Saudi Journal of Biological Sciences 26 (2019) 402–412

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

The effect of *Codonopis bulleynana* Forest ex Diels on chronically constipated mice

Yunpeng Luan [a,b,*], Dechang Mao [b], Aiwei Guo [b], Yong Cao [c], Jielong Zhou [b], Fei Xiang [d], Zhi Xiong [e]

[a] Key Laboratory for Forest Resources Conservation and Utilization in the Southwest Mountains of China, Ministry of Education, Southwest Forestry University, PR China
[b] Department of Life Technology Teaching and Research, School of Life Science, Southwest Forestry University, PR China
[c] Department of Life Technology Teaching and Research, College of Life Sciences, Southwest Forestry University, PR China
[d] Yuxi Genor Biotechnology Co., Ltd, PR China
[e] Light Industry and Food Engineering College, SWFU, Bailongsi, PR China

ARTICLE INFO

Article history:
Received 7 July 2018
Revised 13 November 2018
Accepted 15 November 2018
Available online 16 November 2018

Keyword:
Codonopsis bulleyana
Long-term constipation
16S rDNA
Intestinal microbiota

ABSTRACT

To verify the laxative effect of *Codonopsis bulleyana* and its effect on intestinal microbiota, a long-term constipation model was established using 3.0 mg/kg loperamide hydrochloride, after which, the long-term constipation model was administered by 0.2 g/ml high-dose Codonopsis bulleyana water extract. The therapeutic effects were observed by measuring defecation amount and feces moisture content. The composition of intestinal microbiota was detected and analyzed using16S rDNA sequencing technology. The results showed that Codonopsis bulleyana water extract can increase stool quantity and promote intestinal tract movement in constipated mice. Obvious changes were shown in intestinal microbiota of chronically constipated mice treated with *Codonopsis bulleyana* water extract as the proportion of beneficial bacteria increased in the model treated by *Codonopsis bulleyana*. *Codonopsis bulleyana* water extract alleviates constipation symptoms caused by loperamide hydrochloride and improves the intestinal microbiota in constipated mice.

1. Introduction

With the increased modern living standards, dietary structures have changed, and various chronic diseases have threatened human health. So far, constipation has become a common clinical symptom. Constipation means the situation in which defecation frequency is reduced to less than 2–3 times per week, there exists difficulty in defecation (strenuous defecation takes more than 30 min at a time), and the feces is dry and small (Yanfeng, 2013; Feng et al., 2018; Khattak et al., 2018), When constipation occurs, feces will stay in the intestine for a long time, producing harmful substances such as H_2S and amines under the fermentation of massive microbiota in the intestinal tract. Intestinal microbiota, as an important part of the human body, plays an important role in the host health. In addition to defecation difficulties and fecal dryness, intestinal microbiota also changes significantly in case of constipation, with proportion of beneficial bacteria flora decreased and proportion of some neutral bacteria and harmful bacteria increased. Studies have shown that (Lin et al., 2014; Chassard et al., 2012; Chuanlei et al., 2018), intestinal microbiota of constipated patients have significantly decreased beneficial bacteria such as Bifidobacterium, Bacteroides, Lactobacillus but significantly increased pathogenic bacteria like Fusobacterium, Enterobacter regardless of animal experiments, clinical trials, or epidemiological studies (Xu et al., 2004; Shu-cheng et al., 2009; Kassim et al., 2018). Long-term constipation not only affects the individual's normal life, but also increases the risk of cardiovascular and cerebrovascular diseases and colorectal cancer (Linsheng et al., 2017).

Codonopsis bulleyana Forrest ex Diels is a Codonopsis plant of campanulaceae, which is mainly distributed in central, western and southern Yunan on hillside grassland and shrub at an elevation of 2800–4200 m. Enjoying a long history of cultivation and consumption, it is a unique plant species in Yunnan used as tonic (Pinhua et al., 2013). *Codonopsis bulleyana*, containing many

* Corresponding author at: Key Laboratory for Forest Resources Conservation and Utilization in the Southwest Mountains of China, Ministry of Education, Southwest Forestry University, 300 Bailong Temple, Panlong, Kunming, Yunnan 652400, PR China.
E-mail address: 1820059756@qq.com (Y. Luan).

Peer review under responsibility of King Saud University.

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https://doi.org/10.1016/j.sjbs.2018.11.009
1319-562X/

nutrients such as amino acids, vitamins, etc. (Pinhua et al., 2015) is often eaten as a mild tonic plant in southwestern China. Current studies on *Codonopsis bulleyana* are mainly focused on efficacy verification. There is less research on disease treatment mechanism of *Codonopsis bulleyana*. Studies have shown that (Ruoli et al., 1999; Yunpeng et al., 2017; Zijun et al., 2009; Qiongfen et al., 2002; Luan et al., 2018a, 2018b; Shabi et al., 2018), *Codonopsis bulleyana* can improve intestinal function, regulate immune function, and enhance cardiovascular system, which can inhibit colorectal cancer in mice. Constipation can be treated by surgery, physical method, etc. As the study on intestinal microbiota is gradually deepened, probiotics (Waller et al., 2011; Jayasimhan et al., 2013; Ojetti et al., 2014), synbiotics (Huang et al., 2018; D'Souza and Rahaman, 2018), etc. are used to treat constipation, which can achieve the purpose by changing composition structure of intestinal microbiota or promoting production of beneficial substances in intestinal microbiota. At present, there are few research reports on the effect of *Codonopsis bulleyana* on intestinal microbiota. In this study, long-term constipation model in mice was established using loperamide hydrochloride and treated with Codonopsis bulleyana water extract to detect the difference in intestinal microbiota between treatment group and model group. It is hoped that differential microorganisms between the two can be found to provide data basis for constipation treatment using *Codonopsis bulleyana* and development of functional foods composed of *Codonopsis bulleyana*.

2. Material and method

2.1. Material

Codonopsis bulleyana, Collected from Yiliang county, Yunnan province, China, KM mice (SPF-grade, 4-wk-old male, 20–25 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China, license number: SCXK 2016-0002). Food and water were available ad libitum, and the animals were housed under controlled environmental conditions. All experimental manipulations were undertaken in accordance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals, published by the National Science Council, China.

Commercially available Loperamide Hydrochloride capsules (specification, 2mg/tablet; Xian Janssen Pharmaceutical Ltd.).

2.2. Method

2.2.1. The establishment of long-term constipation model

Sixty Kunming mice with identical growth were selected and bred for one week in the laboratory. The mice were divided into two groups: the model group (group M; 40 mice in total) and the blank group (group B, 20 mice in total). Group M was intragastrically administered with 3.0 mg/(Kg · d) loperamide hydrochloride suspension, with 0.05 ml/10 g sterile normal saline for group B. With reference to data (Yu et al., 2016; Ljuan et al., 2017; Xiaoli et al., 2015; Mahmood et al., 2018), the mice weight and defecation amount were recorded every other day. On 22d, 3 mice were randomly selected from each group to detect the intestinal propulsion function and intestinal feces volume.

