Bacterial lactase genes diversity in intestinal mucosa of dysbacterial diarrhea mice treated with Qiweibaizhu powder

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Abstract

The current research tried to investigate the effect of Qiweibaizhu powder (QWBZP) on intestinal mucosa bacterial lactase gene from dysbacterial diarrhea mice, as the good therapeutic mechanism of QWBZP on antibiotics-induced diarrhea. Dysbacterial diarrhea mice model was constructed by gastric perfusion with mixture of cephadrine capsules and gentamicin sulfate ($23.33 \text{ mL kg}^{-1} \text{ day}^{-1}$) for 5 days. After the success of establishing diarrhea, the mice in treatment group were gavaged with QWBZP for 3 days. Intestinal mucosa in each group was collected, and diversity of bacterial lactase genes in intestinal mucosa of mice was carried out by Miseq metagenome sequencing. The results showed the Chao1, ACE, Simpson and Shannon indices in treatment group were lower than model group and were similar to control group. The same result was obtained from the operational taxonomic units (OTUs). There were 298, 435 and 254 OTUs in the control group, model group and treatment group, respectively. Principal component analysis (PCA) indicated that samples distribution in both normal and treatment groups were relatively intensive, distances among individuals were small, while opposite results were observed in model group. Moreover, antibiotics increased the diversity and abundance of bacterial lactase genes at phylum and genus levels. However, they decreased and were similar to control group after treating with QWBZP. Our results indicate that QWBZP has a positive effect on the recovery of bacterial lactase gene diversity to normal level. In addition, QWBZP increase the abundance of *Lysobacter* and *Eukaryota*.

Keywords Qiweibaizhu powder · Dysbacterial diarrhea · Intestinal mucosa · Lactase gene · Diversity · Antibiotics

Introduction

Lactase, a disaccharidase also known as β -galactosidase, widely present in the mammalian intestinal mucosa microvilli surface, can hydrolyze lactose into two easily soluble monosaccharides, namely glucose and galactose, and play a key role in lactose digestion and absorption (Carter and Attel 2013; Ruchkina et al. 2013; Vandenplas 2015). Human and animal lactase are mainly secreted by intestinal mucosa, distributed in the intestinal contents and mucosa,

and mucosal lactase is the main source of intestinal lactase (Wiecek et al. 2014; Sun et al. 2015). Intestinal lactase is encoded and translated from lactase gene, and its activity level is controlled by genes. Microorganisms are the main source of lactase-producing, many intestinal bacteria also produce lactase by fermentation, such as *Escherichia coli*, *Lactobacillus* sp., *Bifidobacterium* sp., *Bacillus* sp. (Rhimi et al. 2009; Juajun et al. 2011; Ma et al. 2017). Clinically, the patients with symptoms of abdominal distension, borborygmus and diarrhea associated with lactase deficiency (LD) or the decrease of lactase activity show an increasing trend.

In addition to the physiological decrease associated with age, Mammalian lactase activity is also related to a variety of factors (Tran et al. 2011). Studies have shown that antibiotics can inhibit and damage intestinal lactase activity leading to diarrhea (Peng and Ren 2011). And diarrhea is often accompanied by dysbacteriosis (Liu and Sun 2014; Cao et al. 2017). Our previous study also showed that antibiotics reduced the diversity of bacterial lactase gene and changed its community structure in intestinal contents (Long et al.

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2017b). As an important part of mucosa, microorganisms may affect the activities of lactase.

Starting from the whole, traditional Chinese medicine has the potential advantage of regulating the function of spleen or stomach and the immunity of the body, and restoring the intestinal microbiota. It can directly or indirectly regulate intestinal microbiota and improve various intestinal enzyme activities because of its rich chemical component and wide pharmacological effects (Yao et al. 2012; Ma et al. 2014; He et al. 2017a). For example, compound radix pulsatillae can significantly enhance intestinal mucosal lactase activity in diarrhea mice, indicating that the therapeutic mechanism of pulsatillae on diarrhea may be related to the regulation of lactase activity (Zhang et al. 2010). Sijunzi Decoction can improve lactase activity in mice and play an important role in the mechanism of treating diarrhea (Zhang et al. 2009).

