Diversity of intestinal bacterial lactase gene in antibiotics-induced diarrhea mice treated with Chinese herbs compound Qi Wei Bai Zhu San

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Abstract

The current investigation is trying to study the impact of the mixture of Chinese herbs Qi Wei Bai Zhu San (QWBZS) on bacterial lactase gene from antibiotics-induced diarrhea (AAD) mice, as the good curative effect of QWBZS on diarrhea. Mice (6 mice per group) were randomly selected as control, model and treatment groups. To induce diarrhea, mice in both model and treatment groups were intragastrically injected with mixture of gentamycin sulfate and cefradine ($23.33 \text{ mL kg}^{-1} \text{ day}^{-1}$) twice per day and continuously for totally 5 days. After the success of establishing diarrhea model, the mice in treatment group were gavaged with QWBZS for 3 days. Intestinal contents in all three groups were then collected and DNA was extracted in aseptic environment for the following sequencing. The results showed that mice from QWBZS treatment group had obviously detectable levels of intestinal bacteria, such as Actinobacteria, Firmicutes and Proteobacteria, which produce phyla lactase specifically. In comparison with other groups, the mice in treatment group had more abundant expression of lactase gene from *Acidovorax* sp. KKs102, *Stenotrophomonas* sp. LMG11000, *Pseudomonas oleovorans*, *Eggerthella* and *Burkholderia*. Interestingly, the Shannon index decreased significantly after the treatment with QWBZS ($P < 0.01$ or $P < 0.05$). 63.1% of lactase genes detected in the mice in treatment group were unclassified, and 32.8% of them were non-homologous to any fragments in the gene bank, which means that most of lactase-producing bacteria are novel. Our results indicate that treatment with QWBZS did not increase the diversity of bacterial lactase gene. Its curative effect on diarrhea may be relevant to its role in facilitating the growth of novel or some key lactase-producing strains.

Keywords Antibiotics-induced diarrhea · Lactase · Qi Wei Bai Zhu San · Intestine microbiota · High-throughput sequencing

Introduction

According to the theory of Chinese traditional medicine, people with malfunctions of spleen or stomach are easier to suffer from diarrhea than others do (Qiao 2014). Compound traditional Chinese medicine is the essence of traditional Chinese medicine, such as Qi Wei Bai Zhu San (QWBZS). With the stringent design, accurate and appropriate compatibility and the function of regulating intestinal microecology,

QWBZS is a millennium ancient prescription to treat diarrhea, especially pediatric diarrhea (Cai et al. 2013; Wang et al. 2014). It is composed of Sijunzitang and is a kind of broth cooked with radix codonopsis, poria cocos, atractylodes macrocephala koidz, radix glycyrrhizae, radix aucklandiae, agastache rugosa and radix puerariae (Yang and Yang 2010; Wang et al. 2013). Previous studies demonstrated that QWBZS can adjust intestinal *Lactobacillus* and *Bifidobacterium*, reverse the effect of antibiotics on activities of enzymes, such as lactase, xylanase, amylase and protease in mice and promote the growth of yeast and improve mucosa structure of small intestine in antibiotics-induced diarrhea (Guo et al. 2013; Guo et al. 2015a; Liu et al. 2014; Tan et al. 2012).

Diarrhea, a health condition with the symptom featured by over three times of watery stools daily, can be caused by malfunction of spleen or stomach and over-dose antibiotics.

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With the overuse of antibiotics, antibiotics-induced diarrhea (AAD) has become the most common iatrogenic diarrhea. There are several possible pathogenesis of antibiotics-induced diarrhea (Zhou 2004): breaking the balance of intestinal bacteria, interfering the glycometabolism and bile acid metabolism or decreasing the activity of disaccharidase, such as lactase. According to a previous study (Long et al. 2017a), a mix of antibiotics for diarrhea modeling in mice reduced the density of bacterial lactase gene and led to the decreased Chao1 and ACE indexes ($P < 0.05$) and the abundance of some bacteria genera producing lactase.

