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Original Article

Flora regulation of BuzhongYiqi decoction in patients with food allergies after surgery for anal fistula

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Abstract: Aim: This study aimed to investigate the effects of BuzhongYiqi decoction (BYD) on the wound flora and explore the mechanism of BYD in promoting wound healing by metagenomics with Miseq sequencing platform. Methods: 12 patients meeting the inclusion criteria were recruited after surgery for anal fistula and randomly divided into BYD group (BYD + food allergy; n=6), and positive control group (food allergy; n=6). Six patients without food allergy were assigned into negative control group. Results: 1) At the phylum level, the proportion of Cyanobacteria and Fusobacteria in BYD group was significantly lower than that in positive control group, while that of Bacteroidetes and Proteobacteria was significantly higher ($P<0.05$). The proportion of Fusobacteria in BYD group was significantly lower than those in negative control group ($P<0.05$). The proportion of Cyanobacteria and Fusobacteria in BYD group was significantly lower than in positive control group ($P<0.05$). The proportion of Fusobacteria in negative control group was significantly higher than in positive control group ($P<0.05$). 2) At the genus level, the proportion of Corynebacterium, Dermatophilus, CandidatusRhodoluna, Saccharopolyspora, Emticicia, Bacillus, Clostridium, Bradyrhizobium, Rubellimicrobium, Alcaligenes, Comamonas, Morganella, Serratia, Stenotrophomonas in positive control group was significantly higher than in negative control group, but that of Eggerthella and Dialister in positive control group was significantly lower than in negative control group ($P<0.05$). The proportion of Eggerthella, Dorea, Dialister, Janthinobacterium and Bilophila in positive control group was significantly higher than in BYD group ($P<0.05$), but that of Rhodococcus and Serratia in positive control group was significantly lower than in BYD group ($P<0.05$). There was significant difference in the time to wound healing among these three groups ($P<0.05$). Conclusion: BYD may regulate the microbial diversity and population structure and make their metabolites to promote wound healing in patients with food allergy after surgery for anal fistula.

Keywords: Metagenomics, flora, BuzhongYiqi decoction, post-operative wound, food allergy

Introduction

Anal fistula accounts for 35% of anorectal diseases in China [1]. After surgery for anal fistula, the wound is connected to the anus or gut, opened and easy to contaminate. Thus, the wound healing after surgery for anal fistula is closely related to the focal flora (especially the intestinal flora). The types of microorganisms are diverse in human body. Traditional methods used in studies on microorganisms only depict no more than 1% of microorganisms, and remaining microorganisms have not been covered. In 1998, Schloss et al [2] proposed the concept of metagenomics which can be used to identify the microorganisms that are not identified with traditional methods. In 2009, Round et al [3] proposed that comprehensive factors including living style and genetics might act synergistically to cause the imbalance of gut microflora and immune dysregulation may cause inflammatory bowel disease (IBD) [4, 5]. In IBD, the dominant Firmicutes and Bacteroidetes in the intestine reduce [6], bacteria invading the intestinal mucosa increase, especially the Escherichia col [7], and some anaerobes as well as facultative anaerobes, which are not or rarely found in healthy population [8].

Table 1. Food allergy in patients

Patient number	Group	Sex	Age (years)	Type of food allergy
1	BYD Group	Male	26	Crab +, egg white/yolk ++, milk ++
2	BYD Group	Male	30	Egg white/yolk ++
3	BYD Group	Male	59	Tomato + , crab +, egg white/yolk ++, milk +++, rice +
4	BYD Group	Male	34	Tomato ++, shrimp +, egg white/yolk +
5	BYD Group	Female	40	Egg white/yolk ++, mushroom +
6	BYD Group	Male	32	Egg white/yolk ++
7	Positive control group	Male	65	Egg white/yolk +
8	Positive control group	Female	24	Egg white/yolk +++
9	Positive control group	Female	22	Tomato +, egg white/yolk ++
10	Positive control group	Male	30	Egg white/yolk +++ milk +
11	Positive control group	Male	37	Tomato +, wheat +++, egg white/yolk ++
12	Positive control group	Male	43	Milk ++, egg white/yolk ++