QWBZP is a millennium ancient prescription to treat various diarrheas and intestinal diseases with significant effect (Peng et al. 2014). Modern pharmacological studies and clinical practice have shown that QWBZP can significantly regulate intestinal microbiota of mice with dysbacteriosis, and has a good repair effect on the small intestine mucosal structure, but it does not increase the diversity of bacterial lactase gene in the intestinal contents, its curative effect on diarrhea mainly relevant to some key lactase-producing strains (Tan et al. 2013; Zhang et al. 2013; He et al. 2017b).

Compared with the intestinal contents, mucous has many advantages, such as completed in structure, sound in self-immune, stable in flora, and less affected by the external environment. In this study, we aimed to investigate the impact of QWBZP on the diversity of bacterial lactase genes in intestinal mucosa and further explored the curative effect of QWBZP on dysbacterial diarrhea induced by antibiotics.

Materials and methods

Materials

Animals

Eighteen one-month-old Kunming mice with license number SCXK (Xiang) 2013-0004 were purchased from Hunan Slaccas Jingda Laboratory Animal Company (Hunan, China). All procedures involving animals were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Hunan University of Chinese Medicine.

Reagents

Cephadrine capsules (batch number: 151101) were purchased from Suzhou Chung-Hwa Chemical and

Pharmaceutical Industrial Co., Ltd. Gentamicin sulfate injection (batch number: 5150307) was purchased from Yichang Humanwell Pharmaceutical Co., Ltd. Prepared as an antibiotic mixture at a concentration of 62.5 g L⁻¹ with sterile saline according to the ratio of 1:2, and stored at 4 °C (Xiao et al. 2015). Solutions such as lysozyme, protease K, Tris-saturated phenol–chloroform–isoamyl alcohol (25:24:1) and TE buffer were purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd. Other solutions such as CTAB/NaCl, 5 mol L⁻¹ NaCl, 0.1 mol L⁻¹ PBS buffer and 10% SDS were prepared in the laboratory.

Medicine

According to the Chinese Pharmacopoeia 2010, QWBZP was composed of ginseng 6 g, costustoot 6 g, poriacocos 10 g, roasted rhizome atractylodes macrocephalae 10 g, pueraria 10 g, agastache 10 g and liquarice 3 g. Which were purchased as prescription from the first hospital of Hunan University of Chinese Medicine. Weighing appropriate medicine according to the above ratio and soaking the medicine for half an hour covered with cold water, decocting the medicine with big fire until it boils, then with soft fire, decoction twice and each time for 20–30 min. The two times liquid medicine should be mixed to make 100% dose of traditional decoction of QWBZP and frozen at 4 °C for following experiment (Zhang et al. 2014).

Methods

The mice (equal number of male and female) were randomly divided into three groups: control group (tlcm), model group (tlmm) and treatment group (tlqm), six mice (three males and three females) in each group after 2 days of adaptive feeding. The mice in both model group and treatment group were administered 0.35 mL antibiotics mixture composed of gentamycin sulfate and cefradine (23.33 mL·kg⁻¹·day⁻¹). Correspondingly, the mice in control group were gavaged with 0.35 mL sterile water, twice a day for 5 days (Zeng et al. 2012). When symptoms (specifically watery stool, declined activity, curled up, arched back trembling and poor appetite) were induced in mice, it indicated the success of establishing diarrheal model. Then, the mice in the treatment were administered QWBZP decoction at the dose of 0.16 g kg⁻¹ day⁻¹ for 3 days (Guo et al. 2015). On a sterile operation platform, mice were sacrificed using cervical vertebra dislocation. Then, intestinal mucosa was scraped with cover slips and added 2 times weight of saline after squeezing out the chymus, cutting open the intestinal tract and cleaning the intestinal wall with saline. Finally, intestinal mucosa samples of each two mice (one male and one female) in every group were selected and immediately frozen at 4 °C for following experiment (Jin et al. 2012).