2.2.2. The effect of the extract of odor extract on the model

After successful establishment of the model, the group M mice were divided into two groups: the long-term constipation model group (CCa group) and the long-term constipation treatment group (CCaT group). The dose of loperamide was changed to 1.5 mg/(Kg·d) for CCa group to maintain long-term constipation in mice. CCaT group was intragastric ally administered with *Codonopsis bulleyana* water extract (including 2 g/ml crude drug) at a high dose of 0.1 ml/10 g. The mice weight and defecation amount were recorded every 4 days. On the 15th day of treatment, 3 mice were randomly sacrificed in each group to determine the intestinal propulsion function and intestinal feces volume. The mice in the CCaT group were sacrificed 45 days after intragastric administration, with intestine contents collected to detect intestinal microbiota.

2.3. Analysis of Intestinal mirobiota

The mice were sacrificed by cervical dislocation. The whole mice body was disinfected with 75% medical alcohol. The abdominal cavity was cut along the white line on the mice abdomen. The intestinal tissues of mice were completely removed and placed in ice-cold PBS solution to wash residual blood. After removal of the mesentery, excess water was absorbed with aseptic filter paper, followed by dissection of the intestinal tract of the mice to collect intestinal contents for use.

The 1 g intestinal contents were taken to extract the mice intestinal microbial genome DNA using Omega Stool DNA Kit. After passing the 1.5% agarose gel detection, it was ready for use. Primer 341F with barcode: CCTACGGGNGGCWGCAG; 806R: GGACTACHVGGGTATCTAAT were used to amplify16S rDNAV3 + V4 region in the DNA sample. For the amplification system, 50 μL reaction system contained 5 μL of 10 × KOD Buffer, 5 μL of 2.5 mM dNTPs, 1.5 μL of primer (5 μM), 1 μL of KOD polymerase, and 100 ng of template DNA. The amplification conditions were set as pre-denaturation at 95 °C for 2 min, subsequent denaturation at 98 °C for 10 s, annealing at 62 °C for 30 s, extension at 68 °C for 30 s for 27 cycles, and final extension at 68 °C for 10 min. The PCR amplification product was recovered after gel cutting and quantified using a QuantiFluorTM fluorometer. The purified amplification products were mixed in equal amounts, connected with sequencing joint to build a sequencing library for Hiseq 2500 PE250 sequencing on a computer.

Barcode sequence and primer sequence were truncated from the sequence obtained from the sequencing, followed by splicing, quality control and filtering to remove low-quality tags and chimeras that did not meet the length requirement. A high-quality Clean Tage was obtained, and the OTUs cluster analysis was performed based on Clean Tage. The OUT-representative sequence is compared to the GeenGene database. For the results, intestinal microbiota species and abundance were analyzed at the phylum level and the family level to compare differences in intestinal microbiota between the groups.

2.4. Statistical analysis of data

2.4.1. Statistical formula

Calculate feces moisture content and small intestinal movement function according to Formula (1), Formula (2).

$$\text{Feces moisture content} \quad \omega = \frac{M_W - M_d}{M_W} \times 100\% \tag{1}$$

where M_W is feces wet weight and M_d is feces dry weight.

$$\text{Intestinal propulsion rate} \quad W = \frac{L - L'}{L} \times 100\% \tag{2}$$

where L is the total small intestine length of the mice; L′ is the charcoal powder movement distance in the small intestine, that is, the distance from the gastric cardia end to the most significant end of the charcoal powder.

Calculate the Alpha diversity index using mothur software. The formula is as follows:

$$S_{Chao1} = S_{obs} + \frac{n_1(n_1-1)}{2(n_2+1)} \quad (3)$$

where

$S_{chao}1$ = Number of estimated OTUs; S_{obs} = Number of actually observed OTUs; n1 = Number of OTUs containing only one sequence (e.g. "singletons"); n2 = Number of OTUs containing only two sequences (e.g. "doubletons").

$$S_{ACE} = \begin{cases} S_{abund} + \frac{S_{rare}}{C_{ACE}} + \frac{n1}{C_{ACE}}\hat{\gamma}^2_{ACE}, for\ \hat{\gamma}^2 < 0.80 \\ S_{abund} + \frac{S_{rare}}{C_{ACE}} + \frac{n1}{C_{ACE}}\tilde{\gamma}^2_{ACE}, for\ \hat{\gamma}^2 \geqslant 0.80 \end{cases} \quad (4)$$

where

$$N_{rare} = \sum_{i=1}^{abund} in_i \quad C_{ACE} = 1 - \frac{n1}{N_{rare}}$$

$$\hat{\gamma}^2_{ACE} = max\left[\frac{S_{rare}}{C_{ACE}} \frac{\sum_{i=1}^{abund} i(i-1)n_i}{N_{rare}(N_{rare}-1)} - 1, 0\right]$$

$$\tilde{\gamma}^2_{ACE} = \max\left[\hat{\gamma}^2_{ACE}\left\{1 + \frac{N_{rare}(1-C_{ACE})\sum_{i=1}^{abund} i(i-1)n_i}{N_{rare}(N_{rare}-C_{ACE})}\right\}\right]$$

n_i = number of OTUs with i sequences; S_{rare} = number of OTUs with "abund" or less sequence; S_{abund} = number of OTUs with more than "abund" sequence; abund = threshold for "dominant" OTU, with default being 10.

$$H_{shannon} = -\sum_{i=1}^{S_{obs}} \frac{n_i}{N} \ln \frac{n_i}{N} \quad (5)$$

where

S_{obs} = number of actual observed OTUs; n_i = number of OTUs with i-sequences; N = number of all individuals, namely the total number of sequences here.

$$D_{Simpson} = \frac{\sum_{i=1}^{S_{obs}} n_i(n_i-1)}{N(N-1)} \quad (6)$$

where

S_{obs} = number of actually observed OTUs; n_i = number of OTUs containing only one sequence (e.g. "singletons"); N = number of all individuals, namely the total number of sequences here.

2.4.2. Statistical methods

SPSS 17.0 software was used for statistical analysis. One-Way ANOVA was used in statistics. Comparison between groups was performed using LSD multiple comparisons. Detection of significant abundance difference characteristics, screening of groups with significant abundance differences and determination of the influence of each component (species) abundance on discrepancy effect were performed using non-parametric factorial Kruskal-Wallis (KW) sum-rank test and Linear Discriminant Analysis (LDA) software LEfSe. Data roguing and parameter settings were performed using software FLASH, Trimmomatic and software platform: Usearch (version 7.0 http://drive5.com/uparse/). RDP classifier Bayesian algorithm was used in taxonomic analysis of OUT representative sequence with 97% similarity with reference to16S bacterial and archaeal ribosomal database Silva (Release 128 http://www.arb-silva.de).

