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Original Research Article

Molecular Diversity of Intestinal Microbiota in Dysbacteria Diarrheal Mice Associated with Ultra-micro Qiweibaizhusan

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Keywords

Qiwei-baizhusan; Ultra-micro Chinese medicine; ARDRA; intestinal microflora; metagenome; molecular diversity.

A B S T R A C T

To study the micro-ecological mechanism of curative effect of ultra-micro Qiweibaizhusan on mice with dysbacteria diarrhea. The mice model with dysbacteria diarrhea was constructed by gavaging mixed antibiotics composing of gentamycin sulfate and cefradine for 5 days. And then, all the mice were fed with sterile water, 100% dose of traditional Qiweibaizhusan and 50% dose of ultra-micro Qiweibaizhusan for 3 days. At the same time, normal group mice were fed with sterile water. The faecal samples respectively were collected from intestinal tracts of mice. The metagenome DNA of intestinal microbiota was extracted and the 16S rDNA was amplified from these DNA samples through a set of bacterial universal primers. The PCR products were digested by *Hha* I、*Rsa* I、*Sam* I and *Hind* III. And the molecular diversity of intestinal microbiota in mice was analyzed by Amplifed Ribosomal DNA Restriction Analysis (ARDRA) .The bands of 16S rDNA from intestinal microbiota in dysbacteria diarrheal model mice were 3. And the bands of 16S rDNA from intestinal microbiota in dysbacteria diarrheal mice cured with both 100% dose of traditional Qiweibaizhusan and 50% dose of ultra-micro Qiweibaizhusan were 5. The bands of 16S rDNA from intestinal microbiota in normal group mice were 7. The diversity of intestinal microbiotia in model mice was 0.7324. The diversity of intestinal microbiotia in mice cured with both 50% dose of ultra-micro Qiweibaizhusan and 100% dose of traditional Qiweibaizhusan was 1.2207. The diversity of intestinal microbiotia in normal mice was 1.7090. Compared with the control group, the similarity of intestinal microbiotia in model mice was 40%, the similarity of intestinal microbiotia in mice treated with 50% dose of ultra-micro Qiweibaizhusan was 67%, and the similarity of intestinal microbiotia in mice treated with 100% dose of traditional Qiweibaizhusan was 50%. Both the 100% dose of traditional Qiweibaizhusan and the 50% dose of ultra-micro Qiweibaizhusan could regulate intestinal microflora in mice with dysbacteria diarrhea, and restore the diversity of intestinal microbes to cure diarrhea with dysbacteriosis caused by antibiotics. The therapeutic effect of 50% dose of ultra-micro Qiweibaizhusan could reach the curative level of 100% dose of traditional Qiweibaizhusan. Microecological thesis and technology was used to evaluate the effect of Chinese medicine. Ultra-micro Chinese medicine can save medicinal material.

Introduction

The Chinese medicine plays an important role in the prevention and treatment of the disease process. And the applications of the ultra-micro powder Chinese herbal

pieces have a very important significance on modernization of Chinese medicine (Wu et al. 2009). The mechanism of curative effect as the primary issue of Chinese medicine was not clear, which should be researched by the modern science and technology, especially new theories and technologies in the modern life science. It would promote the modernization and internationalization of Chinese medicine. There are almost inconceivable number of microorganisms in the adult human intestine, the size of the population is up to 10^{14}, which is a huge micro-ecological system (Backhed et al. 2005). The intestinal microbes are taxonomically complex and constitute an ecologically dynamic community (microbiota) that has been believed to possess a strong impact on human physiology, such as heavily involved in the metabolism of nutrients and organic substrates, the contribution to the phenomena of colonization resistance to pathogens, the modulation of intestinal immune response and the development of intestinal epithelium, vasculature, and lymphoid tissue (Rakoff-Nahoum et al. 2004; Hattori et al. 2009). The intestinal microbes and enzymes were directly involved in the transformation of oral Chinese medicine ingredient, played important roles in the effect of Chinese medicine on diseases (Bae et al. 2003). It is very significant to explore the micro-ecological mechanism of curative effect of ultra-micro Chinese medicine through the intestinal microbes.

Qiweibaizhusan was created by QianYi, a famous doctor in pediatric clinics of Chinese medicine in the northern song dynasty. Qiweibaizhusan can strengthen the spleen, produce saliva could promote the circulation of qi and relieve distension. Qiweibaizhusan was used to treat spleen and stomach deficiency, body fluid internal friction, vomiting and diarrhea frequently, thirst with desire for drinks, and in particular the treatment of pediatric diarrhea. At the moment the curative mechanisms of Qiweibaizhusan were Mainlm from the research in immunology (Yang et al. 2005; Wang 2007).

