Regulation and analysis of the diversity of intestinal microbiota in SD rats by Danggui Buxue Tang (DBT) fermented with *Bacillus subtilis*

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Abstract

**Purpose:** To investigate the effect of Danggui Buxue Tang (DBT) on intestinal microbiota diversity after fermentation by *Bacillus subtilis*.

**Methods:** *B. subtilis* was used to ferment DBT. Sprague Dawley (SD) rats were randomly divided into the following four groups with six rats in each group: the control group, DBT nonfermentation group, *B. subtilis* group, and DBT fermentation group. Rats were fed continuously for 14 days. The 16S rRNA of faecal samples was analysed by high-throughput Illumina sequencing.

**Results:** In total, 3483 operational taxonomical units (OTUs) were identified in this study, and 1236 OTUs were shared among all samples. Moreover, the most abundant phyla identified in this study were *Bacteroidetes* (29.65–38.19%) and *Firmicutes* (48.30–67.04%). The F/B ratios of the DBT nonfermentation group (1.07%) and the DBT fermentation group (1.78%) were slightly lower than those of the control group (2.29%). *Lactobacillus* was most upregulated in the DBT fermentation group (38.4%), followed by the DBT nonfermentation group (18.97%), control group (14.61%), and probiotics group (8.39%). Moreover, the pathogenic bacteria *Alistipes* and *Parabacteroides* were found to be downregulated in the DBT fermentation group (the percentages of *Alistipes* and *Parabacteroides* were as follows: control group, 8.09% and 0.16%; DBT nonfermentation group, 4.31% and 0.37%; DBT fermentation group, 1.96 and 0.09%; and probiotics group, 6.25% and 0.12%, respectively).

**Conclusion:** This study is the first to research systematically the effects of DBT on the diversity of rat intestinal microbiota before and after fermentation. The structural characteristics of complex bacterial community in each group were clearly analysed, and DBT significantly increases probiotics and inhibits pathogenic bacterial growth in the intestinal tract of rats after fermentation, which plays a significant role in maintaining the balance of the intestinal microbiota of the rats. This research provides new insights into the development and utilization of traditional Chinese medicine.

**Keywords:** Danggui Buxue Tang, Fermentation, Intestinal microbiota

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Introduction

The intestinal microbiota participates in a variety of metabolic processes of the host, including sugar metabolism and fat metabolism. Many metabolic diseases, such as obesity and diabetes, are closely related to changes in the structure of the intestinal microbiota (Koh et al. 2018; Huang et al. 2019). Innate and adaptive immune systems require an interaction between the body and microorganisms during development, and different microbial taxa generally play various physiological roles and perform different molecular functions in the intestines of animals (Kau et al. 2011). The daily diet of animals plays an important role in the structure and balance of intestinal microbial groups, and the key role of intestinal microbial groups in combating the colonization and excessive growth of pathogenic microorganisms in the body has gradually attracted attention (Kamada et al. 2013). Probiotics indirectly regulate the homeostasis of the body through molecular mechanisms related to homeostasis, including the intestinal epithelial barrier, the immune system, and substances necessary to compete with pathogens for growth (Macpherson and Harris 2004; Gourbeyre et al. 2011; Bermudez-Brito et al. 2012). In addition, many studies have shown that probiotics improve the overall health of animals by balancing the intestinal microecosystem. Bacillus subtilis is one of the two types of bacillus used to inoculate forage allowed by the Ministry of Agriculture and Rural Affairs of China, and it is a dominant biological population widely present in soil and plants. As a common probiotic in the production of fermented foods, B. subtilis significantly improves the growth, immune performance, and intestinal microbiota of animals, and it has been widely used to inhibit the propagation of foodborne pathogens (Stein et al. 2005; Chen et al. 2013; Eom et al. 2014; Piewngam et al. 2018).