3. Result

3.1. The establishment of constipation model

During the modeling process, the body weight of group M and group B mice showed an increasing trend in the first week of modeling, but the increase in body weight was smaller in group M. The body weight of group M mice decreased in the second week, which did not occur in group B. The body weight of group M mice showed an upward trend by the second weekend, approaching that of group B on the third weekend. Afterwards, group M mice had heavier body weight than group B, with the weight 1.16 times more than the latter by week 24 (Fig. 1). The defecation amount was similar between group M and B at the initial stage of modeling but was significantly reduced in group M on the fifteenth day of modeling (Table 1, Fig. 2). The feces moisture content also showed such phenomena. The feces moisture content of group M was calculated according to formula (1) on the fifteen day of modeling, which was decreased to 18.57%, 52.46% of that in the blank group (Table 2, Fig. 3).

During the modeling process, formula (2) was used to calculate the small intestine movement capacity. Small intestine movement function was decreased with the progress of modeling. Small

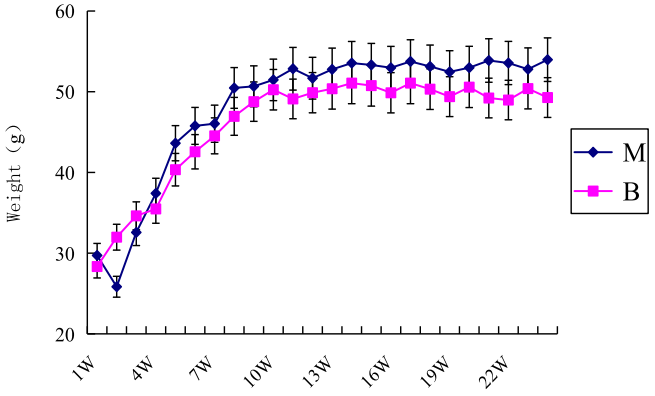


Fig. 1. Weight change during model establishment (W: Week).

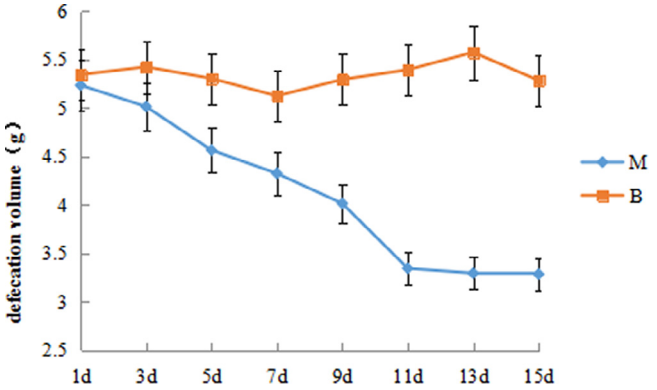


Fig. 2. Changes in defecation amount during model establishment.

Table 1
Changes in defecation amount during model establishment (unit: g).

	1d	3d	5d	7d	9d	11d	13d	15d
M	5.23 ± 0.31	5.01 ± 0.27	4.56 ± 0.26	4.32 ± 0.21	4.01 ± 0.29	3.34 ± 0.28	3.29 ± 0.21	3.28 ± 0.22
B	5.34 ± 0.29	5.42 ± 0.25	5.3 ± 0.26	5.12 ± 0.28	5.29 ± 0.31	5.31 ± 0.22	5.57 ± 0.32	5.28 ± 0.34

Table 2
Feces moisture content during modeling (%).

	1d	3d	5d	7d	9d	11d	13d	15d
M	30.28 ± 1.03	27.17 ± 0.98	28.13 ± 1.25	23.34 ± 1.13	21.45 ± 1.35	19.22 ± 1.29	19.01 ± 1.33	18.57 ± 1.14
B	35.27 ± 1.20	36.33 ± 1.34	35.34 ± 0.96	34.26 ± 1.38	35.37 ± 1.25	36.28 ± 1.27	34.14 ± 1.36	35.4 ± 1.21

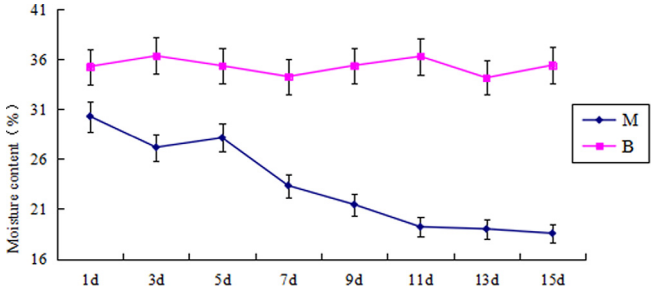


Fig. 3. Changes in feces moisture content during modeling.

intestine movement capacity of group M is 0.55 of group B on 22d (Table 3, Fig. 4). The intestinal feces volume in group M increased with the change of modeling time. The degree of adhesion of intestine feces to the colorectal intestinal wall was increased with the increased modeling time. The intestine feces volume of group M is 1.73 times that of group B on 22d (Table 3, Fig. 5).

Based on relevant literature data (Schloss et al., 2009; Edgar, 2010; Qiang et al., 2017; Chuanlei et al., 2018), the defecation amount, intestinal feces volume, intestinal propulsion rate, feces moisture content in group B was respectively 1.61, 0.58, 1.82, 1.91 times that of group M. The group M indicators are almost the same as those reported in the literature. Thus, it can be inferred that the experimental model has been established successfully (See Fig. 6).

3.2. Effect of Codonopsis bulleyana extract on model mice

After successful establishment of the model, the mice in group M were divided into long-term constipation model group (CCa group) and long-term constipation treatment group (CCaT group). CCaT group was given high-dose *Codonopsis bulleyana* water extract (Mahmood et al., 2018). Defecation amount of CCaT group intragastrically administered for 15 days and blank group (group B) is higher than that in CCa group (Table 4, Fig. 7), and increased feces moisture content and small intestine movement function, i.e. propulsion rate are shown in CCaT group (Table 5, Fig. 8, Table 6, Fig. 9) (see Table 7).

3.3. 16S rDNA sequencing analysis of intestinal microbiota

3.3.1. Statistical analysis and diversity analysis of intestinal microbial sequence OTUs

As chimera sequence will be produced in PCR amplification during high-throughput sequencing, the chimera sequence is removed according to the method described in literature (Yuan et al., 2017; Linsheng et al., 2017). The sequenced results are shown in Table 8, revealing high proportion of high-quality sequences in the

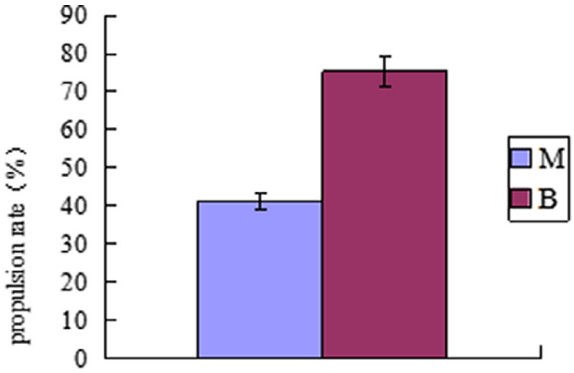


Fig. 4. Small intestine movement function of mice on 22d of modeling.