ARDRA (amplified ribosomal DNA restriction analysis) is the application of RFLP (restriction fragment length polymorphism) in the ribosomal DNA sequences. RFLP is according to the difference of DNA fragment restriction site in different individual or population, using restriction endonuclease to get restriction fragments which are different in length, type and the number, then analyze the restriction fragments. The restriction fragments are complex and difficult to analyze. It is difficult to study microbial diversity. ARDRA , which is combined by PCR and RFLP, is applied to rDNA restriction fragment.

Materials and Methods

Experimental animals

16 Kunming mice (SPF grade, half of male and female) were provided by Experimental Animal Center of Hunan University of Chinese Medicine, license number: SCXK 2009-0004. The weight of each mouse was about 20±2 g. Mouse food was provided by Experimental Animal Center of Hunan University of Chinese Medicine. The mice were randomly divided into 4 groups (4 mice per group): normal group, model group, 100% dose of traditional Qiweibaizhusan group and 50% dose of ultra-micro Qiweibaizhusan group.

Drug treatment

The concentration of gentamycin sulfate and cefradine was 62.5g.L^{-1}. The normal group′s mice were treated with cool boiled water (0.35 mL/mouse) by oral administration with gavage, and the rest of group′s mice were treated with antibiotics mixture (0.35 mL/mouse) by oral administration with gavage, twice a day, for 5 days. Qiweibaizhusan was prepared according to the Chinese Pharmacopoeia, which was composed of ginseng (Shanxi Province) of 6 g, ginseng (Yunnan Province) of 6 g, poria cocos (Yunnan Province) of 10 g, roasted rhizoma atractylodis macrocephalae (Zhejiang Province) of 10 g, agastache (Guangdong Province) of 10 g, pueraria (Hunan Province) of 10 g, liquarice (Neimenggu Province) of 3 g. All herbs were provided by The First Affiliated Hospital of Hunan University of Chinese Medicine. The traditional Qiweibaizhusan water decoction were prepared in appropriate concentration (Yu 2007) and stored at 4ºC. The ultra-micro Qiweibaizhusan were made into water decoction (100% dose) after filter and centrifuge, the concentration was the same as traditional Qiweibaizhusan water decoction, then diluted to 50% dose and stored at 4°C.

The normal group and the model group were treated with cool boiled water during the treatment, the other animals were treated with oral water decoction of Chinese medicine according to the clinical equivalent dosage of mice, the 100% traditional Qiweibaizhusan group were treated with dose of 0.16 $g \cdot kg^{-1} \cdot d^{-1}$ and the 50% dose of ultra-micro Qiweibaizhusan group were treated with dose of 0.08 $g \cdot kg^{-1} \cdot d^{-1}$. All the drugs were orally administered to the mice by a gavage twice a day, for 3 days.

Extraction of intestinal contents

All the mice were sacrificed on the morning of the ninth day after treatment. Mouse intestinal (jejunum to the rectum) and gastric contents were collected in a sterile environment.

Metagenome DNA extraction of intestinal microflora

0.2 g of intestinal contents were weighed in a sterile environment and were homogenized in 30 mL of 0.1 mol/L phosphate buffer solution (PBS) (8 mmol/L Na_2HPO_4, 137 mmol/L NaCl, 2.7 mmol/L KCl, 1.5 mmol/L KH_2PO_4), then were centrifuged for 2 min at 200 g, after washed twice by sterile water the supernatant were collected and transferred to fresh tubes and centrifuged for 8 min at 10000 g. The pellets were washed once with PBS, twice with acetone and three times with PBS, then resuspended in 4 mL TE buffer (pH 8.0) (Zhong et al. 2003). 500 μL of bacteria resuspension were mixed with 45 μL of TE buffer, 5 μL of proteinase K and 20 μL of lysozyme, incubated at 37°C for 30 min, then mixed with 30 μL of 10% SDS, incubated at 37°C for 40 min (vibrated every 10 min), the mixture were incubated at 65 °C for 10 min after which 100 μL of 5 mol/L NaCl and 80 μL of CTAB/NaCl were added and mixed well. An equal volume of Tris-saturated phenol-chloroform-isoamyl alcohol (25:24:1) were added to the sample, mixed well, and the samples were then centrifuged for 3 min at 12000g. The supernatant were transferred to fresh tubes, centrifuged for 3 min at 12000g after an equal volume of chloroform-isoamyl alcohol (24:1) were added and mixed well. The supernatant were transferred to fresh tubes, 1/10 volume of 3 mol/L sodium acetate and double