Traditional Chinese medicine (TCM) has been used in China for thousands of years (Zheng et al. 2010). One of the classic compounds, Danggui Buxue Tang (DBT), is composed of two simple medicinal materials, Astragali Radix and Angelica Sinensis Radix, at a ratio of 5:1. DBT has been widely used in China for more than 800 years (Xie et al. 2012). The results of pharmacological studies show that DBT has many effects. For example, DBT replenishes blood by promoting haematopoiesis and stimulating cardiovascular circulation, and it improves secretion plenishes blood by promoting haematopoiesis and stimulating cardiovascular circulation, and it improves secretion and intestinal microbiota and related immune active substances in the body. In addition, DBT has antioxidant activity and immune activity (Dong et al. 2006; Gao et al. 2008). Furthermore, the use of “medicine food homology” (MFH) for dietary regulation will be a new trend. Using the renewable resources of MFH to develop and produce new drugs or health care products has a broad prospect (Hou and Jiang 2013; Song and Jiang 2018).

Microbial fermentation technology has been widely used in the food industry, including in the production of beer and wine. Therefore, during the fermentation process, the relevant metabolic activities of microorganisms are indispensable. The status of TCM is equally important. To fully exploit the potential efficacy value of TCM, fermentation has an extremely important role in standardizing efficacy. However, the effect of DBT fermentation liquid on intestinal microbiota has not been reported (Sang et al. 2012).

Here, we provided and compared the microbial communities of rats with three different diets (DBT non-fermentation liquid, DBT fermentation liquid, and B. subtilis), and the 16S rRNA genes of the faecal samples were analysed to better understand the differences in the structure of the faecal microbial communities among the four groups. The aim of this study was to explore the positive effects of TCM fermentation broth on intestinal microbiota and lay a theoretical foundation for the expansion of TCM research.

Materials and methods

Plant materials and preparation of DBT

Plant materials

The Astragali Radix and Angelica Sinensis Radix were acquired from Minxian in the Gansu Province. For the preparation of DBT, Astragali Radix and Angelica Sinensis Radix were weighed accurately at a ratio of 5:1, crushed evenly, mixed by vortexing, and filtered through a sieve.

Fermented DBT and B. subtilis bacterial preparations

The B. subtilis used in this study was purchased from the China Centre of Industrial Culture Collection (CICC 10089). The fermented liquid medium (FLM) contained 6% dried DBT powder, 0.95% glucose, 1.52% peptone, 0.3% yeast extract powder, 0.05% K2HPO4, 0.07% MgSO4, and 0.02% CaCO3 in 100 ml of distilled water (initial pH 7.2), and it was made in 250 ml flasks. The stock culture was grown in LB broth in a shaker at 160 rpm and 37 °C for 24 h until the logarithmic phase. Liquid-state fermentation and bacterial culture (grown in LB) were performed by culturing 2.92% (v/v) pre cultured B. subtilis (3.6 × 10⁸ CFU/ml) in a shaker at 160 rpm and 37 °C for 35.8 h to obtain the fermentation product (B. subtilis, 6.8 × 10⁸ CFU/ml). The fermentation product was then obtained after freeze-drying. DBT (unfermented product) was also obtained in the same manner but without bacteria present in the process.

Animals and experimental design

Female Sprague Dawley (SD) rats weighing 180–220 g were obtained from the Lanzhou Veterinary Research Institute of the Chinese Academy of Agricultural Sciences.
After 1 week of adaptation, rats were randomly divided into the following four groups (n = 6): the control group (CG) received 3 ml of normal saline; the DBT fermentation group (FG) was intragastrically administered 2 g/kg DBT fermentation product with 3 ml of normal saline; the DBT nonfermentation group (WG) received 2 g/kg DBT with 3 ml of normal saline; and the probiotics group (PG) was orally administered B. subtilis (6.8 × 10⁸ CFU/per rat) with 3 ml of normal saline. The study period was 2 weeks, and the oral treatments for all rats were administered daily. Each group of rats was given the same diet (SPF-grade fodder) during the treatment, and faecal samples were collected from each rat after 2 weeks. Sterile forceps were used for sample handling, and an average of 100–200 mg of faeces was collected per rat. Immediately after collection, samples were frozen at −80 °C for DNA extraction. All of rats in this study were cared for according to the specifications of the Ethics Committee of Lanzhou Institute of Husbandry and Pharmaceutical Sciences of Chinese Academy of Agriculture Sciences for the Care and Use of Laboratory Animals (2018).