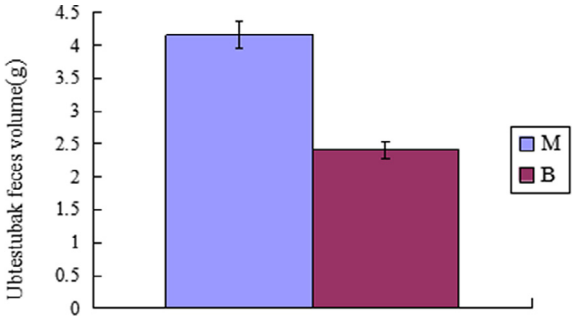
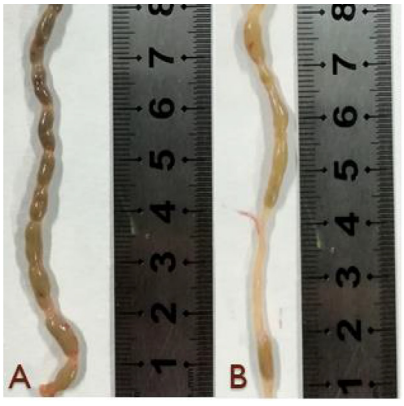


Fig. 5. intestine feces volume on 22d of modeling.



Note: A is the model group (M), B is the blank group (B)

Fig. 6. Model group and blank group. Note: A is the model group (M), B is the blank group (B).

Table 3
Mice movement function and intestinal feces volume on 22d of modeling.

	Total length of the small intestine	Charcoal powder advance length	Propulsion rate(%)	Intestinal feces volume (g)
M	70	29	41.42	2.41 ± 0.18
B	73	55	75.34	4.16 ± 0.21

Table 4
Changes in defecation amount in mice after intragastric administration of *Codonopsis bulleyana* water extract (unit: g).

	1d	3d	5d	7d	9d	11d	13d	15d
B	5.44 ± 0.19	5.61 ± 0.24	5.39 ± 0.25	5.31 ± 0.33	5.27 ± 0.29	5.45 ± 0.21	5.19 ± 0.27	5.27 ± 0.31
CCa	3.59 ± 0.21	3.37 ± 0.24	3.44 ± 0.19	3.51 ± 0.18	3.43 ± 0.25	3.46 ± 0.27	3.39 ± 0.31	3.42 ± 0.26
CCaT	3.44 ± 0.21	3.51 ± 0.27	3.78 ± 0.19	3.96 ± 0.25	4.14 ± 0.31	4.51 ± 0.28	4.68 ± 0.19	4.85 ± 0.24

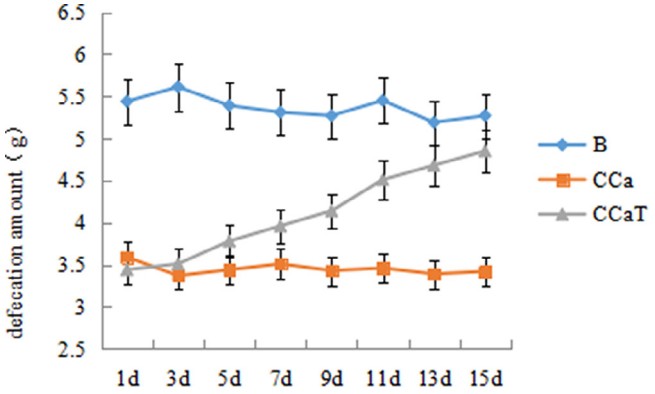


Fig. 7. Changes in defecation amount in mice after intragastric administration of *Codonopsis bulleyana* water extract.

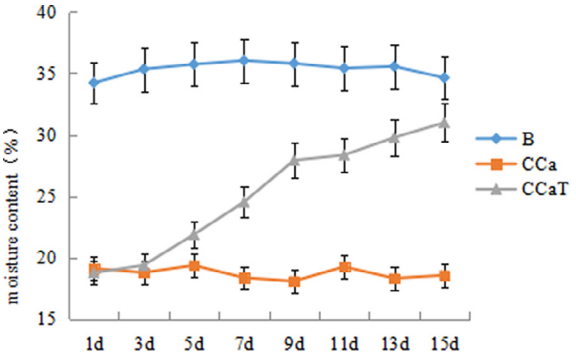


Fig. 8. Change in feces moisture content in mice after intragastric administration of *Codonopsis bulleyana* water extract.

experimental group that exceeds 95%. The high-quality sequences are concentrated in 306–473 bp in terms of length, indicating that the obtained sequences are bacterial sequences and can be used for subsequent analysis (see Fig. 10).

The high-quality sequences were clustered based on similarity 0.97. There were 456 consistent OTUs in the three experimental groups, of which there were 660 total OTUs in group B, 649 total OTUs in CCa group, and 705 total OTUs in the CCaT group. In terms of OUT total number, CCaT ranks the top, followed by CCa and B groups. Among all OTUs, group B shares 496 OTUs with CCa and 524 OTUs with CCaT group, while CCa and CCaT group share 537 OTUs (Fig. 11).

Alpha diversity analysis can reflect abundance and evenness within a specific region or ecosystem. Alpha diversity index includes ACE, Chao1, Shannon, and Simpson indexes. The values of ACE, Chao1, Shannon, and Simpson can indicate diversity abundance. A higher Alpha diversity indicates better abundance of bacterial species, more uniform structure of intestinal bacteria and more stable flora (Dandan et al., 2016). Beta diversity is to make comparative analysis of microflora composition of different samples.

It can be seen from Alpha diversity index that, Chao1, ACE and Shannon suggest abundance of intestinal microbiota in the three experimental groups following the high to low order of CCaT, B, CCa (Table 9, Fig. 12). This indicates that *Codonopsis bulleyana* water extract can increase the diversity of chronically constipated mice.

Weighted and unweighted Unifrac indexes between groups are demonstrated in the form of a heat map using Pheatmap package in R language. (see Fig. 13 The color shades and numerical values in the heat map represent the differences among the samples. A larger value indicates greater difference between samples, and more diverse microbial composition. Comparison of these two indexes reveals differences in microorganism composition among the three groups: B, CCa, and CCaT.

PCA (Principal Component Analysis) and PCoA analysis were performed based on OTU species abundance information

Table 6
Intestine propulsion rate of mice after intragastric administration of *Codonopsis bulleyana* water extract (%).

	Total length of the small intestine	Charcoal powder advance length	Propulsion rate (%)
B	72	52	72.22
CCa	71	31	43.66
CCaT	71	47	66.20

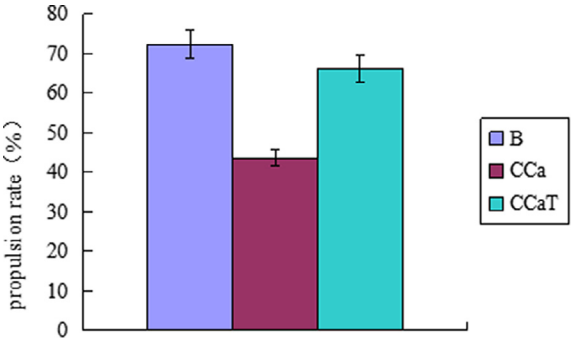


Fig. 9. Intestine propulsion rate of mice after intragastric administration of *Codonopsis bulleyana* water extract.

