Bacterial Characteristics in Intestinal Contents of Antibiotic-Associated Diarrhea Mice Treated with Qiweibaizhu Powder

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Background: Qiweibaizhu powder (QWBZP) is a classical prescription of traditional Chinese medicine (TCM) to treat diarrhea in pediatric patients. Its use in health care practices and interventions has shown its effect on antibiotic-associated diarrhea (AAD). It is known that the occurrence of AAD is related to an imbalance of intestinal microbiology. Previous studies found that QWBZP could regulate the amount of some cultured microbes and the activities of lactase and sucrase in AAD mice. In order to investigate the treatment mechanism of QWBZP on AAD, we studied the effect of QWBZP on intestinal bacteria in a community of AAD mice.

Material/Methods: AAD mice were established by administrating the mixture of gentamycin sulfate and cefradine at the dose of 23.33 mL·kg⁻¹·d⁻¹ for 5 days. Then the AAD mice were gavaged with QWBZP decoction for 4 days and gradually recovered to a normal status. On the tenth day, the intestinal contents of mice were collected, and then the DNA was extracted for 16S rRNA sequencing followed by analysis.

Results: The analysis of bacterial 16S rRNA sequencing showed the Simpson index was decreased and the Shannon index was increased in AAD mice treated with QWBZP compared to the model group; there was no significant difference between the control group and the treatment group (P>0.05). Principle co-ordinates analysis (PCoA) indicated that there was a shorter distance between the control group and the treatment group than that between the control group and model group. At the phylum level, use of antibiotics decreased the relative abundance of Actinobacteria, Bacteroidetes, and Proteobacteria, but increased the abundance of Firmicutes and Verrucomicrobia, and the reverse changes occurred after treated with QWBZP. At the genus level, the abundance of Bacteroides and Ochrobacitrum increased in the model group, while an opposite result was observed in the treatment group. Moreover, the relative abundance of Osillospira decreased in the model group and increased in the treatment group. Genus Dorea, Coprococcus and Blautia in the model group were higher than those in the control group and further increased in the treatment group.

Conclusions: These results indicated that QWBZP improved the diarrhea syndrome with restoring the diversity and adjusting the structures of bacteria in mice intestine, which might reveal the therapeutic mechanism of QWBZP on treating AAD.

MeSH Keywords: Bacteria • Diarrhea • Enterocolitis, Pseudomembranous • Medicine, Chinese Traditional • Qiweibaizhu Powder

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All these aforementioned studies revealed that QWBZP can regulate intestinal micro-ecology of mice with AAD. In this study, the effect of QWBZP on the diversity and structure of the bacterial community in AAD mice was investigated by metagenomic analysis using 16S rRNA high-throughput sequencing, which may help identify the related characteristic bacteria and further elucidate the therapy mechanism of QWBZP on AAD.

**Material and Methods**

**Reagents and medicine**

Cephradine capsules (batch number: 40115028) were purchased from Suzhou Chung-HWA Chemical & Pharmaceutical Industrial Co., Ltd. Gentamycin sulfate injection (batch number: 1150425A6) was produced by Yichang Humanwell Pharmaceutical Co., Ltd. The antibiotics mixture was prepared with cephadine capsules and gentamycin sulfate at 1:2 (wt:vol) 1 hour before use. Herbs for QWBZP were purchased from The First Affiliated Hospital of Hunan University of Chinese Medicine. All the herbs were processed as follows: immersed in water in a gallipot for 30 minutes before heating to boil, and then stewed gently for 30 minutes. The decoction was filtered to separate the residue. Water was added to the residue again for a second extraction. The filtrates obtained from the 2 cycles were mixed and concentrated and stored at 4°C for future experiments.

**Animal models of diarrhea and treatment**

Healthy adult mice were purchased from Hunan Slaccas Jingda Laboratory Animal Company. All involved animals were handled in accordance with the protocols approved by the Institutes Animal Care and Use Committee (the approval number: 00088885). Eighteen mice, with equal number of male and female mice, involved in the experiment were fed normally for 3 days to adapt to the environment before the experiment. To prepare diarrhea models, 6 males and 6 females were randomly selected. They were administrated with the prepared antibiotics mixture twice a day for 5 days (at the dose of 23.33 mL·kg⁻¹·d⁻¹). The remaining 6 mice were used as control group (qck). Mice administrated with antibiotics mixture exhibited symptoms of watery stools gradually, reduced food intake, depressed, erected hair. Then the antibiotics were stopped and then these diarrheal mice were randomly divided into 2 groups with equal number of male and female in each group: model group (qm) and treatment group (qq). From the sixth day to the eighth day, the mice in the treatment group (qq) received 0.16 g·kg⁻¹·d⁻¹ of QWBZP decoction orally twice a day. Mice of the model (qm) and control group (qck) were administrated with an equal volume of...
distilled water. During the experiment, all involved mice were fed as normal. On the ninth day, mice in the 3 groups were sacrificed, their intestinal contents were collected respectively under sterile conditions. Two mice content samples (random selected 1 male and 1 female) in the same group were mixed and put in a sterile tube and stored at –80°C. Then the total 9 metagenome DNA samples was extracted from the 3 groups, according to a previously described procedure.