In clinical practice, delayed wound healing after surgery for anal fistula often occurs in patients with immune dysfunction. Especially in patients with food allergy, wound healing is significantly delayed. Whether delayed wound healing is related to the types and composition of microorganisms in the wound is still unclear. In the present study, anal fistula patients with or without food allergy were recruited to investigate the relationship between wound microorganisms and wound healing after surgery for anal fistula. According to the Traditional Chinese Medicine, weakness of the spleen and the stomach and deficiency of Qi and blood are the major cause of delayed wound healing. Thus, in the present study, BuzhongYiqi Decoction (BYD), a classic Traditional Chinese Medicine, was used in these patients. It has been confirmed that BYD is able to regulate the immune function, exert antibiotic effect on Helicobacter pylori in mice [9], inhibit the proliferation of hepatocellular carcinoma cells [10], regulate the inflammation in allergic asthma mice [11], exert anti-aging in mice [12] and inhibit IgE secretion in mice [13]. Metagenomics based on the IlluminaSolexa high-throughput sequencing platform was employed to investigate the difference in the bacterial flora between patients with and without BYD treatment.

Methods

Patients and grouping

This study has been approved by the Ethics Committee of Yueyang Hospital of Integrated Traditional Chinese and Western Medicine affiliated to Shanghai University of Traditional Chinese Medicine. Informed consent was obtained from each patient. Serum specific IgE (sIgE) was used to screen 14 allergens in total. All allergy tests were done with standardized kits and defined with uniform monitor value. A total of 12 patients with positive serum specific IgE, which meant they were allergic to one or more allergens after surgery for anal fistula were recruited between May 2014 and December 2014 from the Yueyang Hospital of Integrated Chinese and Western Medicine of Shanghai University of Traditional Chinese Medicine. SPSS 18.0 software was used to generate a random number table. Each patient chose a paper with a number, and entered the corresponding group according to the chosen number. 12 patients with food allergy were randomly divided into 2 groups: BYD group (BYD + food allergy; n=6), and positive control group (food allergy; n=6). Six patients in positive control group were allergic to egg white/yolk; egg white/yolk and milk; shrimp and milk; tomato, wheat, and egg white/yolk; milk and egg white/yolk; and milk and egg white/yolk, respectively. Six patients in BYD group were allergic to crab and egg white/yolk; tomato and egg white/yolk; egg white/yolk; tomato, shrimp, and egg white/yolk; egg white/yolk; milk and egg white/yolk, respectively (**Table 1**). At the same time, six patients without allergy after surgeries for anal fistula were assigned into negative control group (n=6).

There were 5 males and 1 female in BYD group, 4 males and 2 females in positive control group, and 5 males and 1 female in negative con-

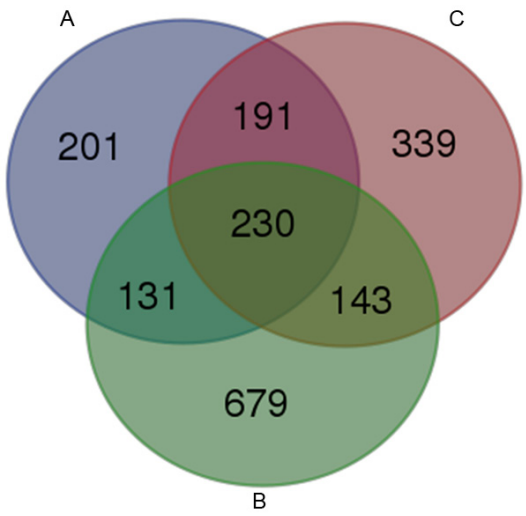


Figure 1. Diagram for OTU. A total of 230 OTU were shared among 3 groups, and the number of unique OTU was 201 in BYD group, 679 in positive control group and 339 in negative control group. A: BYD group; B: Positive control group; C: Negative control group.

trol group. sIgE was used to screen the allergens (n=14). Patients with blood diseases, cerebrovascular and cardiovascular diseases, diabetes mellitus, IBD, tuberculous anal fistula, malignancy, liver diseases, kidney diseases or mental illnesses were excluded from this study.