Table 5
Change in feces moisture content in mice after intragastric administration of *Codonopsis bulleyana* water extract (%).

	1d	3d	5d	7d	9d	11d	13d	15d
B	34.21 ± 1.30	35.32 ± 1.24	35.71 ± 0.86	36.02 ± 1.48	35.78 ± 1.15	35.39 ± 1.37	35.54 ± 1.26	34.62 ± 1.28
CCa	19.13 ± 1.13	18.79 ± 0.96	19.38 ± 1.15	18.37 ± 1.23	18.09 ± 1.15	19.27 ± 1.39	18.32 ± 1.23	18.57 ± 1.24
CCaT	18.77 ± 1.34	19.39 ± 1.19	21.88 ± 1.23	24.52 ± 1.17	27.91 ± 1.21	28.34 ± 1.26	29.76 ± 1.09	30.97 ± 1.31

Table 7
Intestinal feces volume in mice after intragastric administration of *Codonopsis bulleyana* water extract (unit: g).

CCa	CCaT	B
4.23 ± 0.14	2.79 ± 0.19	2.41 ± 0.17

Table 8
Sample sequence statistical table.

Sample name	Effective sequence	High quality sequence	Proportion (%)
B-1	75,875	72,761	95.90
B-2	70,024	67,780	96.80
B-3	62,904	60,632	96.39
CCa-1	62,014	59,938	96.65
CCa-2	57,615	55,585	96.48
CCa-3	48,787	46,748	95.82
CCaT-1	66,584	63,903	95.97
CCaT-2	68,925	66,508	96.49
CCaT-3	67,157	64,900	96.64

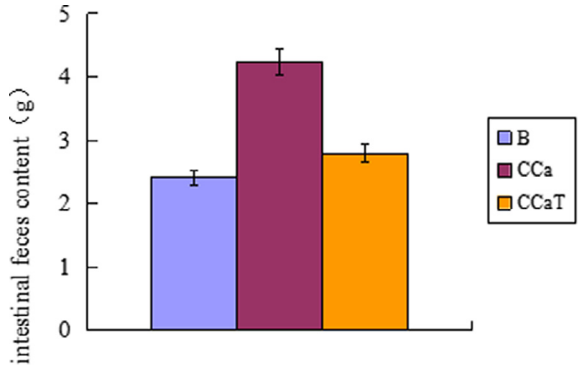


Fig. 10. Intestinal feces volume in mice after intragastric administration of *Codonopsis bulleyana* water extract.

(see Figs 14,15). In the analysis results, more similar sample composition comes with closer distances on PCA and PCoA maps, and different samples will often show respective cluster distribution. PCA analysis was performed on the samples of B, CCa, CCaT experimental groups, respectively. It was found that CCaT clustered well at the levels of phylum and family, with a certain degree of differentiation from B and CCa groups.

3.3.2. Intestinal microbiota composition analysis

On the phylum level, phylum bacteria such as Firmicutes, Bacteroidetes, Proteobacteria, Deferribacteres, Tenericutes, Actinobacteria, etc. are annotated (Fig. 16). At this taxonomic level, Firmicutes is the dominant phylum of the mice intestine in the three groups, with proportion in the rank of CCaT, CCa, B. The proportion of Firmicutes bacteria increased in the constipated mice model established by loperamide. Firmicutes bacteria proportion was higher in Codonopsis bulleyana treatment group than in CCa group, probably because certain compounds in Codonopsis bulleyana can stimulate the growth of Firmicutes bacteria in the intestine. Actinobacteria were highly enriched in CCa group at this taxonomic level. The proportion of Deferribacteres and Tenericutes was lower in the CCa group than in the CCaT and B groups.

At the family taxonomic level, 16S rDNA gene sequence of different mice intestinal microbiota is Lactobacilla-ceae, Lachnospiraceae, Ruminococcaceae, Helicobacteraceae, Prevotellaceae, Rikenellaceae, Bacteroidaceae, better than others (Fig. 17). The composition of mice intestinal microbiota in the CCaT group treated by Codonopsis bulleyana extract was more complicated at this taxonomic level. That is, there were more annotated dominant bacteria in this group than groups CCa and B. Moreover, beneficial bacteria were uniformly distributed in CCaT group with relatively high content, indicating that Codonopsis bulleyana can improve the structural composition of the intestinal microbiota in loperamide-induced constipated mice.

3.3.3. Differential analysis of Intestinal microbiota

Statistical method (Metastats software) was employed to test the differences in microflora abundances between the two sample groups to obtain p-values. Then p-values were corrected using FDR

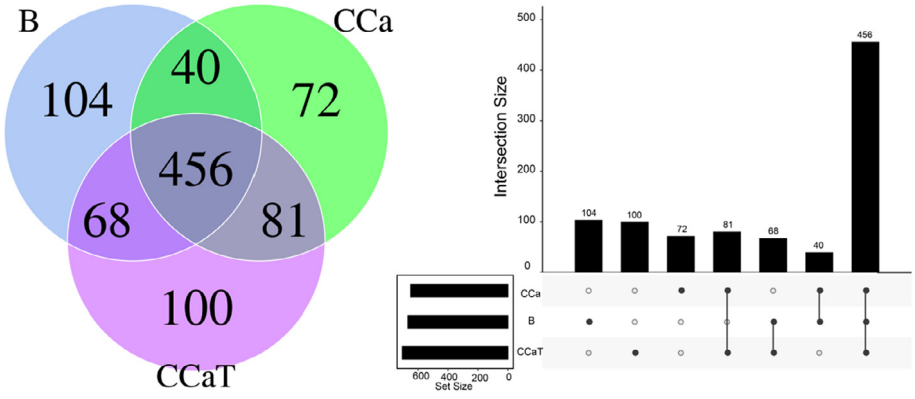


Fig. 11. Venn diagram of OTUs (OTU clustering of sequences with 97% or higher similarity level).

Table 9
Alpha diversity index.