DNA extraction and intestinal microbiota analysis
DNA extraction and high-throughput sequencing were performed according to our previously reported method with some modifications. DNA from different samples was extracted using the E.Z.N.A.®Stool DNA Kit (D4015, Omega, Inc., USA) according to the manufacturer’s instructions. Total DNA was eluted in 50 μl of elution buffer and stored at −80 °C until measurement by PCR by LC-Bio (Hang Zhou, China), and the isolation of DNA was confirmed by 1.2% agarose gel electrophoresis. Before sequencing, the 16S rDNA V3-V4 region of each sample was amplified with a set of primers targeting the 16S rRNA gene region. Sequencing libraries were generated using the NEB Next, Ultra DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer’s recommendations, and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer (Life Technologies, CA, USA) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina MiSeq platform, and 300 bp paired-end reads were generated (Zhang et al. 2018).

Bioinformatics analysis
Samples were sequenced on an Illumina MiSeq platform according to the manufacturer’s recommendations provided by LC-Bio. Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequences. Paired-end reads were merged using FLASH. Quality filtering of the raw tags was performed under specific filtering conditions to obtain high-quality clean tags according to fqtrim (V 0.94). Chimeric sequences were filtered using Search software (v2.3.4). Sequences with ≥ 97% similarity were assigned to the same operational taxonomic units (OTUs) by Vsearch (v2.3.4). Representative sequences were selected for each OTU, and taxonomic data were then assigned to each representative sequence using the Ribosomal Database Project (RDP) classifier. The differences in the dominant OTUs in different groups and multiple sequence alignment were conducted using mafft software (V 7.310) and were used to study the phylogenetic relationship of different OTUs. OTUs abundance information was normalized using a standard sequence number corresponding to the sample with the least sequences. Alpha diversity was applied to analyse the complexity of OTUs diversity for a sample through two indices, including Chao1 and observed OTUs, and all these indices in our samples were calculated with QIIME (version 1.8.0). Beta diversity analysis was used to evaluate differences in the OTUs complexity of samples. Beta diversity was calculated by NMDs, and cluster analysis was calculated by QIIME software (version 1.8.0) (Li et al. 2018).

Statistical analysis
Data were analysed by one-way ANOVA using SPSS 23.0, and the significance test of differences in four groups was performed by LSD multiple comparisons. P < 0.05 was used as the significant level of difference, and the data analysis results were expressed as the mean ± standard deviation.

Results
Abundance and diversity of sample OTUs
The rank abundance curve of bacterial OTUs indicated that in the model used in this study, most OTUs exhibited low-abundance microbial populations in rat faeces. A Venn diagram was generated to aid in the understanding of the OTUs shared between samples (Fig. 1). The analysis results showed that 3483 OTUs were detected in all samples, but only 1236 were shared in the total abundance. In our study, 2649, 2862, 2485, and 1902 OTUs were obtained from the CG, WG, PG, and FG samples, respectively, accounting for 76.06, 82.17, 71.35 and 54.61% of the OTUs, respectively.

Variation in alpha diversity
Alpha diversity was assessed by the Chao1 index and observed OTUs index, and the results are presented in Fig. 2. In all four groups, rarefaction curves of Chao1 indices were close to the saturation platform at a sequencing depth of 10,000 (Fig. 2a). The observed OTUs curves showed a similar trend. The microbial community richness in the WG samples was higher than those in the other three groups (P < 0.05) (Fig. 2b). The microbial community richness in the FG samples was the lowest
among the samples \((P < 0.05)\). Thus, these findings demonstrated that the WG samples had the highest microbial community diversity \((P < 0.05)\) (Table 1).