BYD was administered twice daily for consecutive 2 weeks (after a meal in the morning and evening; n=100 ml for each). The components of BYD include astragalus (15 g), atractylodes (10 g), codonopsispilosula (15 g), angelica (6 g), dried tangerine (6 g), radix bupleuri (5 g), rattletop (5 g) and prepared licorice root (5 g). They were under granule preparation, which was produced with uniform technology, delivered by national pharmaceutical factory. They were concentrating agent of herb. Thus, the drug quality was guaranteed. These granules were purchased from JiangyinTianjiang Pharmaceutical Company and mixed in the Department of Pharmacy of Yueyang Hospital of Integrated Chinese and Western Medicine of Shanghai University of Traditional Chinese Medicine.

Analysis of microbial diversity

DNA extraction and amplification by PCR: 14 days after treatment, a sterilized swab was used to collect the wound secretions which were then transferred into a sterilized tube and stored at -70°C. The whole procedures were avoided by the bacterial contamination. 16s DNA extraction was performed. The concentration and purity of DNA were determined by spectrophotometry. The primers used for the amplification of 16S rDNA V1-V3 were AGAGTTTGATCCTGGCTCAG and ATTACCGCGGCTGCTGG.

MiSeq high-throughput sequencing: 16S library was established with Illumina® TruSeq® DNA PCR-Free Sample Preparation Kit according to the PCR-Free protocol. IlluminaMiSeq PE300 sequencing platform was used to sequence the V1-V3 region of 16S rRNA.

Bioinformatics analysis

On the basis of Barcode, data collected after detection were divided, and sequences of Barcode and primers for PCR were removed. The reads were connected into Raw Tags, and FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/) was used to treat reads. Then, high quality Clean Tags were obtained, and the operational taxonomic unit (OUT) at the similarity of 97% was calculated with Mothur (version 1.27.0) software. The obtained sequences were compared to those from green genes (http://greengenes.lbl.gov/Download/), and the species were determined. According to the OUT data, a-diversity analysis was performed. Addition, principal component analysis, delineation of Venn figure according to OUT distribution and analysis of compositions of flora at phylum level were also performed.

Statistical analysis

Statistical analysis was performed with SPSS version 18.0. Quantitative data with normal distribution and homogeneity of variance were subjected to t test and chi square test. Qualitative data were compared with analysis of variance for multiple comparisons or non-parametric test if abnormal distribution and heterogeneity of variance were present. A value of $P<0.05$ was considered statistically significant.

Results

Results of sequencing

A total of 115739 original sequences were obtained from 18 samples. After quality control, 114956 Clean Tags were obtained (99.32%). A total of 1914 OTU was obtained, and the num-

Table 2. A-diversity analysis

Sample	Shannon	Chao1	Observed species	Goods coverage	Simpson
S1	5.152358197	353.6222222	248	0.974858902	0.931182033
S2	5.117292498	330.0277778	255	0.992446667	0.941785136
S3	3.63267651	372.2195122	226	0.987890797	0.825423306
S4	3.544546367	173.4042553	160	0.988664987	0.710024689
S5	2.427094472	151.2142857	101	0.990464241	0.531956946
S6	3.937379915	285.375	143	0.987189148	0.875378848
S7	5.061265591	545.2361809	526	0.995150981	0.926925693
S8	5.384830535	370.0909091	214	0.952157598	0.943062737
S9	6.426837956	450.0392157	409	0.986317668	0.972930655
S10	5.919236044	451.7027027	360	0.979401408	0.954330738
S11	4.404135284	179.3333333	141	0.980777267	0.866678809
S12	2.897866287	130.25	104	0.981327801	0.645420835
S13	3.970867419	338.0576923	254	0.981846273	0.832235827
S14	5.472797613	337.3818182	274	0.98171926	0.943858786
S15	4.746083395	339.85	267	0.978607192	0.888889741
S16	5.412555058	369.4067797	300	0.989580948	0.944386173
S17	5.322322356	175.1052632	123	0.957786116	0.946611801
S18	4.462368066	254.4166667	213	0.976372712	0.853780582