Simple	Chao1	ACE	Shannon	Simpson
B	907.38 ± 54.93	874.04 ± 73.80	6.25 ± 0.61	0.97 ± 0.02
CCa	801.83 ± 121.47	784.38 ± 102.53	6.03 ± 1.32	0.95 ± 0.04
CCaT	925.10 ± 38.92	894.77 ± 52.40	6.54 ± 0.72	0.97 ± 0.01

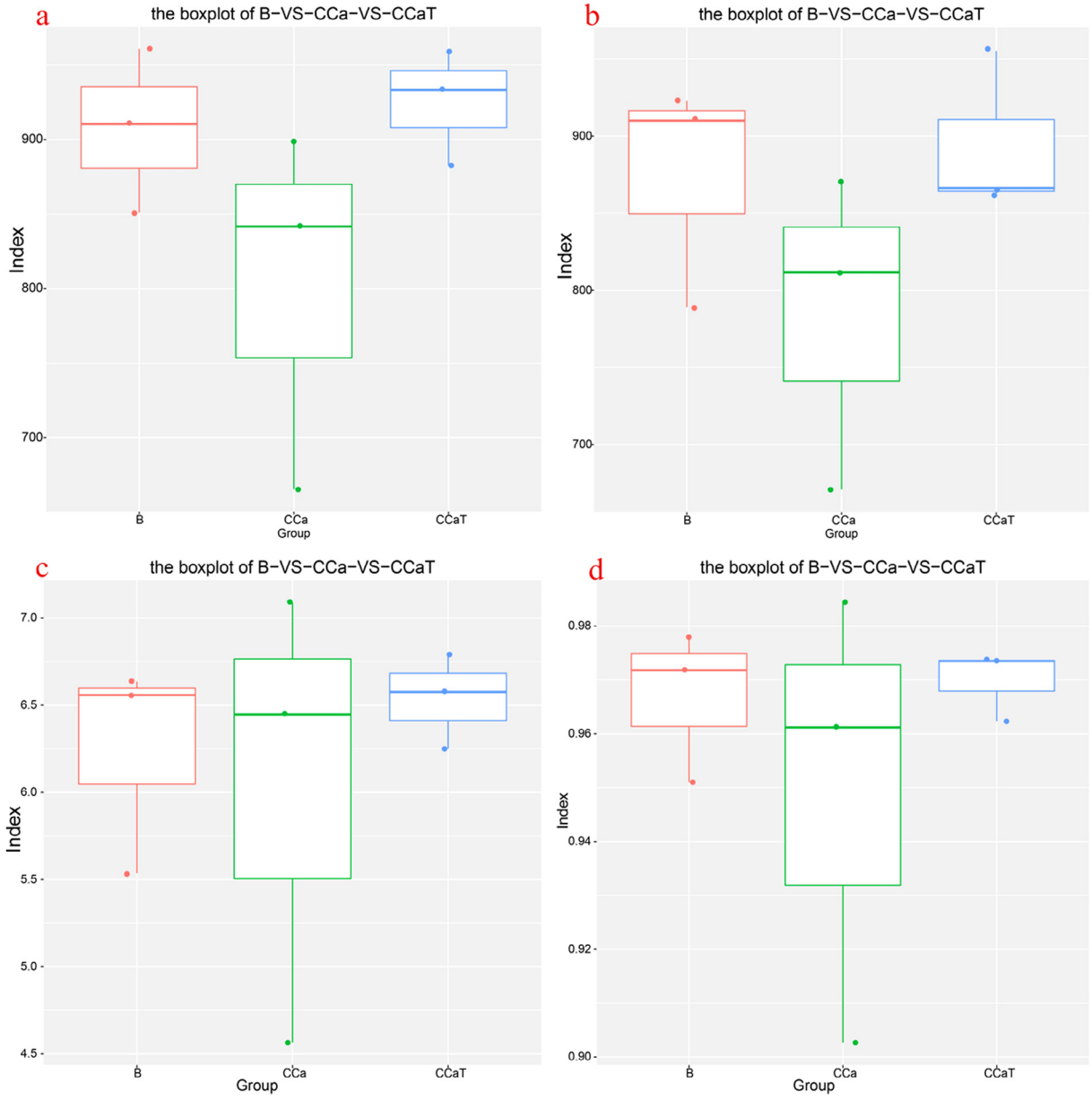


Fig. 12. B-VS-CCa-VS-CCaT Alpha diversity index box plot. a, The box plot of Chao1 index; b, The box plot of ACE; c, The box plot of Shannon index; d, The box plot of Simpson index.

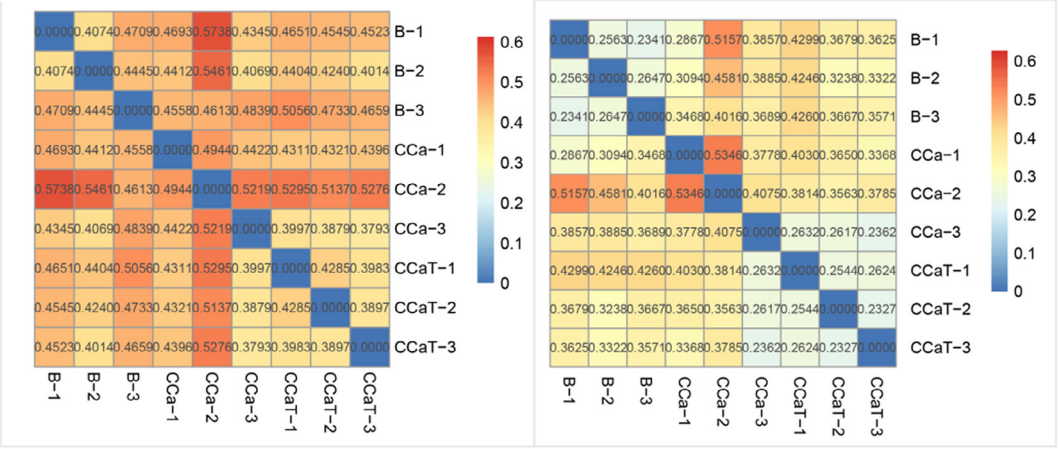


Fig. 13. Heat maps of Unweighted Unifrac index and Weighted Unifrac index between samples.

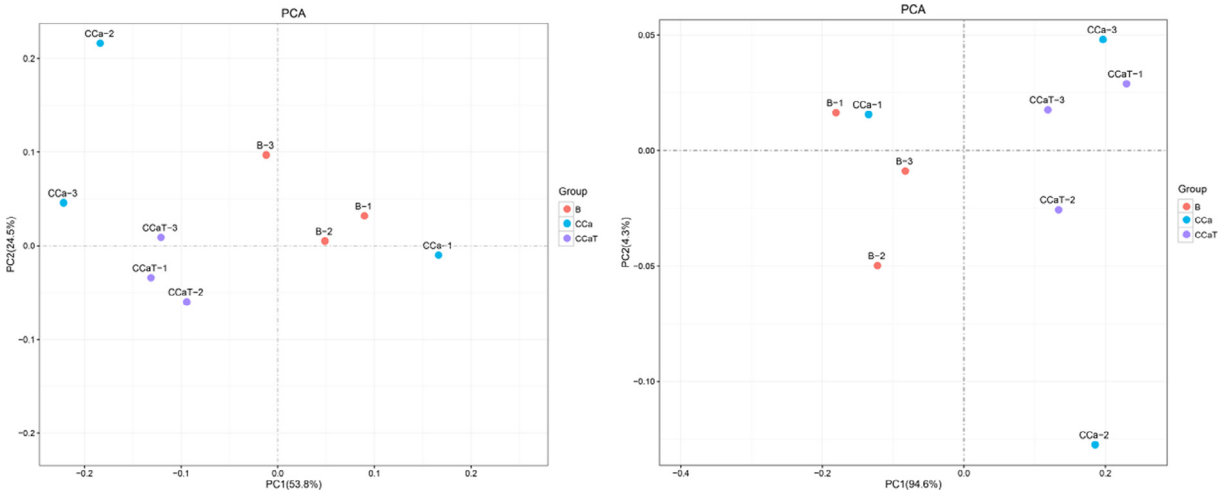


Fig. 14. OTU PCA plot with Phylum level on the left and Family level on the right.