Lactase plays critical role in regulating intestinal function and is mainly produced by Proteobacteria, Actinobacteria or Firmicutes (Alexandre et al. 2013; Ojetti et al. 2010). Antibiotics, especially beta-lactam antibiotics such as cefradine used in our previous study, can destroy the intestinal lactase (Peng and Ren 2011). Due to the deficiency or low activity of lactase, the lactose cannot be absorbed but decomposed by intestinal microbiota, which causes intestinal water and osmotic pressure increase by producing a large number of short-chain fatty acids and hydrogen and eventually induce diarrhea (Hammer and Hammer 2012; Zhang et al. 2014).

16S rDNA sequencing has been widely used in the classification, identification and detection of bacteria, and research of intestinal microbiota has been a focus because of its crucial role in human health. However, there is few study on intestinal bacterial lactase gene while some studies on genetic xylanase and pectase diversity in rumen have been reported (Wang et al. 2011; Yuan 2012). As such, we designed and validated a pair of universal primers used for the amplification of lactase gene (Long et al. 2017b). The diversity of bacterial lactase gene were analyzed by high-throughput sequencing.

The current research aimed to investigate pharmacological mechanisms of QWBZS's effect on the activities of lactases at the genetic level and to provide experimental basis for the treatment of AAD with QWBZS.

Materials and methods

Reagents and medicine

Medicines and antibiotics were purchased from Suzhou Chung-Hwa Chemical & Pharmaceutical Industrial Co. Ltd, or Yichang Humanwell Pharmaceutical Co. Ltd. Protease K, acetone, lysozyme, tris saturated phenol–chloroform–isoamyl alcohol (25:24:1) and TE buffer were purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd. 10% SDS, 0.1 mol L⁻¹ PBS buffer, 5 mol L⁻¹ NaCl and CTAB/NaCl were prepared in the lab. QWBZS broth was made of *Radix codonopsis*, *poria cocos*, *atractylodes macrocephala* koidz, *radix glycyrrhizae*, *radix Aucklandiae*, *agastache*

rugosa and *radix puerariae*, which were purchased as prescription from The First Hospital of Hunan University of Chinese Medicine. The herbs were boiled for 1 h and the broth was collected and stored at 4 °C.

Animals and procedures

Adult mice were purchased from Hunan Slaccas Jingda Laboratory Animal Company with licence number SCKX (Xiang) 2013-0004. All experiments and procedures involving animals were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Hunan University of Chinese Medicine. The experiments included equal number of male and female mice. Mice were randomly selected into three groups (6 mice per group): healthy control group (control), diarrhea model group (model) and diarrhea treatment group (treatment). Mice of control group were gavaged with 0.35 mL of sterile water twice a day for 5 days. To induce diarrhea, mice in both model group and treatment group were intragastrically injected with mixture of gentamycin sulfate and cefradine (23.33 mL kg⁻¹ day⁻¹) twice per day and continuously for totally 5 days by following the procedures described previously (Zeng et al. 2012). Once the diarrhea symptoms (specifically watery stool, reduced intake, erected coat and declined activity) were induced, the mice in treatment group were administered intragastrically with QWBZS broth at the dose of 0.16 g kg⁻¹ day⁻¹ for 3 days (Guo et al. 2015b). Mice were then euthanized and intestinal contents samples of each two mice (one male and one female) in every group were then collected for the following experiments (Zeng et al. 2012).

Table 1 Statistics of sequences intestinal bacteria DNA sequences

Samples	Effective sequences	High quality sequences	Ratio (%)
tlcn1	63,052	61,821	98.05
tlcn2	77,507	76,101	98.19
tlcn3	75,377	73,418	97.40
tlmn1	70,334	68,547	97.46
tlmn2	65,217	63,929	98.03
tlmn3	69,876	68,810	98.47
tlqn1	73,310	72,454	98.83
tlqn2	51,263	50,521	98.55
tlqn3	79,978	78,813	98.54
Total	625,914	614,414	98.16

Effective sequence: index exactly matched sequence. High quality sequence: the effective sequences after filtering and removing chimeric. tlcn1-3, tlmn1-3 and tlqn1-3 are control group samples 1–3, model group samples 1–3 and treatment group samples 1–3, respectively