Polymerase chain reaction (PCR) of intestinal content bacterial gene and 16S rRNA sequencing

Polymerase chain reaction (PCR) was performed targeting the V4 region of the 16S rRNA gene of bacteria using the forward primer 520F (5′-AYTGGGYDTAAAGNG-3′) and reverse primer 802R (5′-TACNVGGGTATCTAATCC-3′). Primers were designed and synthesized by Shanghai Personal Biotechnology Co., Ltd. The PCR mixture (25 μL) consisted of 8.75 μL of sterile ddH2O, 5.0 μL of 5×Q5 reaction buffer, 5.0 μL of 5×Q5 GC high enhancer, 2.0 μL of dNTP (2.5 mM), 2.0 μL of template DNA (20 ng/μL), 1.0 μL of each primer (10 μM), and 0.25 μL of Q5 polymerase (5 U/μL). PCR were carried out as follows: initial denaturation at 98°C for 5 minutes, following 27 cycles at 98°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. PCR products were examined by 2% agarose gel electrophoresis. The purified products were sequenced using Illumina MiSeq system by Shanghai Personal Biotechnology Co., Ltd.

Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics software version 21. Data were presented as mean ± standard deviation (SD). Unpaired Student’s t-tests were used to compare the means of 2 groups, one-way ANOVA analysis were used to compare the means of 3 groups, and P<0.05 was considered to indicate statistical significance.

Results

QWBZP affected the diversity of bacteria in AAD mice intestine

There were totally 1 363 490 effective sequences and 1 254 848 high-quality sequences in all samples acquired by sequencing. The length of high-quality sequences consistent with the expected bacterial product by primers (Figure 1), and the rarefaction curves tending to be plat (Figure 2) indicated that the data could be used for the following analyses.

By paired-end sequencing, a total of 1936 operational taxonomic units (OTUs) were obtained from the 9 samples. There were 970, 952, and 1565 OTUs in the control, model, and treatment groups separately, and 538 OTUs coexisting in the 3 groups. Community diversity indices such as Chao, ACE, Simpson, and Shannon in each sample are listed in Table 1. Compared to the control group, the model group had higher Simpson index and lower Shannon index, the differences between the model and control groups were significant (t=-7.163, P=0.02; t=7.019, P=0.02), and the Simpson and Shannon indices in the treatment group recovered to the level of the control group. There was no significant difference between the 3 groups in Chao and ACE indices (P>0.05).

QWBZP changed the community structure of intestinal bacteria in AAD mice

Principle co-ordinates analysis (PCoA) can reflect dissimilarities of microbial community among the samples. The degree of differentiation in the bacterial community of the 3 groups
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Table 1. Alpha biodiversity indices of bacteria in contents of mice intestine.

| Group   | Chao        | ACE         | Simpson    | Shannon    |
|---------|-------------|-------------|------------|------------|
| qck     | 752.760±114.412 | 753.942±110.852 | 0.035±0.007 | 4.418±0.161 |
| qm      | 677.687±139.170  | 834.488±107.018  | 0.113±0.017* | 2.862±0.348* |
| qq      | 770.143±452.620  | 766.822±437.306  | 0.067±0.025  | 3.736±0.804  |

Data are presented as mean±standard deviation (SD) of 3 samples in each group. Compared to the control group: *P<0.05.

qck – represents the control group; qm – represents the model group; qq – represents the treatment group.

Figure 3. Multiple sample principle co-ordinates analysis (PCoA). PCoA of samples showing 3-dimensional sort graph based on weighted Unifrac. Each dot represents a sample. Dots with the same color belong to the same group. The closer the 2 dots, the smaller difference between bacterial community of the 2 samples. The first principle component axis (PC1) explained 58.22% of the variance in the data, the second principle component axis (PC2) explained 23.09% while the third principle component axis (PC3) explained only 7.19%. qck represents the control group, qm represents the model group, qq represents the treatment group.

QWBZP regulated the relative abundance of intestinal bacteria in AAD mice

Based on the information of the top 100 OTUs in the OTU table compared with the NCBI database, an information graph indicating species evolution and relative abundance was created. The evolution tree gave a visual representation of the bacterial evolution relationship and the differences of the relative abundance among groups. As shown in Figure 5, the control group had a higher proportion of Osilospira, Lactobacillus, Coprobacillus, Coriobacteriaceae, Adlercreutzia, Desulfovibrionaceae, Helicobacteraceae, Rikenellaceae, Prevotella, and Ruminococcaceae than those of the model and treatment groups.