Notes: Data from sequencing were rationale and sequencing basically covers all the microorganisms observed in the samples. Chao1 and observed species were index for Community richness. Shannon and simpson were index of Community diversity. Goods coverage indicated sequencing depth. S1, S2, S3... indicated the number of original data.

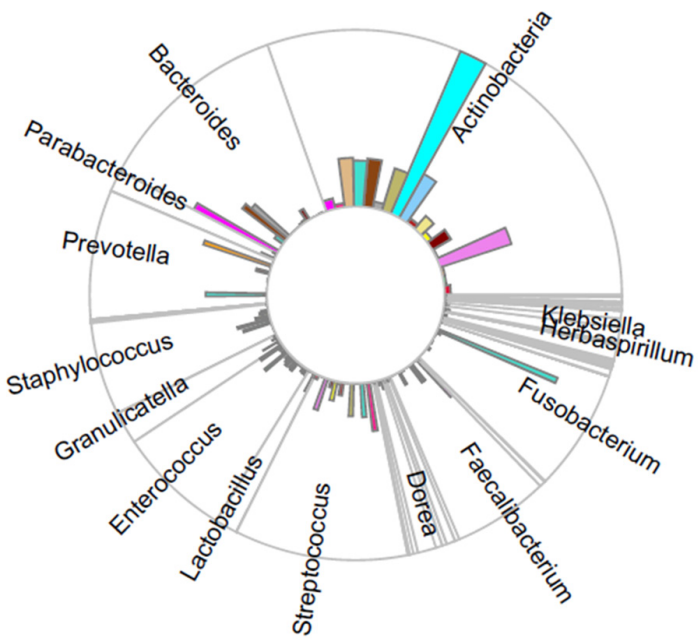


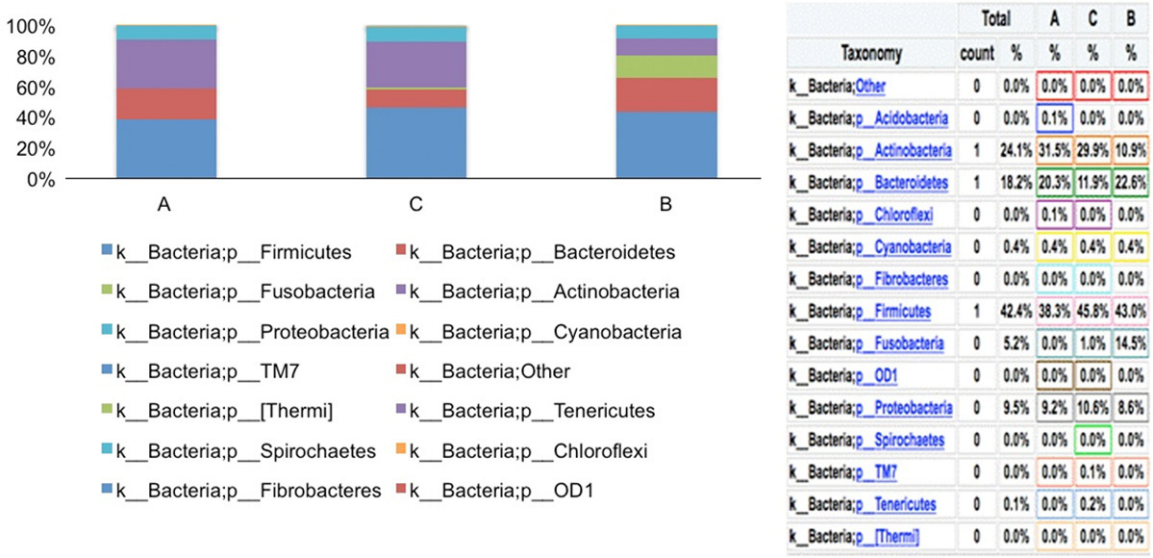
Figure 2. Beta analysis of multiple samples. Actinobacteria, Bacteroidetes, Proteobacteria and Fusobacteria have a high richness.