**Variation in beta diversity**

To facilitate the observation of faecal microbial population differences between groups, the NMDS test based on the unweighted UniFrac distance matrix was applied. The individual samples from rats fed with the same sample solution were more closely clustered, but there were significant differences between the individual samples of different sample solutions given to the stomach, indicating that the different dietary patterns in this study had significant effects on the faecal microbial communities in the four groups (Fig. 3).

**Microbial community structure at the phylum, family, and genus levels**

In the study of taxonomic composition (Fig. 4), the classification of sequences from the samples resulted in more than nine different phyla identified in this study. The most abundant phyla identified in the four groups were *Bacteroidetes* (29.65–38.19%) and *Firmicutes* (48.30–67.04%), both accounting for 96.98% (Fig. 4a). The other phyla present in relatively low abundance were *Actinobacteria*, *Tenericutes*, *Proteobacteria*, *Verrucomicrobia*, and *Cyanobacteria* as well as two unclassified bacteria. In addition, the ratio of *Firmicutes* to *Bacteroidetes* \((F/B)\) was lower in the WG and FG samples than in the CG samples. The F/B ratio was lower in the WG samples than in the FG samples, and it was higher in the PG samples than in the CG samples.

To better understand the structure of the faecal microbiota in rats, we performed faecal sample analysis at the family level (Fig. 4b), and the top 20 bacteria were selected for analysis at this level. The following families showed an increasing trend after different treatments. In the CG samples, *Lachnospiraceae* was the predominant family (31.02%) followed by *Porphyromonadaceae*, *Lactobacillaceae*, and *Prevotellaceae*. *Lachnospiraceae* was also the predominant family in the PG samples (46.00%) followed by *Porphyromonadaceae*, *Lactobacillaceae*, and *Rikenellaceae*. Remarkably, the abundance of *Lactobacillaceae* in the PG samples was higher than that in the other groups \((P < 0.05)\), while the abundance of *Lactobacillaceae* in the FG samples was lower than that in the other groups \((P < 0.05)\), especially in the CG samples.
samples. We also found two unclassified sequences at the family level, namely Bacteroidales and Clostridiales, and they comprised 2.33, 3.94, 1.18, and 1.24% of CG, WG, FG, and PG samples, respectively.

At the genus level, significant differences were observed for most of the samples (Fig. 4c). The unclassified bacteria in the four samples accounted for 34.81, 40.56, 52.88, and 35.25% of the top 20 reads for the CG, WG, PG, and FG samples, respectively. We generated a heatmap to show the genera at different levels in the samples. Lactobacillus was most abundant in the FG samples (38.4%) followed by WG (18.97%), CG (14.61%), and PG (8.39%) (P < 0.01). Surprisingly, there was a significant reduction in the relative abundance of Alistipes (CG, 8.09%; WG, 4.31%; FG, 1.96; and PG, 6.25%), Prevotella (CG, 8.09%; WG, 4.31%; FG, 1.96; and PG, 6.25%), and Bacteroides (P < 0.05) after treatment. Moreover, the abundance of Parabacteroides in the FG samples (0.09%) was significantly less than that of the other three groups (CG, 0.16%; WG, 0.37%; and PG, 0.12%). Thus, the intake of different treatments had a significant influence on the diversity of intestinal microbiota.

### Discussion

The faecal microbiota plays an important key role in the absorption and transformation of nutrients involved in the daily diet (Ghosh et al. 2014). In recent years, related studies have reported that Chinese herbal extracts, probiotics, and fermentation broth have significant effects on intestinal microbiota (Eom et al. 2014, Zhang et al. 2017). In this study, rats were given DBT, B. subtilis, and DBT fermentation product to comprehensively evaluate the effect on intestinal microbiota by comparison. Compared to other samples, the intestinal probiotics were significantly upregulated in the FG samples, and the pathogenic bacteria were significantly downregulated in the FG samples. These findings demonstrated that DBT fermentation product has a significant influence on improving the intestinal environment and balancing the intestinal microbiota.