Metagenome extraction

According to our previous report, metagenome DNA from the intestinal mucosa microorganism was extracted by the following protocols (Wu et al. 2012). 2.0 g of intestinal mucosa was collected in a sterile environment, placed in a germ-free centrifuge tube and homogenized in 5 mL of 0.1 mol L⁻¹ phosphate buffer solution (PBS), then centrifuged at 200×g for 2 min. After washing twice with PBS, the supernatant was transferred into new germ-free tubes and centrifuged at 10,000×g for 8 min. Then, the new sediments were gathered, washed once with PBS, twice with acetone, and three times with PBS, then resuspended in 4 mL TE buffer. After sample pretreatment, 500 µL of suspension was added with 20 µL lysozyme, 5 µL proteinase K, and 45 µL TE buffer, and homogenized in 1.5 mL germ-free EP tubes. Samples were incubated at 37 °C for 30 min, and mixed with 30 µL of 10% SDS, followed by incubation at 37 °C for 40 min, with vortexing once every 10 min. Afterwards, 80 µL of CTAB/NaCl and 100 µL of 5 mol L⁻¹ NaCl were added and mixed well. The mixture was vortexed at 65 °C for 10 min. An equal volume of Tris-saturated phenol–chloroform–isoamyl alcohol (25:24:1) was then added to the sample, mixed well and centrifuged at 10,000×g for 3 min. The supernatant was transferred into new germ-free tubes, mixed with an equal volume of chloroform–isoamyl alcohol (24:1), centrifuged at 10,000×g for 3 min. The supernatant was transferred into fresh germ-free tubes, and an equal volume of chloroform–isoamyl alcohol (24:1) was added and mixed well again. The supernatant was transferred into fresh germ-free tubes after centrifugation at 10,000×g for 3 min, 10⁻¹ volume of 3 mol L⁻¹ sodium acetate and double volume of absolute ethyl alcohol were added, and precipitated at – 20 °C for about 12 h. Samples were centrifuged at 10,000×g for 3 min. The acquired sediment were washed with 70% ethanol, dried and eventually dissolved in 50 µL TE buffer for DNA metagenome extraction.

PCR amplification of intestinal bacterial lactase gene and Miseq metagenome sequencing

Primers were designed for PCR amplification based on the conserved region of the β-galactosidase gene nucleotide sequence of *Escherichia coli* and *Lactobacillus* from NCBI. The forward primer was 5'-TRRGCAACGAATACGGSTG-3' and the reverse primer was 5'-ACCATGAARTTSGTGGTSARCGG-3'. Universal primers were designed and synthesized by Shanghai Personal Biotechnology Co., Ltd. PCR mixture (25 µL) contained 5.0 µL 5×reaction buffer, 5.0 µL 5×GC buffer, 0.5 µL 10 mmol L⁻¹ dNTP, 1.0 µL 10 µmol L⁻¹ forward primer, 1.0 µL 10 µmol L⁻¹ reverse primer, 1.0 µL DNA template, 11.25 µL sterilized ddH₂O, 0.25 µL Q5 high-fidelity DNA

polymerase. PCR conditions were as follow: initial denaturation at 98 °C for 30 s, followed by 32 cycles at 98 °C for 15 s, annealing at 46 °C for 30 s, extension at 72 °C for 30 s, and then 72 °C for 5 min and holding at 4 °C (Long et al. 2017a). Miseq metagenome sequencing was completed by Shanghai Personal Biotechnology Co., Ltd.