Fig. 1 Rank-abundance curve of each sample. The abscissa represents the ordinal of the OTU, and the ordinate represents the abundance of the OTU. The larger the curve span is, the richer the composition of the species is. The more flat the curve, the higher the uniformity of the species composition. t1cn1-3, t1mn1-3 and t1qn1-3 are control group samples 1–3, model group samples 1–3 and treatment group samples 1–3, respectively

Table 2 Alpha biodiversity fares

Group	Chao1	ACE	Shannon
Control group	532.00 ± 48.14	570.22 ± 77.44	5.30 ± 0.24
Model group	517.00 ± 116.25	585.54 ± 148.49	5.12 ± 0.33
Treatment group	401.00 ± 19.67	479.00 ± 41.04	4.46 ± 0.14aB

a: $P < 0.01$ in comparison with the control group. B: $P < 0.05$ in comparison with compared with the model group

DNA extraction

Genomic DNAs from the intestinal contents were extracted by following the protocols (He et al. 2017; Wu et al. 2012). 2.0 g of intestinal feces was weighed in a sterile environment and was homogenized in 5 mL of 0.1 mol L⁻¹ phosphate buffer solution (PBS), followed by centrifugation at 200 g for 2 min. After washing the sediments twice with PBS, the whole supernatants were transferred into new tubes and centrifuged for 8 min at 10,000g. The new sediments were then gathered, washed once with phosphate buffer solution, twice with acetone, and three times with phosphate buffer solution, then resuspended in 4 mL TE buffer. 500 µL of spreadhead were added with 45 µL TE buffer, 5 µL proteinase K and 20 µL lysozyme and homogenized in 1.5 mL germ-free EP tubes. Samples were incubated at 37 °C for 30 min, then mixed with 30 µL of 10% SDS, followed by incubation at 37 °C for 40 min with turning upside down once every 10 min. Afterwards, 100 µL of 5 mol L⁻¹ NaCl and 80 µL of CTAB/NaCl were added and mixed well. The

mixture was reacted at 65 °C for 10 min. An equal volume of Tris saturated phenol–chloroform–isoamyl alcohol (25:24:1) then were added to the sample, mixed well, and were centrifuged at 10,000g for 3 min. The supernatant were transferred to fresh sterile tubes, mixed with an equal volume of chloroform–isoamyl alcohol (24:1), centrifuged at 10,000g for 3 min. The supernatant was transferred into fresh sterile tubes and mixed with an equal volume of chloroform–isoamyl alcohol (24:1) again. After centrifugation at 10,000g for 3 min, the supernatant were transferred into new sterile tubes, added with 1/10 volume of 3 mol L⁻¹ sodium acetate and double volume of absolute ethyl alcohol, and were precipitated at – 20 °C overnight. Samples were centrifuged at 10,000g for 3 min. The acquired sediment were washed with 70% ethanol, dried and eventually dissolved in 50 µL TE buffer.

Fig. 2 PCoA analysis of the samples showing 3D sort graph based on Weighted UniFrac. Each point represents a sample. The points with the same colors belong to the same group. The closer the distance between the two points, the smaller difference between the two samples of microbial community. tlc1-3, tlm1-3 and tlq1-3 are control group samples 1–3, model group samples 1–3 and treatment group samples 1–3, respectively

PCR amplification of intestinal bacterial lactase gene and sequencing

According to a previous report (Long et al. 2017b), the lactase genes were amplified by PCR with the forward primer 5'-TRRGCAACGAATACGGSTG-3' and the reverse primer 5'-ACCATGAARTTSGTGGTSARCGG-3'. The PCR products were purified and applied for sequencing. Universal primers for amplifying bacteria beta-galactosidase gene were designed based on the sequence info from NCBI database and synthesized by Personal Biotechnology Co., Ltd. Shanghai, China. PCR mixture (25 μ L) included 0.25 μ L Q5 high-fidelity DNA polymerase, 5 μ L 5 $\times$ reaction buffer, 5 μ L 5 $\times$ high GC buffer, 0.5 μ L 10 mmol L⁻¹ dNTP, 1 μ L 10 μ mol L⁻¹ forward primer, 1 μ L 10 μ mol L⁻¹ reverse primer, 1 μ L DNA template and 11.25 μ L sterilized ddH₂O. The PCR conditions were as follow: initial denaturation at 98 °C for 30 s, 32 cycles of 98 °C for 15 s, 46 °C for 30 s and 72 °C for 30 s, extension at 72 °C for 5 min and holding at 4 °C. PCR products were first examined by 2% Agarose gel electrophoresis. PCR products of bacteria lactase genes were purified, and were then sequenced by Illumina Miseq in Personal Biotechnology Co., Ltd.