Moreover, the difference in the relative abundance of Osilospira, Rikenellaceae, and Ruminococcaceae between the model and control groups was significant (t=2.898, P=0.044; t=3.225, P=0.032; t = –3.499, P=0.025). Compared with the control and treatment groups, the model group had the highest abundance of Clostridiaceae, Peptostreptococcaceae, Epulipiscium, Ochrobactrum, Verrucomicrobiaceae, Akkermansia, Porphyromonadaceae, Bacteroides, and Parabacteroides, while the differences in the relative abundance of Clostridiaceae, Peptostreptococcaceae, and Bacteroides among the 3 groups were significant (P=0.015; P=0.001; P=0.002). The relative abundance of Coprococcus, Blautia, Dorea, Alcaligenaceae, and Sutterella in the treatment group was markedly higher than those of the control and model groups. There was great difference in the abundance of Alcaligenaceae, and Sutterella between the model and control groups (t=3.751, P=0.020; t=3.766, P=0.020), while no statistical difference appeared between the control group and treatment group.

was identified. The distinctiveness of distances between the samples of the 3 groups was greater than the difference between samples of the same group. Figure 3 shows that the deviation of dots representing the model group from that of the control group was great, while the distances between the dots of the control group and treatment group were short, this demonstrated that QWBZP can adjust community structure of bacteria to a normal level.

QWBZP modulated the growth of certain intestinal bacteria in AAD mice

Histograms illustrating the gut microbial community structure revealed the microbial species and their relative abundance. There were 14 detected phyla with relative abundance over 0.1% in all samples. Bacteroidetes and Firmicutes were the dominant phyla, followed by Actinobacteria, Proteobacteria, and Verrucomicrobia in the 3 groups (Figure 4A). Compared to other groups, the model group had the highest abundance of Firmicutes and Verrucomicrobia, and the lowest abundance of Actinobacteria, Bacteroidetes, and Proteobacteria, but there was no significant difference among the groups. There were 41 genera identified in all samples. The proportion of S24–7 unclassified was the highest in the control and treatment group while Bacteroides was the dominant genus in the model group (Figure 4B).

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QWBZP has been used safely and effectively to treat diarrhea in the past thousand years. The clinical application of QWBZP also confirmed its curative effect on AAD. In present study, there were more OTUs obtained, higher Shannon index and lower Simpson index in treatment group compared to the model group, PCoA indicated a shorter distance between the control and treatment groups than that between the control and model groups. All the information indicated that QWBZP could increase the diversity of bacteria and played a key role in regulating bacterial community in the treated AAD mice.

The current work also showed that the relative abundance of Proteobacteria and Actinobacteria in treatment group was higher than that in the model group, this indicated that QWBZP could promote the proliferation of Proteobacteria and Actinobacteria. Lu revealed that Actinobacteria, Firmicutes and Proteobacteria were the main phyla producing lactase [19], the decrease of lactase activity was associated with AAD. It is possible that QWBZP can promote proliferation of some bacteria producing lactase.

To investigate bacterial communities associated with the QWBZP therapy for AAD, we focused on identifying bacterial genera. Figure 5 indicated that the treatment group had lower proportion of Bacteroides, Ochrobactrum, Lactobacillus and higher abundance of Prevotella, Oscillospira, Dorea, Coprococcus, Blautia than those in the model group. Among these bacterial genera, Bacteroides is a commensal genus in people intestinal tract includes some opportunistic pathogens leading to endogenous infection when the ecological balance in intestine is broken, such as Bacteroides fragilis causing diarrhea [11–13]. In the study, Bacteroides was the most abundance genus in the model group, the relative abundance of Bacteroides in treatment group decreased to a quarter of the model group and restored to the control group level, the information implied that QWBZP could regulate the imbalance of Bacteroides strains to the relative balance. Lactobacillus, recognized as probiotics, can produce lactic acid improving the intestinal environment to prevent the adhesion of harmful bacteria and secret lactase to decompose lactose [20,21], but the abundance of Lactobacillus furtherly decreased in the treatment group, this showed that QWBZP have little effect on proliferation of...
**Lactobacillus. Ochrobactrum** must survive in the aerobic condition with the strong drug resistance to β-lactam antibiotics, which relative abundance in model group was higher than that in the control group also indicated its resistance to antibiotics, and QWBZP could inhibit its growth.

Genera **Oscillospira, Dorea, Coprococcus, and Blautia** are members of phylum **Firmicutes**, they are strictly anaerobic and can ferment carbohydrate to provide nutrition to the host [22–27]. Compared to other groups, the relative abundance of these bacteria in treatment group was the highest, it suggested that QWBZP could proliferate these bacteria groups to maintain gut integrity.

**Conclusions**

Our analysis indicated that QWBZP had an effect on the diversity and constitution of the bacterial community in AAD mice. The relative abundance of bacteria was adjusted by QWBZP. The prescription inhibited the growth of **Ochrobactrum** and **Bacteroides** and promoted the growth of **Oscillospira, Dorea, Coprococcus**, and **Blautia**. The results revealed there are relations between the efficacy of QWBZP and the intestinal bacteria. These findings can provide a reference for AAD treatment and the therapeutic mechanism of QWBZP. However, further studies should be performed to clarify what role bacteria play during AAD treatment with QWBZP.
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