapeutic strategy against inflammatory diseases (43). The strain *Lactobacillus reuteri* WHH1689 has no lactose utilization capability and exhibits a high survival rate during storage at room temperature in drinkable yogurts (44). In addition, this strain has shown great resistance to conditions that simulate the gastrointestinal tract, including strong adherence to HT-29 cells and inhibitory activities against *Escherichia coli*, *S. flexneri*, *Salmonella paratyphi* β and *Staphylococcus aureus* (44). *Faecalibacterium prausnitzii* can inhibit the release of IL-8 and exert anti-inflammatory effects (45). *Faecalibacterium prausnitzii* can also inhibit the invasion of pathogenic bacteria via colonization resistance (46). Therefore, further research and clinical intervention evaluations are required to investigate the use of probiotics for the prevention and treatment of *Shigella* infection. One of the biomarkers in the IP group was Lachnospiraceae bacterium COE1. The members of the Lachnospiraceae family are known to influence the development of obesity and diabetes in mice with a genetic susceptibility to obesity (47). In addition, long-term high-fat feeding causes obesity-related inflammation of the ileum and colon, and increases the expression of catenin, a colon cancer risk factor, in the colon accompanied by an increase of Lachnospiraceae and Streptococcaceae abundance in the hindgut of C57BL/6 mice (48). Therefore, the increased abundance of Lachnospiraceae may affect intestinal metabolites, which reflect the intestinal metabolic response to *Shigella* infection.

In conclusion, the present results suggested that *S. flexneri* infection in mice can influence the profile of the gut microbiota, and the change of some specific taxa may reflect the results of *Shigella*-microbiota interaction, such as the decrease abundance of probiotic *Lactobacillus* and the increased

abundance of Prevotellaceae. The oral and IP challenges of *S. flexneri* exerted different effects on the intestinal flora, including on the diversity, relative abundance and composition of the microbiota. The present results suggested that intestinal flora may serve as a barrier to *Shigella* transoral infection, while parenteral infection results in serious clinical manifestations due to the absence of gut microbiome inhibition. In future studies, metabonomics, metagenome and transcriptomics are required to characterize the precise mechanism of interaction between *Shigella*, the host and the gut microbiota.

Acknowledgements

Not applicable.

Funding

This study was financially supported by a grant from The Open Project of Key Laboratory of Environmental Factors and Cancer of Fujian Medical University (grant no. GWSZD-201801).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JY and WC participated in raising the animals, DNA extraction and drafting of the manuscript. PX performed the statistical analysis and bioinformatics analysis. WZ participated in the study design and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The current study was approved by The Institutional Animal Care and Use Committee of Fujian Medical University. All animal care and experimental procedures were conducted according to the institutional ethical guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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