Gene diversity analysis and statistical analysis

The sequencing results were blasted using software available online, including Qiime (<http://qiime.org/>) (Caporaso et al. 2010) and Mothur (<http://www.mothur.org/>). Lactase genes' abundance and diversity were measured by Rank abundance curve and Alpha diversity index (Pitta et al. 2010, 2014;

Shannon 1997; Mahaffee and Kloepper 1997). PCoA figure was generated according to UniFrac analysis and used for species evolution analysis (Lozupone and Knight 2005). The source and abundance of bacterial lactase genes at the specific taxonomic levels were presented by figure of Species evolution and abundance information. Species composition ratio was measured by pie charts. Differences among groups were considered significant when *P*-values less than 0.05.

Results

Quality of extracted DNA and statistics of sequences

Before sequencing, the quality of genomic DNAs in all samples was measured by OD260/OD280. All of the samples had OD260/OD280 above 1.8. There were totally 625,914 effective sequences and 614,414 high quality sequences acquired by sequencing. High quality sequences account for around 98% of effective sequences in each sample. The results were shown in Table 1. These results indicated that the samples were suitable for the following analyses.

Richness and homogeneity of bacterial lactase gene

To investigate the impact of QWBZS treatment on diarrhea, the richness and homogeneity of the lactase genes were determined by Rank-abundance curve and Alpha index. As shown in Fig. 1, among three groups of mice, the treatment group had lowest abundance values. It indicates that the treatment decreased the richness of bacterial lactase genes

Fig. 3 Species evolution and abundance information. The number of sequences of different samples on a branch is presented by a pie chart with different colors: the larger the area of the pie chart, the more the number of sequences at the branch. The different colors represent the different samples. The larger the fan-shaped area of a color indicates that the number of sequences corresponding to the sample on the branch is greater than that of the other samples. tlcn: control group; tlmn: model group; tlqn: treatment group

in the intestines of mice. This is confirmed by the Alpha index result: the Chao 1 index was the highest in control group, followed by model group and treated group, while the difference had no significance ($P > 0.05$). However, the Shannon index of lactase genes was significantly lower in the QWBZS treatment group than those in model group and control group ($P < 0.05$ or $P < 0.01$) (Table 2).

Difference analysis of bacterial community producing lactase

The method PCoA was applied for difference analysis of bacterial community. The results show that the clusters from treatment group were distinctly separated from others in control and model groups, which suggests that the diversity of bacteria in QWBZS-treated mice differed from the mice in other two groups. There was no significant difference, however, between the control group and model group. The

percentages attributing to the variations of PC1, PC2 and PC3 were 44.82, 25.72 and 10.9%, respectively (Fig. 2).

Community structure of lactase-producing microbiota and lactase gene abundance

The evolution of lactase genes was investigated by evolution tree which show the similarity of the genes. As shown Fig. 3, intestinal lactase was mainly produced by Actinobacteria, Firmicutes and Proteobacteria, in particular Proteobacteria, in which Enterobacteriaceae and Pseudomonas were major species for lactase gene. In comparison with control group and model group, QWBZS treatment increased the abundance of lactase gene originated from *Acidovorax* sp. KKS102, *Stenotrophomonas* sp. LMG11000 and *Pseudomonas oleovorans* (Fig. 3).