Analysis of the four groups of faecal samples indicated that Firmicutes accounted for the largest proportion of all categories in the total microbiota. Previous studies have shown that Firmicutes plays a key role in improving the nutritional absorption of the daily diet and energy conversion of animals (Turnbaugh et al. 2008; Jumpertz et al. 2011). Although the diet patterns of rats in the four experimental groups were different, Firmicutes was still the most important category of all stool samples (48.30–67.04%) in our study, and the differences between the groups were not significant.

Bacteroidetes and Firmicutes play an important role in the regulation of colon metabolism, and they can further decompose and absorb undigested food residues in the intestine through a series of systematic energy metabolism mechanisms (Candela et al. 2010). In addition, related studies have indicated that reducing the proportion of F/B in the main bacteria effectively prevents further development of obesity affected by diet (Turnbaugh et al. 2006). As a TCM for tonics, DBT has a significant stimulating effect on the immune functions of animals, and Astragalia Radix polysaccharide, a main active ingredient of DBT, has a certain effect on the body weight of mice fed a high-fat diet (Mao et al. 2009). In the present study, we noted that the F/B ratios of the WG and FG samples were lower than those of the CG samples (WG, 1.07 ± 0.43; FG, 1.78 ± 0.93; and CG, 2.29 ± 1.79). In addition, the F/B ratios of the PG samples showed an upward trend (3.45 ± 2.69), but the difference in means was not significant (P = 0.13). Nevertheless, relevant studies have indicated that polysaccharides and metabolites can inhibit the growth of Firmicutes and thus balance Bacteroidetes in the intestine (He et al. 2016). Bacteroidetes can further digest and absorb dietary polysaccharides and other complex energy substances in the large intestine and then convert them into short-chain fatty acids for reuse as energy substances in animals (Becker et al. 2014).

### Table 1 The influence of different preparations on biodiversity of the three treatment groups

| Samples | Chao1       | Observed OTUs       |
|---------|-------------|---------------------|
| CG      | 1137.87 ± 224.19a | 735.67 ± 140.38a |
| WG      | 1181.81 ± 178.28b | 1003.33 ± 120.69b |
| FG      | 918.97 ± 141.11c | 584.83 ± 139.93c |
| PG      | 1248.20 ± 91.17a | 863.50 ± 61.22ab  |

The results are expressed as the mean ± SD (n = 6). Different letters in the same column indicate significant differences (P < 0.05) among different groups.
SCFAs are metabolites of the intestinal microbiota. The daily diet pattern of the animal body has a certain effect on the structure of the intestinal microbiota and affects the composition ratio and concentration of SCFAs. SCFA-producing bacteria breakdown fibrous foods to obtain energy substances, and high concentrations of SCFAs in faeces promote this behaviour of bacteria and then play a certain role in resisting host colon disease (Sanz et al. 2005).

In addition, butyric acid is produced in the later stages of fermentation, which is related to the conversion of other bacterial metabolites formed during fermentation (Świątecka et al. 2011). By far, most bacteria related to butyric acid concentration in the caecum are related to Firmicutes. In the present study, the WG and FG samples promoted the growth of Bacteroides and inhibited the proliferation of Firmicutes. However, the internal pathways and mechanisms need to be comprehensively elucidated by genomic technology.