volume of anhydrous ethanol were added, and tubes were placed at -20°C all night. Tubes were then centrifuged for 10 min at 12000g, The pellets were washed with 70% ethanol, dried and finally resuspended in 50 µL TE buffer(Ausubel, 2008). Samples were electrophoresed in an agarose gel (0.7%) and were visualized under UV light after staining with ethidium bromide, and then photographed.

Universal primer PCR of metagenome DNA

Universal primer 27F(5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Weisburg et al. 1993) were provided by GenScript (Nanjing) Co., Ltd.. dNTP Mixture and Taq Enzyme were provided by TIANGEN BIOTECH (BEIJING) Co., Ltd.. PCR amplification reaction were prepared containing 5 µL of 10×Taq Buffer, 4 µL of 2.5 mmol/L dNTPs Mixture, 1 µL of 10 µmol/L 27F, 1 µL of 10 µmol/L 1492R, 1 µl of 2.5 U/µL Taq Enzyme, 2 µL of Template DNA and 36 µL ddH_2O. Cycling parameters were 1 cycle of 5 min at 94 °C followed by 35 cycles of 45 sec at 94 °C, 45 sec at 52ºC, 90 sec at 72°C. The last cycle was followed by 1 cycle of 10 min at 72°C. PCR products were electrophoresed in an agarose gel (1%) and were visualized under UV light after staining with ethidium bromide, and then photographed.

Amplified ribosomal DNA restriction analysis

PCR products were incubated with *Hha* I, *Rsa*I, *Sam* I and *Hind* III at 37°C for 3h. Incubated products were electrophoresed in an agarose gel (2%) and were visualized under UV light after staining with ethidium bromide, and then photographed.

Polymorphic analysis

The comparison of digested DNA products was performed on the basis of the presence (1) or absence (0) of fragments by using Quantity One software. Bands that faint or less than 100bp are not into account. The pair-wise genetic distance matrix was used for constructing the dendrogram using the UPGMA (Unweighted Pair Group Method with Arithmetic Averages) algorithm.

Diversity and similarity index

The Shannon - Wiener index (H) is a general diversity index that is positively correlated with species richness and evenness, $H=-\sum|ni/N\ln(ni/N)|$. ni is the individual number, N is total individual number in community. Shannon - Wiener index show that more species in community represents more complexity, namely the higher value of H, the more information the community contains. The Similarity was calculated by Sorenson Pairwise Similarity Coefficien (Cs). $Cs = 2j/(a+b)\times100$, S is Sorenson Pairwise Similarity Coefficien; A is the band number of a sample; B is the band number of another sample; J is the band number of two lane Shared. The Sorenson index of Completely different two map 0, and the Sorenson index of completely the same two DNA map is 100%.

Results and Discussion

Metagenome DNA extracted from intestinal flora in mice

Metagenome DNA were extracted from intestinal contents in 15 mice (1 died). And metagenome DNA were electrophoresed in an agarose gel (0.7%) and were visualized under UV light after staining with ethidium bromide (Fig.1).

All of DNA specimens examined were similar, generally demonstrated a band with sizes corresponding to about 23 kb. Gel electrophoresis indicated that DNA concentrations were not the same in different mice because of the discrepancies in the brightness of the bands.

Bacterial universal primer PCR of metagenome DNA

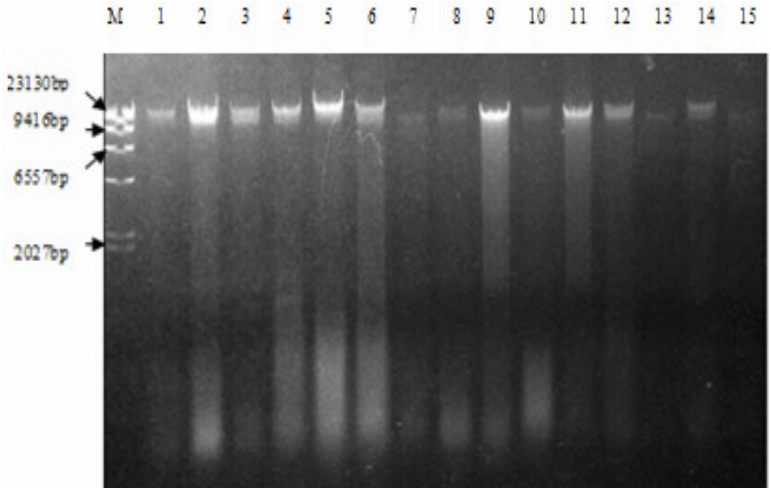
PCR products were electrophoresed in an agarose gel (1%) and were visualized under UV light (Fig.2). Gel electrophoresis resolved one fragment approximately 1.5 kb in size representing the 16S rDNA which had good repeatability and stability.