Overall, thirty species of bacteria were detected at genus level. *Rhodococcus*, *Paenibacillus* and *Ensifer* were detected only in control group, while bacteria of

Table 3 The abundance of bacterial lactase genes from known species at genus level

Genus	Control group	Model group	Treatment group
<i>Corynebacterium</i>	9.63E-04 ± 9.59E-05	9.78E-04 ± 6.91E-05	4.35E-04 ± 2.61E-05
<i>Mycobacterium</i>	8.53E-04 ± 2.80E-04	3.98E-04 ± 6.46E-04	-
<i>Rhodococcus</i>	1.28E-04 ± 2.22E-04	-	-
<i>Frankia</i>	1.03E-04 ± 4.7E-05	4.5E-05 ± 6.4E-05	-
<i>Modestobacter</i>	2.29E-04 ± 3.29E-04	2.8E-05 ± 4.8E-05	1.2E-05 ± 1.1E-05
<i>Microbacterium</i>	1.57E-04 ± 2.71E-04	1.842E-03 ± 3.132E-03	4.2E-05 ± 4.9E-05
<i>Arthrobacter</i>	8.28E-04 ± 6.00E-04	7.23E-04 ± 3.14E-04	5.24E-04 ± 2.41E-04
<i>Micromonospora</i>	-	4.9E-05 ± 4.2E-05	0.5E-05 ± 0.8E-05
<i>Streptomyces</i>	3.99E-04 ± 2.84E-04	4.13E-04 ± 3.40E-04	0.7E-05 ± 1.2E-05
<i>Eggerthella</i>	4.1E-05 ± 2.3E-05	2.2E-05 ± 0.9E-05A	4.32E-04 ± 3.98E-04AB
<i>Paenibacillus</i>	0.8E-05 ± 1.3E-05	-	-
<i>Ruminococcus</i>	-	0.6E-05 ± 1.0E-05	-
<i>Bradyrhizobium</i>	2.5E-05 ± 1.0E-05	2.7E-05 ± 2.5E-05	4.6E-05 ± 2.8E-05
<i>Agrobacterium</i>	4.2E-05 ± 1.6E-05	3.9E-05 ± 3.9E-05	1.0E-05 ± 0.8E-05
<i>Ensifer</i>	4.6E-05 ± 8.0E-05	-	-
<i>Rhizobium</i>	-	0.80E-04 ± 1.39E-04	1.4E-05 ± 1.4E-05
<i>Methylococcus</i>	-	0.67E-04 ± 1.16E-04	-
<i>Novosphingobium</i>	-	4.07E-04 ± 4.47E-04	1.2E-05 ± 1.1E-05
<i>Burkholderia</i>	8.7E-05 ± 1.9E-05	5.5E-05 ± 3.9E-05	0.15E-04 ± 1.5E-04A
<i>Acidovorax</i>	2.49E-04 ± 0.74E-04	2.18E-04 ± 2.03E-04	1.063E-03 ± 0.745E-05
<i>Azoarcus</i>	2.95E-04 ± 1.54E-04	1.01E-04 ± 1.33E-04	0.26E-04 ± 3.3E-04A
<i>Citrobacter</i>	3.56E-04 ± 3.32E-04	5.6E-05 ± 8.3E-05	3.7E-05 ± 4.8E-05
<i>Enterobacter</i>	1.27E-04 ± 0.56E-04	1.28E-04 ± 0.69E-04	3.0E-05 ± 2.2E-05
<i>Escherichia</i>	0.804E-03 ± 1.378E-03	0.99E-04 ± 1.01E-04	0.5E-05 ± 0.9E-05
<i>Klebsiella</i>	4.14E-04 ± 2.73E-04	8.96E-04 ± 6.41E-04	6.91E-04 ± 5.00E-04
<i>Serratia</i>	1.3E-05 ± 1.2E-05	2.63E-04 ± 4.56E-04	-
<i>Pseudomonas</i>	1.446E-02 ± 0.809E-02	1.768E-02 ± 1.062E-02	1.95E-02 ± 1.206E-02
<i>Stenotrophomonas</i>	-	2.2E-05 ± 2.6E-05	1.06E-04 ± 0.94E-04
<i>Fretibacterium</i>	0.6E-05 ± 1.0E-05	3.18E-04 ± 2.62E-04A	5.3E-05 ± 6.1E-05

A: $P < 0.05$ in comparison with the control group; B: $P < 0.05$ in comparison with the model group

Ruminococcus and *Methylococcus* were exclusively identified in model group mice. In addition, bacteria of *Mycobacterium*, *Frankia*, *Sphingobium* and *Serratia* were undetectable in treatment group mice. Except for *Eggerthella*, *Burkholderia* and *Azoarcus*, the abundance of lactase genes in most of the originated genus in all three groups showed no significant difference. The abundance of lactase genes in *Eggerthella* and *Burkholderia* were seen to be increased while those in *Azoarcus* were shown to be decreased in treatment group mice compared to the control group and model group (Table 3).