Lactobacillus is one of the most widely studied and used probiotics (Soto et al. 2014). Lactobacillus relieves lactose intolerance, prevents intestinal infection, stimulates the immune system to enhance response, reduces inflammation, reduces allergic reactions, promotes gastrointestinal motility, and prevents depression (Levri et al. 2005; Bravo et al. 2011). In addition, Lactobacillus promotes antihypertensive activities, prevents colon cancer, and reduces the incidence of cardiovascular disease. In our study, Lactobacillus was the most predominant genus. Some related studies have shown that several TCMs of the tonic category promote the proliferation of probiotics in the intestine, inhibit the proliferation of pathogenic bacteria, and regulate the intestinal microbiota (Yu et al. 2019). As a TCM for tonics, DBT had a significant stimulating effect on Lactobacillus in our study, and the FG samples had a stronger effect than the WG samples ($P < 0.05$). Moreover, Parabacteroides is a genus of Bacteroides and is a drug-resistant pathogen that has been found to cause intra-abdominal infections. Alistipes is also a pathogen that is involved in the progression of colitis and colon cancer. Langille et al. (2014) also reported a significant increase in the abundance of Alistipes in weak mice. Our study showed that the abundance of these two pathogens was significantly reduced in the FG samples compared to the other three groups, which was consistent with previous reports. For example, after treatment of ulcerative colitis in rats induced by trinitrobenzene sulfonic acid using a red ginseng decoction, the number of beneficial bacteria in the intestines of rats, such as Bifidobacterium animalis, Bifidobacterium
longum, and Lactobacillus, is significantly upregulated, while the number of pathogenic bacteria, such as Escherichia coli, is significantly reduced (Guo et al. 2015). In the present study, the PG samples showed the opposite result for Lactobacillus. In addition, we also found that the number of Prevotella in the other three groups decreased compared to the CG samples and that the PG samples were particularly significant (P < 0.01). It has been reported that the number of Prevotella has a strong positive correlation with animal body weight. Probiotics colonize the intestines and help to digest food residues, produce nutrients, balance intestinal microbiota, inhibit pathogenic bacterial growth, inhibit bacterial reproduction, and stimulate the body’s immune system (Hill et al. 2014). However, with the development of sequencing technology, some related reports have indicated that additional supplementation with probiotics does not have a probiotic effect on the body (Zmora et al. 2018). Therefore, we postulate that this phenomenon may be related to the type of probiotics and the internal environment of the animal, which requires further exploration.

The intestinal microbiota of rats fed DBT fermentation product was analysed for the first time by high-throughput sequencing technology, and this analysis provided a clearer understanding of the structure of the intestinal microbiota of rats after experimental treatment. However, in the process of sequence analysis of samples, some inevitable errors in sample preparation, library preparation, and bioinformatics analysis are prone to occur (Lawrence et al. 2013). Thus, improvement of the experimental method can reduce the occurrence of experimental errors as much as possible (Williams et al. 1999; Akbari et al. 2005). However, studies on the identification of faecal microbial populations are not adequate for comprehensive and systematic analysis of faecal microbial populations. The lack of 16S rRNA gene sequences in the available databases also hinders the development of sequencing technology. In addition, short-read high-throughput sequencing has weaknesses, including the generated “noise.” In general, the quality of sequencing in this study was reliable (Table S1). Although we analysed specific microbial microbiota in rat stool samples, there are still many strains in the samples that need further classification and functional identification (Quince et al. 2011).

In conclusion, we performed 16S rRNA-based microbial analysis of the bacteria in rats given different dietary treatments by high-throughput Illumina sequencing technology. We found many differences in the intestinal microbiota structure and changes in composition among the groups in this study. Although the bacterial community may be affected by many factors, the structural characteristics of the complex bacterial community in the faeces of the four groups were well explained in this study. In particular, the DBT fermentation product significantly improves the microbiota balance, increases the number of beneficial bacteria, and inhibits the growth of pathogenic bacteria. However, the mechanism by which the fermentation broth balances the intestinal microbiota remains unclear. Therefore, further metagenomics and metabolomics research should be undertaken to fully understand the series of potential metabolic regulatory mechanisms of specific functional gut microbiota related to animals.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s13213-020-01563-y.

Additional file 1: Table S1. Data of reliability statistics for high-throughput sequencing.

Authors’ contributions
The authors read and approved the final manuscript.

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Ethics approval and consent to participate
All of rats in this study were cared for according to the specifications of the Ethics Committee of Lanzhou Institute of Husbandry and Pharmaceutical Sciences of Chinese Academy of Agriculture Sciences for the Care and Use of Laboratory Animals (2018).

Competing interests
The authors declare that they have no conflict of interest.

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