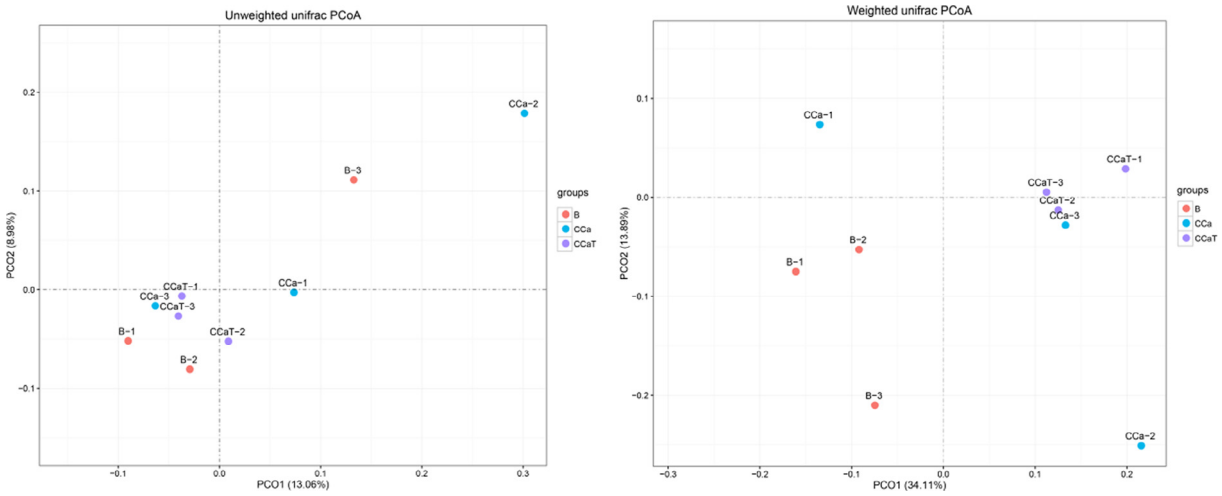


Fig. 15. PCoA analysis based on unweighted Unifrac distance and weighted Unifrac distance.

to obtain q-values. Finally, significance of difference was assessed through screening of p-values or q-values to identify the species that cause differences in composition of the two sample groups.

At the family level, Anaerotruncus ($P < 0.05$), Prevotellaceae, Lactobacillaceae ($P < 0.05$), Ruminococcaceae ($P > 0.05$) showed a downward trend in intestinal microbiota of chronically constipated mice, i.e. CCa group, while Clostridiaceae ($P > 0.05$) showed an upward trend. The proportion of bacteria such as Anaerotruncus, Prevotellaceae, Ruminococcaceae and Bifidobacterium increased, while that of Enterobacteriaceae decreased.

In comparison among B, CCa and CCaT groups, there is one OUT unit with significant difference in abundance in group B, three OUT units with significant difference in abundance in group CCa, and 4 OUT units with significant differences in abundance in group CCaT (Fig. 18). The LEfSe–based evolutionary branch diagram (Fig. 18) shows the differences among intestinal bacteria in B, CCa, and CCaT groups.

4. Discussion

Codonopsis bulleyana is an often-consumed cheap tonic medicinal material whose main function is to lubricate the intestine and relax the bowls. Some researchers treated the constipation model established by loperamide using *Codonopsis bulleyana* water extract, finding that constipated mice's intestinal feces was reduced with feces moisture content increased after the treatment, indicating that *Codonopsis bulleyana* water extract can significantly relieve constipation symptoms (Yunpeng et al., 2017). It was found in our experiment that *Codonopsis bulleyana* water extract can increase defecation amount, reduce intestinal feces volume of constipated mice, and increase the intestinal propulsion rate in the model group. Combined with the existing literature, these results demonstrate laxative effect of *Codonopsis bulleyana* water extract.

Intestinal microbiota imbalance is one major cause of constipation. When constipation occurs, the number of beneficial bacteria decreases, while the proportion of neutral bacteria and harmful bacteria increases. Intestine provides an ideal place for the survival of microorganisms, as the large area of the host intestine, appropriate and constant temperature, and abundant nutrients available for microorganisms provide an ideal environment for the massive intestinal microbiota. Intestinal microbiota structure shows similar variation characteristics regardless of constipation types. A group researchers found that abundance of Actinobscteria was significantly increased in the intestine of patients with chronic functional constipation compared with healthy persons, while abundance of Proteobacteria was significantly reduced (Linsheng et al., 2017). Moreover, 20 genera were found to have significant differences

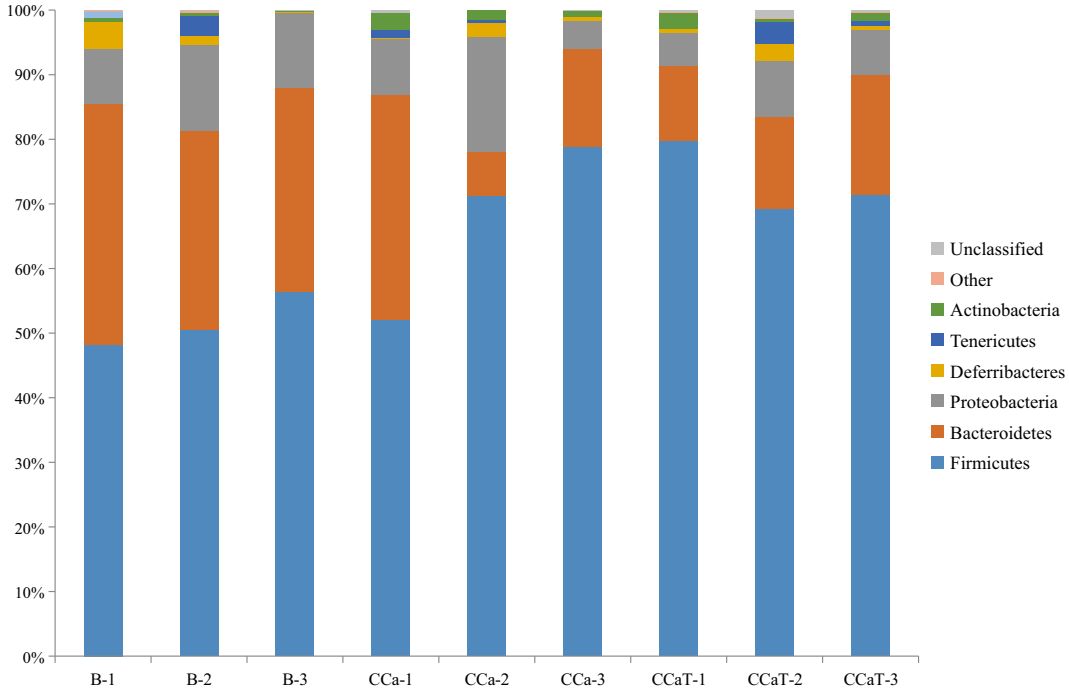


Fig. 16. Intestinal microbial composition in each group of mice (phylum level).