ber of OTU was 753 in BYD group, 1183 in positive control group and 903 in negative control group (**Figure 1**).

Analysis on the basis of OTU richness

Chao1 and observed species are indicators used for the calculation of community richness. Shannon and simpson are indicators used for the calculation of community diversity. Goods coverage is used to evaluate the depth of sequencing (**Table 2**).

Beta analysis was used to analyze the amplitude changes, according to the information database of microbial species including Taxonomic system relationship tree including provided by NCBI (ftp://ftp.ncbi.nih.gov/pub/taxonomy/). Abundance of each OUT and corresponding taxonomic information were regressed into the database, which can help understand evolutionary relationship and abundance changes all tested microorganisms. Beta analysis of multiple samples was shown in **Figure 2**. Distribution of flora in the wound of 3 groups at the phylum level shown in **Figure 3**.



	Total		A	C	B
Taxonomy	count	%	%	%	%
k__Bacteria;Other	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Acidobacteria	0	0.0%	0.1%	0.0%	0.0%
k__Bacteria;p__Actinobacteria	1	24.1%	31.5%	29.9%	10.9%
k__Bacteria;p__Bacteroidetes	1	18.2%	20.3%	11.9%	22.6%
k__Bacteria;p__Chloroflexi	0	0.0%	0.1%	0.0%	0.0%
k__Bacteria;p__Cyanobacteria	0	0.4%	0.4%	0.4%	0.4%
k__Bacteria;p__Fibrobacteres	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes	1	42.4%	38.3%	45.8%	43.0%
k__Bacteria;p__Fusobacteria	0	5.2%	0.0%	1.0%	14.5%
k__Bacteria;p__OD1	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria	0	9.5%	9.2%	10.6%	8.6%
k__Bacteria;p__Spirochaetes	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__TM7	0	0.0%	0.0%	0.1%	0.0%
k__Bacteria;p__Tenericutes	0	0.1%	0.0%	0.2%	0.0%
k__Bacteria;p__[Thermi]	0	0.0%	0.0%	0.0%	0.0%

Figure 3. Distribution of flora in the wound of three groups at phylum level. At the phylum level, a majority of microorganisms belong to Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria, Fusobacteria, Cyanobacteria and Tenericutes; and very few belong to Fibrobacteres, Chloroflexi, Acidobacteria and Tenericutes.

Table 3. The proportion of 4Phylumsin BYD and positive control group

Taxon	Proportion		P
Phylum	BYD group	Positive control group	
Bacteroidetes	20.25%	14.15%	0.03
Cyanobacteria	0.46%	11.88%	0.03
Fusobacteria	0.04%	5.29%	0.03
Proteobacteria	12.98%	11.44%	0.01

Notes: The proportion of Cyanobacteria and Fusobacteria in BYD group was significantly lower than in positive control group ($P<0.05$). The proportions of Bacteroidetes and Proteobacteria in BYD group were significantly higher than in positive control group ($P<0.05$).