Bioinformatics and statistical analysis

The abundance and diversity of bacterial lactase genes in the intestinal mucosa were measured by alpha diversity analysis which contained Chao1, ACE, Simpson, and Shannon indices based on the operational taxonomic units (OTUs) (Mahaffee and Kloepper 1997; Shannon 1997; Pitta et al. 2010, 2014). Principle component analysis (PCA) (Ramette 2007) and Hierarchical clustering (HC) were used to analyze the main distribution characteristics and the similarity of community samples. The SPSS 21.0 software (IBM Corp, Armonk, NY, USA) was used to analyze the measurement data. Pairwise samples *t* test was applied to compare the statistical significance of differences, with *P* < 0.05 or *P* < 0.01.

Results

Effects of QWBZP on bacterial lactase genes diversity in the intestinal mucosa

To investigate the impact of QWBZP treatment on dysbacterial diarrhea, the alpha diversity analysis was estimated. The results showed that Chao1, ACE, Simpson and Shannon indices were higher in the model group than those both in control and treatment groups. Among them, there was significant difference in Simpson index (Fig. 1; *P* < 0.05), which indicated that antibiotics increased the diversity of bacterial lactase genes in the intestinal mucosal community. After treatment with QWBZP, we found that Chao1, ACE, Simpson and Shannon indices were similar to the normal group, and there is no significant difference (*P* > 0.05), which suggests that QWBZP helps the recovery of mucosal bacteria lactase genes diversity in model mice to normal level.

Effects of QWBZP on bacterial lactase genes structure in the intestinal mucosa

PCA analysis was applied for OTUs results of bacterial lactase genes sequencing. Each point represented a sample, and the points with different colors belonged to the different groups. The closer the distance between two points was, the higher the similarity of gene sequence structure between two samples, and the smaller the difference was. It could be seen that the percentage contributed to variation of PC1 and PC2 was 99.99%, and these provided

Fig. 1 Effects of QWBZP on the intestinal mucosa bacterial lactase genes diversity. * $p < 0.05$; ** $p < 0.01$

the vast majority of raw data information (Fig. 2a). The results also showed that samples between the control group and the model group were significantly separated, and the distance between the two groups was relatively great, indicating that antibiotic modeling had a significant impact on the structure of bacterial lactase genes in the intestinal mucosa. After treatment with QWBZP, the distance of samples was tightly concentrated between treatment and control groups compared to samples between treatment and model groups. These findings suggest that QWBZP has good curative effect on bacterial lactase genes in intestinal mucosa.

To further investigate the similarity of bacterial lactase genes, Hierarchical clustering was used based on UniFrac distance matrix. In particular, the distance length between samples in each group was measured. According to the similarity between samples, the shorter the branch length between two samples was, the higher the similarity between two samples. As shown from Fig. 2b, tlcm1, tlcm2, tlcm3 and tlqm2 are clustered into a category, tlmm2 and tlmm3 are clustered into a category, tlqm1, tlqm3 and tlmm1 are clustered separately. These results showed that the similarity of gene sequences from the control group was higher compared to the sequences from the model group, indicating that antibiotics may destroy the structure of intestinal mucosa bacterial lactase genes. After treatment with QWBZP, tlqm2 could be well grouped with the normal group, indicating that the difference of lactase genes sequence between normal and treatment group mice is small. However, tlqm1 and

tlqm3 are not clustered with the normal group, which may be related to individual differences in mice.

Effects of QWBZP on bacterial lactase genes OTUs number in the intestinal mucosa

Based on sequence homogeneity, the sequences with over 97% similarity were aligned and grouped into individual OTUs, and the results were analyzed by Qiime software. There were 298, 435 and 254 OTUs expressed in mice from control group, model group and treatment group, respectively. Among them, 151 were identical (Fig. 3). It indicates that antibiotic increased the number of OTUs of bacterial lactase gene in intestinal mucosa, and they decreased significantly to normal level after treatment with QWBZP.