Amplified ribosomal DNA restriction analysis

The initial study describing the applicability of ARDRA for the identification of species based on 16S rRNA gene sequence. Every group of PCR products were digested by *Hha* I, *Rsa* I, *Sam* I and *Hind* III, The restriction patterns obtained by electrophoresis were shown in Fig. 3. Restriction digestion of the amplified 16S rDNA revealed different profiles among the groups. From the restriction map, each 16S rDNA restriction fragment length polymorphism type was an OTUs, and diversity of OTUs could estimate the minimum number of bacteria in isolate. The number of bands derived from ARDRA patterns analysised by Quantity One software(Fig.4). Table 1 summarized the OTUs of metagenome 16S rDNA derived from ARDRA patterns. Restriction patterns were related to the community diversity. The abundance of the community was indicated by the richness of restriction patterns, and the species quantity were indicated by the brightness of bands (Zhang 2011). The restriction patterns of normal group were more than other groups. It indicated that the community of normal group were more rich. The restriction patterns of the model group were less than other groups, and it showed that the intestinal flora of model group was imbalance. The amount of bacteria greatly reduced. The restriction patterns of the 100% dose of traditional Qiweibaizhusan group and the 50% dose of ultra-micro Qiweibaizhusan group were richer than the model group but not the same rich as the normal group. It indicated that mice intestinal flora recovered but couldn't reach the normal level after treatment of Qiweibaizhusan. The differences between restriction patterns in model group and the two Qiweibaizhusan group were that both the 100% dose of traditional Qiweibaizhusan group and the 50% dose of ultra-micro Qiweibaizhusan group got a band at about 300bp place. The 50% dose of ultra-micro Qiweibaizhusan group got a band at about 400bp place and the 100% dose of traditional Qiweibaizhusan group got a band at about 500 bp place. This showed that different pharmaceutical method had different influence to intestinal microbes.

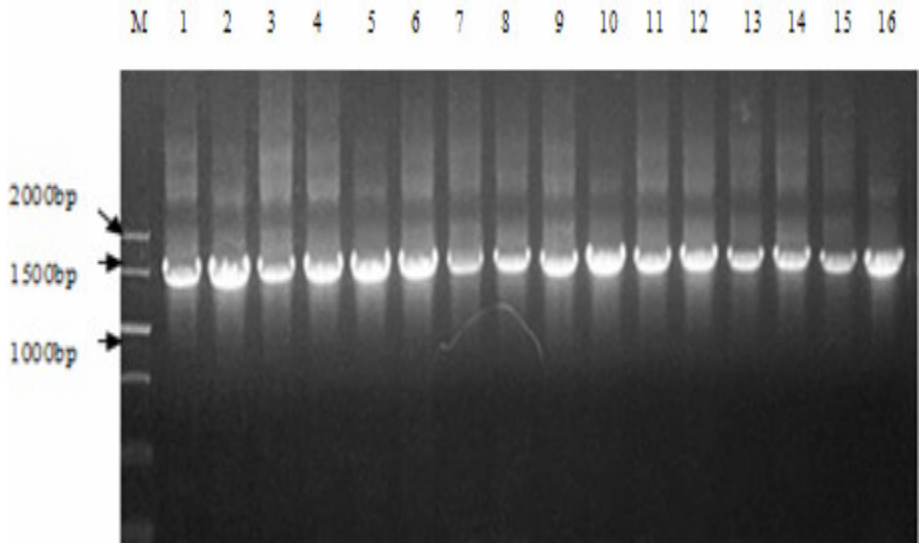
The ARDRA patterns were analysed by using UPGMA method, and similarity values between the ARDRA patterns were calculated and the Phylogenetic tree showed in Fig.5. 4 groups classified into 2 clusters: the normal group, about 25%; the model group, the 100% dose of traditional Qiweibaizhusan group and the 50% dose of ultra-micro Qiweibaizhusan group were clustered to a group, about 75%. It was indicated that there were low similarity of 4 groups bacterial community, and the model group, the 100% dose of traditional