Table 4. The proportion of Fusobacteria in BYD and negative control group

Taxon	Proportion		*P* value
Phylum	BYD group	Negative control group	
Fusobacteria	0.01%	0.78%	0.03

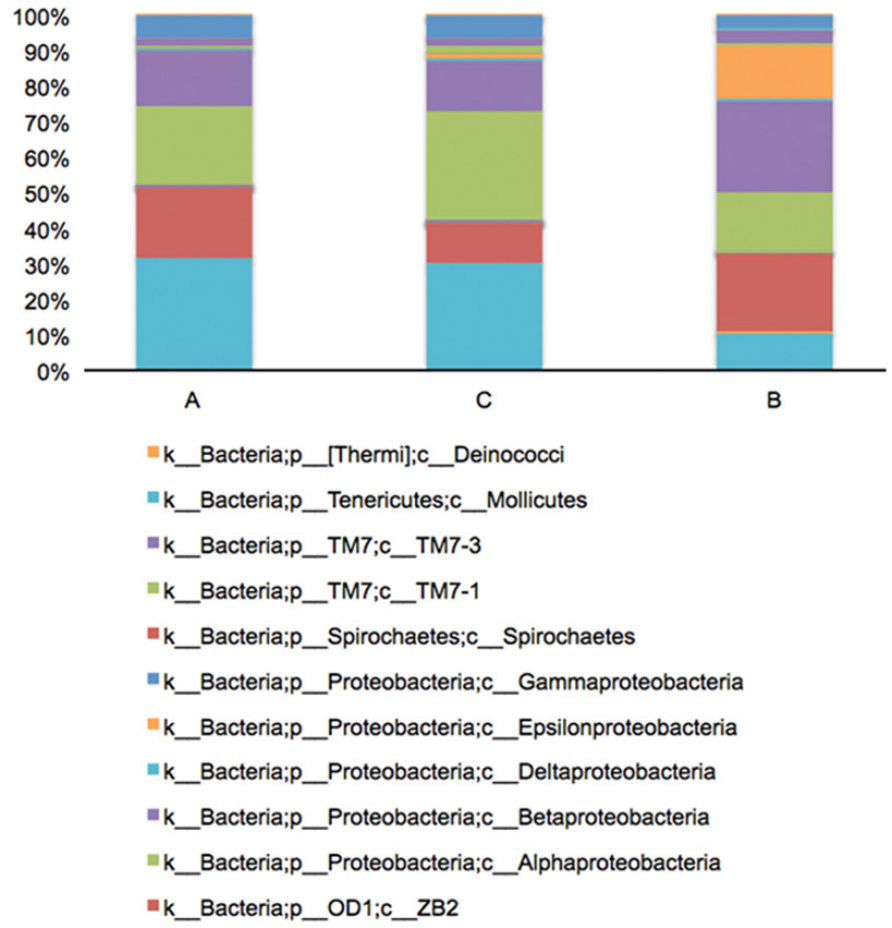
Notes: The proportion of Fusobacteria in BYD group was significantly lower than in negative control group ($P<0.05$).

Firmicutes had the highest richness in three groups, and the richness of Firmicutes was 42.99%, 38.33% and 45.76%, respectively, in BYD, positive control and negative control groups, respectively. In positive control group, the following higher richness was found in Bacteroidetes (22.55%), Fusobacteria (14.51%), Actinobacteria (10.87%) and Proteobacteria (8.59%). In BYD group and negative control group, the following higher richness was found in Actinobacteria (31.51% and 29.93%), Bacteroidetes (20.26% and 11.86%), Proteobacteria (9.22% and 10.57%) and Fusobacteria (0.02% and 1.02%).

At the phylum level, significant difference in microorganisms was present between individuals. The proportion of Firmicutes with the highest richness in the samples ranged from 8.78% to 86.31%. In positive control group, Actinobacteria had the second highest proportion and its proportion in the samples ranged from 2.69% to 78.54%.

Significant differences in the Bacteroidetes, Cyanobacteria, Fusobacteria and Proteobacteria were observed between BYD group and positive control group ($P<0.05$). The proportion of Cyanobacteria and Fusobacteria in BYD group was significantly lower than in positive control group (0.46% vs 1.18% and 0.03% vs 5.28%, respectively). The proportion of Bacteroidetes and Proteobacteria in BYD group was significantly higher than in positive control group (20.25% vs 14.14% and 12.97% vs 11.44%, respectively) (**Table 3**).