Effects of QWBZP on bacterial lactase genes abundance in the intestinal mucosa

The source and abundance of lactase gene in each sample were counted at the phylum and genus levels, respectively. At the phylum level, the difference in the proportion of lactase-producing bacteria between treatment and control groups was less than that between treatment and model groups. The intestinal mucosa bacterial lactase genes were mainly originated from Proteobacteria both in control and treatment group. After being induced by antibiotics, the relative abundance of Proteobacteria decreased and the abundance of unclassified bacteria increased. At the genus level,

Fig. 2 Effects of QWBZP on the intestinal mucosa bacterial lactase genes structure. tlcmm: control group; tlm: model group; tlmq: treatment group

we got the same result. A total of 8 genera were detected among three groups, there were 5, 3 and 8 genera in control group, model group and treatment group, respectively, and 3 of them were identical. In addition, 5 of these genera were identical in treatment and control groups. The relative difference of genera in control and treatment groups was less than that in control and model groups. In comparison with control and treatment groups, model group increased the abundance of lactase gene originated from *Citrobacter* and unclassified bacteria, and reduced the abundance of *Stenotrophomonas* (Fig. 4).

To confirm our finding, further analysis was provided. In Fig. 4, the ordinate represents taxonomic units with significant differences between groups, and the abscissa shows the logarithm scores of the LDA difference analysis corresponding to the taxonomic units based on bar graphs and sorted by score values to describe the difference between groups. The different color of bar indicates the higher abundance sample corresponding to the taxonomic unit. The longer the bar chart length, the more the difference of taxonomic units

Fig. 3 Effects of QWBZP on the intestinal mucosa bacterial lactase genes OTUs number. tlcmm: control group; tlm: model group; tlmq: treatment group

became. As indicated in Fig. 4, the lactase gene abundance of bacteria was highly present in model group compared with that from control and treatment groups, and the abundance of *Phyllobacteriaceae* and *Mesorhizobium* in control group were significantly higher than those both in model and treatment groups. Furthermore, QWBZP increased the abundance of bacterial lactase genes from *Lysobacter* and *Eukaryota*.

Effects of QWBZP on bacterial lactase genes similarity in the intestinal mucosa

The difference based on the UniFrac distance value in different groups can be displayed by the box plots. The specific distribution characteristics of each group data can be presented according to median value, upper and lower quartiles, maximum and minimum values. The abscissa represents the comparison in intra-group or in inter-group, the ordinate indicates the corresponding distance value, and box size corresponds to the difference of sample gene sequence. The results showed that the difference in inter-sample was minimum in the control group, followed by the treatment group, and the difference was maximum in the model group. What's more, the difference in intra-group

Fig. 4 Effects of QWBZP on the intestinal mucosa bacterial lactase genes abundance. tlcmm: control group; tlm: model group; tlmq: treatment group

Fig. 5 Effects of QWBZP on the intestinal mucosa bacterial lactase genes similarity. tlcmm: control group; tlm: model group; tlmq: treatment group

was higher than that in inter-group (Fig. 5). It indicates that there was significant difference in genes sequence similarity between control and model groups. After treatment with QWBZP, lactase genes sequence in the treatment group was close to the sample in the control group, their difference was small, and their similarity was high, which suggested that the mice returned to normal.

Discussion

QWBZP helped to recover the diversity of bacterial lactase genes in intestinal mucosa