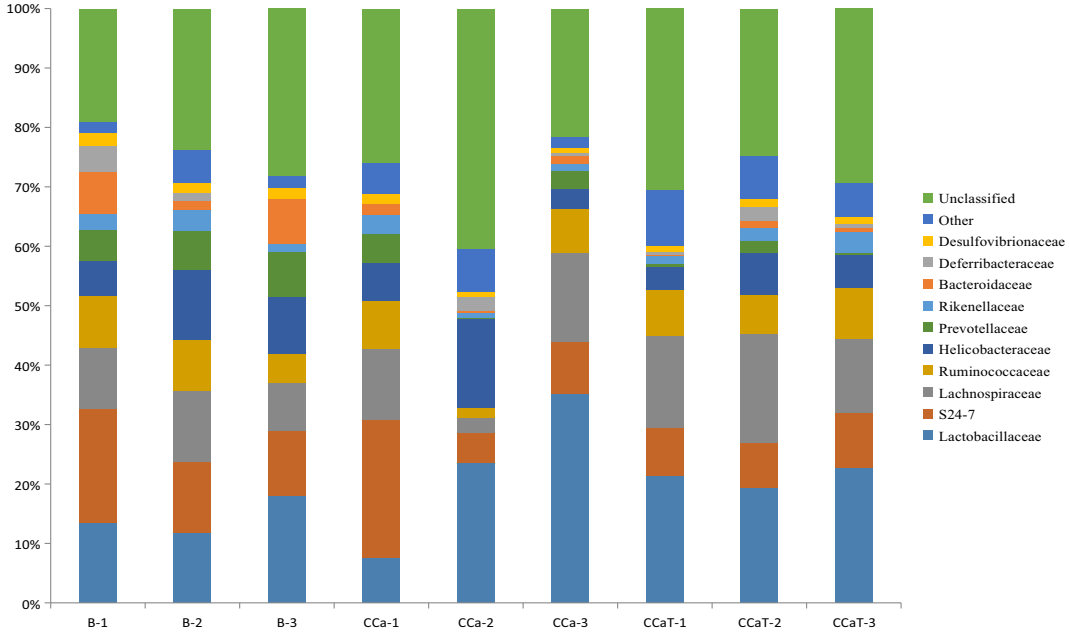


Fig. 17. Intestinal microbial composition in each group of mice (family level).

at the genus level. Intestinal bacterial diversity was significantly reduced in mice with spleen deficiency constipation (Dandan et al., 2016). Constipation symptoms can be alleviated by adjusting the composition of intestinal microbiota. Such change in the intestinal microbiota may be a result of changes in the intestinal environment. In case of constipation, the feces stay in the intestines for a long time, producing harmful substances under the action of certain microorganisms in the intestine, which will kill some sensitive bacteria. Some harmful bacteria insensitive to these metabolites will multiply into the dominant flora in the intestine. When appropriate exogenous regulatory substances are given, the intestinal environment changes and harmful bacteria gradually decrease, which in turn makes beneficial bacteria the dominant bacteria.

Long-term constipation-induced intestinal cancer may concern the composition of the intestinal microbiota, which may be improved by *Codonopsis bulleyana* water extract. It was found in our experiment that intestinal microbiota changed in constipation model mice, with some harmful bacteria turned into dominant bacteria. Compared with the blank group mice, some microorganisms only enriched in intestinal cancer were also enriched in the model group (Gao et al., 2014). In the group treated by *Codonopsis*

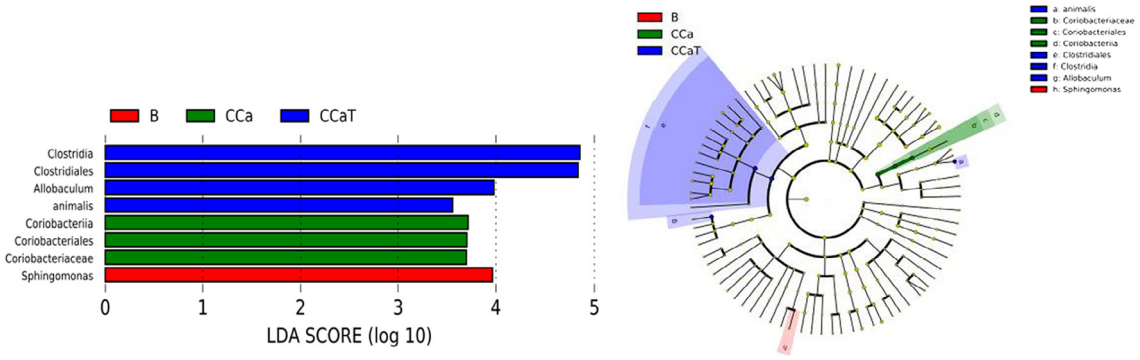


Fig. 18. LEFse differential analysis diagram, the left is LEfSe analysis between the three groups of mice, LDA value> ±2.0, the length of the bar chart represents LDA value; the right is a LEfSe-based evolutionary branch diagram analysis, showcasing species with significant differences in abundance between groups B, CCa and CCaT. B: blank control group; CCa: long-term constipation model group; CCaT: long-term constipation treatment group.

bulleyana water extract, *Codonopsis bulleyana* water extract can improve composition structure of intestinal microbiota, which is because some metabolites produced by Codonopsis bulleyana water extract under the effect of intestinal microbiota induces changes in intestinal microbiota in chronically constipated mice.

Previous studies have found that *Codonopsis bulleyana* is a non-toxic food with multiple effects (Pinhua et al., 2015; Pinhua et al., 2013; Ruoli et al., 1999; Yunpeng et al., 2017; Zijun et al., 2009; Qiongfen et al., 2002; Luan et al., 2018a, 2018b; Qiongfen et al., 2003; Ruoli et al., 1999). In this experiment, we found that *Codonopsis bulleyana* can promote intestinal motility and improve the intestinal microbiota composition of the constipation model. This indicates that *Codonopsis bulleyana* is a food material that can be potentially developed into functional food.

Acknowledgements

This study was funded by National Natural Science Foundation of China (NSFC) (31860254/61363061/31660029) and Advanced and Characteristic Key Biological Disciplines of Yunnan Province (project serial code: 50097505). The thesis of "Scientific and Technological Innovation Team Construction Project for Protection and Utilization of Under-forest Biological Resources (project serial code: 51400605)" was completed through joint support.

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