A total of 14 phylums were detected in BYD group and negative control group, significant



Taxonomy	Total count	Total %	A %	C %	B %
k__Bacteria;Other;Other	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Acidobacteria;c__Solibacteres	0	0.0%	0.1%	0.0%	0.0%
k__Bacteria;p__Acidobacteria;c__[Chloracidobacteria]	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Actinobacteria;c__Acidimicrobiia	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Actinobacteria;c__Actinobacteria	1	24.0%	31.5%	29.9%	10.4%
k__Bacteria;p__Actinobacteria;c__Coriobacteriia	0	0.2%	0.0%	0.0%	0.4%
k__Bacteria;p__Bacteroidetes;Other	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Bacteroidetes;c__Bacteroidia	1	18.0%	20.2%	11.4%	22.5%
k__Bacteria;p__Bacteroidetes;c__Cytophagia	0	0.0%	0.0%	0.1%	0.0%
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia	0	0.1%	0.0%	0.3%	0.0%
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia	0	0.0%	0.0%	0.1%	0.0%
k__Bacteria;p__Bacteroidetes;c__[Saprospirae]	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Chloroflexi;c__Anaerolineae	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Chloroflexi;c__TK10	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Cyanobacteria;c__4C0d-2	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Cyanobacteria;c__Chloroplast	0	0.4%	0.4%	0.4%	0.4%
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae	0	0.0%	0.1%	0.0%	0.0%
k__Bacteria;p__Fibrobacteres;c__Fibrobacteria	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;Other	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Firmicutes;c__Bacilli	1	23.0%	21.9%	30.9%	16.1%
k__Bacteria;p__Firmicutes;c__Clostridia	1	18.4%	15.5%	13.8%	26.0%
k__Bacteria;p__Firmicutes;c__Erysipelotrichi	0	1.0%	1.0%	1.1%	0.9%
k__Bacteria;p__Fusobacteria;c__Fusobacteriia	0	5.2%	0.0%	1.0%	14.5%
k__Bacteria;p__OD1;c__SM2F11	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__OD1;c__ZB2	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria	0	1.5%	0.9%	2.5%	0.9%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria	0	2.5%	1.9%	2.0%	3.8%
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria	0	0.1%	0.0%	0.0%	0.2%
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria	0	5.4%	6.4%	6.0%	3.7%
k__Bacteria;p__Spirochaetes;c__Spirochaetes	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__TM7;c__TM7-1	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__TM7;c__TM7-3	0	0.0%	0.0%	0.0%	0.0%
k__Bacteria;p__Tenericutes;c__Mollicutes	0	0.1%	0.0%	0.2%	0.0%
k__Bacteria;p__[Thermi];c__Deinococci	0	0.0%	0.0%	0.0%	0.0%

Figure 4. Distribution of flora in the wound of three groups at class level. At the class level, a majority of microorganisms belong to Bacilli and Clostridia.