In recent years, molecular ecology research focusing on the functional gene diversity of encoding related enzymes has been rapidly developed, which provided a new starting point for understanding the ecological function of microorganisms from the perspective of functional genes, and attained more realistic mean in the ecological function of microorganisms using functional gene diversity including abundance and structure (Zhang et al. 2006; Yang et al. 2009). The current research aimed to investigate the diversity of intestinal microbiota used lactase gene and applied second-generation Miseq metagenome sequencing, which provided a new way for researchers to evaluate the effect of Chinese herbal compound on the diversity of intestinal microbiota at the molecular level. The results show that the α -diversity index of bacterial lactase gene in QWBZP group was lower than model group and was similar to control group. We have got the same result in OTUs number, the number of OTUs (435) in the model group was significantly higher than that in the control group (298) and QWBZP group (254), and the OTUs number in QWBZP group was close to normal group. It indicated that treatment with QWBZP could help recover the diversity of bacterial lactase gene to normal level, and cure the dysbacterial diarrhea. These results are in sharp contrast to our previous study, the bacterial lactase gene diversity in the intestinal contents was not recovered after treatment with QWBZP (He et al. 2017b). A possible reason may be related to the different composition of intestinal microbiota between contents and mucosa.

QWBZP effectively restored the community structure of bacterial lactase-producing genes in intestinal mucosa

The method PCA was applied for the analysis of bacterial community structure. From our results, the distribution was relatively concentrated in both control and QWBZP groups, the sample distance was close, and the similarity of gene structure was high. While in model group, the sample distance was relatively great, and the difference was larger in inter-group. Furthermore, the distance was also far way between model group and control/QWBZP group, indicating the poor similarity between model and other two groups.

To further investigate the homogenous bacterial lactase gene, HC was used. The results showed that QWBZP group was clustered with control group, while the model group was classified as a single group. In box plot, the size was smaller both in control and QWBZP groups, but larger in model group. Moreover, the box size was obviously smaller in inter-group and larger in intra-group. These findings suggest that antibiotic modeling has destroyed the structure of bacterial lactase genes and there was significant difference in lactase genes between model and control/QWBZP groups. These results are consistent with our previous study that antibiotic modeling changed the structure of bacterial lactase genes (Long et al. 2017b, 2018). In addition, the difference in lactase genes between control and QWBZP group was small, and the similarity in gene structure was high, suggesting that treatment with QWBZP could help to restore the community structure of bacterial lactase-producing genes to normal level. This result is superior to the curative effect of QWBZP on bacterial lactase gene in the contents (He et al. 2017b). We hypothesize that this is related to the repair effect of QWBZP on the structure in the intestinal mucosa of mice with dysbacterial diarrhea (Zhang et al. 2013; Liu et al. 2014).

QWBZP effectively regulated the abundance of bacterial lactase genes in intestinal mucosa

The species abundance of phylum and genus were compared among three groups. In comparison with control group, the composition and abundance of species was similarity to QWBZP group at the phylum and genus levels. The model group decreased the abundance of lactase gene originated from *Proteobacteria* and *Stenotrophomonas*, and increased many unclassified lactase genes. In specific taxonomic units, antibiotics modeling increased the abundance of *Bacteria*, and the abundance of *Lysobacter* and *Eukaryotawas* increased after treatment with QWBZP.

In our previous study, the genera of lactase detected in the intestinal contents were far beyond these in the intestinal mucosa, suggesting that there are more

lactase-producing strains in the intestinal contents. After treatment with QWBZP, the number of bacterial lactase genes in the intestinal contents of model mice was significantly decreased ($P < 0.01$). QWBZP effectively restored some lactase gene species or certain mutant genes (He et al. 2017b). In our opinion, some possible reasons for this could be the difference in the main source of lactase-producing strains between contents and mucosa, the stability of bacterial colonization in intestinal mucosa, and the function of mucosal self-immunity (Long et al. 2018).

In summary, antibiotics destroyed the structure of bacterial lactase genes in the intestinal mucosa, and increased their diversity. After treatment with QWBZP, the diversity of bacterial lactase genes in intestinal mucosa restore to normal level. Our study suggests that the probable mechanism of QWBZP on dysbacterial diarrhea would be the recovery of some key mucosal bacterial lactase-producing genes diversity and structure.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Ethical approval was obtained from the Animal Ethics and Welfare Committee of Hunan University of Chinese Medicine.

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