Fig.1 Electrophoresis for metagenome DNA extracted from intestinal microflora in mice



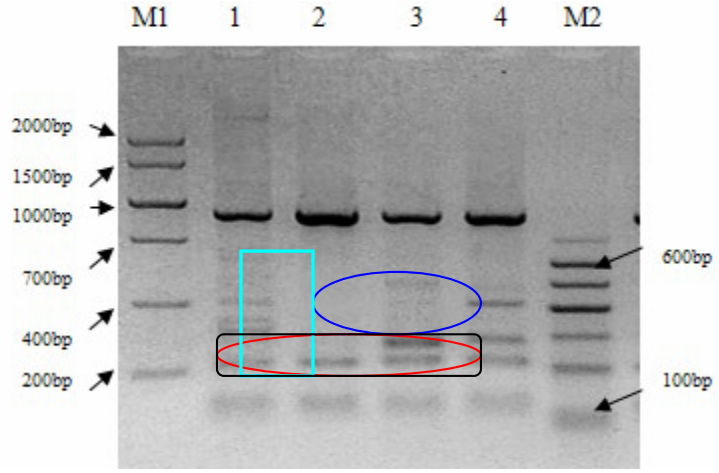
Lane 1-3: Metagenome DNA of the normal group; Lane 4-7: Metagenome DNA of the model group; Lane 8-11: Metagenome DNA of the 100% dose of traditional Qiweibaizhusan group; Lane 12-15: Metagenome DNA of the 50% dose of ultra-micro Qiweibaizhusan group; M: λDNA/*Hind* Ⅲ

Fig.2 Electrophoresis for 16S rDNA amplified from metagenome DNA of Intestinal flora in mice



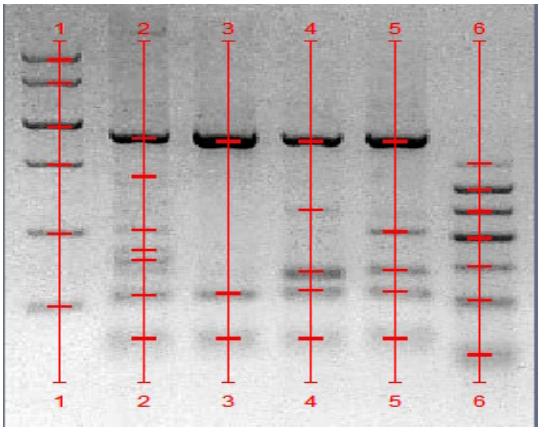
Lane 1-3: PCR products of the normal group; Lane 4-7: PCR products of the model group; Lane 8-11: PCR products of the 100% dose of traditional Qiweibaizhusan group; Lane 12-15: PCR products of the 50% dose of ultra-micro Qiweibaizhusan group; Lane 16: PCR products of *E.coli* for positive control; M: λDNA/*Hind* III

Fig.3 Electrophoresis for ARDRA of 16S rDNA from intestinal microflora in mice



Lane 1: ARDRA for the normal group; Lane 2: ARDRA for the model group; Lane 3: ARDRA for the 100% dose of traditional Qiweibaizhusan group; Lane 4: ARDRA for the 50% dose of ultra-micro Qiweibaizhusan group; M1、M2：Marker

Fig.4 The number of bands derived from ARDRA patterns analysised by Quantity One software



Lane 1: Marker ;Lane 2: ARDRA for the normal group; Lane 3: ARDRA for the model group; Lane 4: ARDRA for the 100% dose of traditional Qiweibaizhusan group; Lane5: ARDRA for the 50% dose of ultra-micro Qiweibaizhusan group; Lane 6:Marker

Table.1 The OTUs of metagenome 16S rDNA derived from ARDRA patterns in mice

	The normal group	The model group	The 100% dose of traditional Qiweibaizhusan group	The 50% dose of ultra-micro Qiweibaizhusan group
OTUs	7	3	5	5

Qiweibaizhusan group and the 50% dose of ultra-micro Qiweibaizhusan group were more similar to each other than the normal group.