Besides those of known bacteria, other lactase genes in unclassified bacteria or bacteria without blast hit were found to be in the level of phylum and genus. The percentage of unclassified and no blast hit bacteria were 63.1 and 32.8%, respectively (Fig. 4).

Discussion

In China, it is very common to treat diarrhea with Chinese traditional medicines (herbs). As reported, their effects were partly due to the regulation of lactase activities (Zhang et al. 2009, 2010). Our previous study showed that the lactase activity was decreased in mice with antibiotics-induced diarrhea. Therefore, our current study aimed to figure out whether QWBZS, a common medicine used for treating diarrhea, affects the lactase activity and further prove that the diarrhea is strongly associated with change in lactase activity.

Our results showed that as with control group and model group, the QWBZS treatment group had no significant difference in Chao index and ACE index of intestinal bacteria lactase genes, whereas Shannon index in the QWBZS treatment group was decreased significantly ($P < 0.01$ or

Fig. 4 Percentage of different lactase-producing bacteria. **a** In the level of phylum; **b** in the level of genus

$P < 0.05$). As shown in PCoA figure, samples in treatment group were clearly distinguished from those of control group and model group. These results indicated that QWBZS treatment decreased the richness of lactase genes of intestinal bacterial contents and changed the composition of bacteria community for producing lactase.

Bacterial lactase activities in the intestine can be affected by the quantity and different species of bacteria producing lactase, as well as the variation (including mutations) of lactase gene. The species producing lactase can be divided into three types on the basis of lactase-producing capability: high lactase-producing strains, low lactase-producing strains and inactive lactase-producing strains (Tan and Shi 2008; Jiang et al. 2014). Our current results showed that while the majority of species expressing lactase genes were

unclassified, Actinobacteria, Firmicutes and Proteobacteria were the major known phyla for producing lactases in gut. Among the known lactase-producing genus, *Mycobacterium*, *Frankia*, *Sphingobium* and *Serratia* could not be detected in the treatment group. These results suggested that most of the identified bacteria expressing lactase genes in our study are novel and that *Mycobacterium*, *Frankia*, *Sphingobium* and *Serratia* make little contribution to lactase activity. Elgueza-bal et al. also reported no association was found between *Mycobacterium avium* subsp. paratuberculosis infection and lactase gene expression (Elgueza-bal et al. 2012).

It is known that lactase activities can be influenced by not only bacteria species but also their gene abundance. At the genus level, the abundance of lactase genes was shown to be higher in *Corynebacterium*, *Arthrobacter*, *Eggerthella*, *Acidovorax*, *Klebsiella* and *Pseudomonas*. Treatment with QWBZS increased the abundance of lactase gene originated from *Acidovorax* sp. KKs102, *Stenotrophomonas* sp. LMG11000, *Pseudomonas oleovorans*, *Eggerthella* and *Burkholderia*, but decreased that from *Azoarcus*. A recent study by Wang et al. (2017) reported that the metabolite by *Eggerthella* showed an inhibitory effect on growth of human colon cancer line. In addition, the relative abundance of *Eggerthella* was reduced in Rett syndrome subjects as compared to healthy controls (Strati et al. 2016). These results suggested that *Eggerthella* is one of the major strains with high level of lactase gene expression and lactase production.

In summary, our study suggests that the probable mechanism of QWBZS improving lactase activity would be the increase in the abundance of lactase genes by some key lactase-producing species rather than an increase in the lactase gene diversity. In addition, a large number of novel bacteria expressing lactase genes were found in our study and they are worth being explored in future studies.

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Compliance with ethical standards

Conflict of interest No conflict of interest was declared.

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