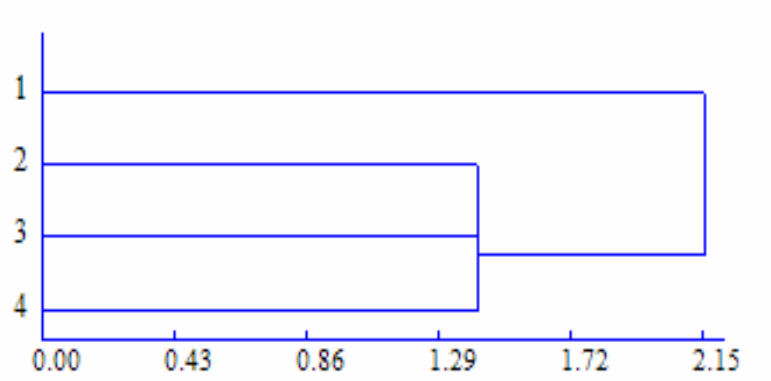
Diversity and similarity index

Bacterial compositions were compared by calculating the diversity index and similarity index (Tab.2). Compared with the normal group, the similarity index was 100%, the other 3 groups were not the same as the normal group. The similarity index of the 50% dose of ultra-micro Qiweibaizhusan group was 67%, the 100% dose of traditional Qiweibaizhusan group was 50% and the model group was just 40%. The diversity index ranged from 0.7324 to 1.7090, The highest diversity index was found in the normal group, the model group was the lowest, although the diversity index of the 100% dose of traditional Qiweibaizhusan group and the 50% dose of ultra-micro Qiweibaizhusan group were the same(1.2207), which indicated that the intestinal microflora of the normal group was the stablest, the amount of bacteria in the model group greatly reduced. After being treated with the 100% dose of traditional Qiweibaizhusan and the 50% dose of ultra-micro Qiweibaizhusan, the H value increased from 0.7324 to 1.2207 and indicated that the diversity of intestinal flora increased.

Bacterial 16S rDNA sequence is highly conserved sequence, which carried a large amount of information. Higher the diversity index of the intestinal flora is, greater the stability of the intestinal microflora is and more hard the balance to be broken. After being treated with the 100% dose of traditional Qiweibaizhusan and the 50% dose of ultra-micro Qiweibaizhusan, the restriction patterns and the diversity index increased. Experimental results showed that the number of lactobacillus and bifidobacterium increased obviously in intestine after treatment (Tan et al, 2012) which indicated that the treatment of Qiweibaizhusan could increase the abundance of intestinal microflora. But compared with normal group, the restriction patterns were slightly less, diversity index decreased, and species group of intestinal microflora failed to meet normal level. This might be some of lavage antibiotics make some intestinal bacterial species to death, or make some original intestinal bacteria in the gut can not colonize and survive by changing the mucous membrane of the intestinal wall or the environment in intestine. The treatment of Qiweibaizhusan made intestinal bacteria such as lactobacillus and bifidobacterium multiply vigorously, which could inhibit the growth of harmful bacteria, take attachment site of some intestinal microorganisms and reduce the abundance and diversity of intestinal species.

The diversity of intestinal species after treatment was less than normal mice, but diarrhea caused by dysbacteriosis had been cured. This may be because diarrhea is the result of a variety of microbial interaction in intestine, the member is intestinal bacteria and harmful bacteria. The treatment of Qiweibaizhusan make intestinal bacteria multiply vigorously, inhibit the growth of harmful bacteria, adjust the intestinal micro-ecology and cure diarrhea. After the treatment of ultra-micro Qiweibaizhusan, the intestinal flora in mice was restored, but it could't reach the normal level. The treatment of ultra-micro Qiweibaizhusan could make intestinal bacteria multiply vigorously and improve the intestinal microbial diversity.

Fig.5 Phylogenetic tree derived from ARDRA patterns using UPGMA method



1: the normal group;2: the model group; 3: the 100% dose of traditional Qiweibaizhusan group; 4: the 100% dose of ultra-micro Qiweibaizhusan group

Table.2 Diversity (H) and Similarity (Cs) of fecal samples in each group

group	Diversity H	SimilarityCs(%)
the normal group	1.7090	100
the model group	0.7324	40
the 100% dose of traditional Qiweibaizhusan group	1.2207	50
the 50% dose of ultra-micro Qiweibaizhusan group	1.2207	67

It was possible that microbes represented by missing bands have no direct correlation with diarrhea, microbes represented by those more than model group bands are related to diarrhea. Diarrhea is cured by adjusting the species and quantity of bacteria related to diarrhea, but the specific species has to sequencing in further analysis.

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