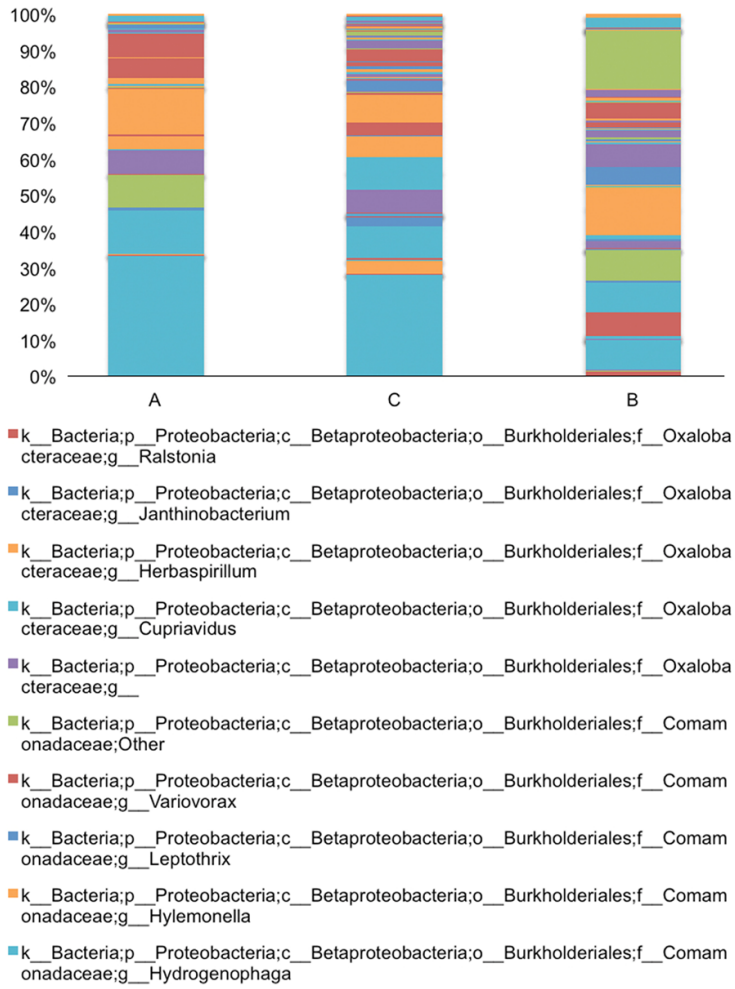


Figure 5. Distribution of flora in the wound of three groups at genus level. At the genus level, the proportion of Streptococcus, Enterococcus, Staphylococcus, Faecalibacterium, Finegoldia and Peptoniphilus independently account for 2 or higher.

difference was found in only Fusobacteria between two groups (0.04% in BYD group and 0.78% in negative control group). No significant differences were observed in remaining 13 phylums (P>0.05) (**Table 4**).

The proportion of Fibrobacteres in negative control group was significantly higher than in positive control group (P<0.05), but there were no marked differences in other phylums.

Distribution of flora in the wound of three groups at class and genus levels was shown in **Figure 4**. In respect of richness, Firmicutes was the dominant phylum of microorganisms in the wound of three groups. At the class level, Bacilli and Clostridia were the dominant, and very few were Erysipelotrichi (**Figure 4**). Firmicutes include 69 genuses which are mainly Streptococcus, Enterococcus, Staphylococcus, Faecalibacterium, Finegoldia and Peptoniphilus, accounting for 2% or higher (**Figure 5**).

At the genus level, the proportion of Corynebacterium, Dermatophilus, CandidatusRhodoluna, Saccharopolyspora, Emticicia, Bacillus, Clostridium, Bradyrhizobium, Rubellimicrobium, Alcaligenes, Comamonas, Morganella, Serratia and Stenotrophomonas in positive control group was significantly higher than in negative control group, but that of Eggerthella and Dialister in positive control group was significantly lower than in negative control group (**Figure 6**). The microorganisms of above genuses mainly belong to Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria which were significantly different between positive control group and negative control group (P<0.05).

The proportion of Eggerthella, Dorea, Dialister, Janthinobacterium and Bilophila in positive control group was significantly higher than in BYD group (**Figure 7**), but that of Rhodococcus and Serratia in positive control group was significantly lower than in BYD group. Microorganisms of above genuses mainly belong to Actinobacteria, Firmicutes and Proteobacteria, which were significantly different between the two groups (P<0.05).

Time to wound healing

The time to wound healing was 37.50±2.34 days in BYD group, 42.6±5.27 days in positive control group and 32.16±4.26 days in negative control group, showing significant difference among three groups (P<0.05; **Figure 8**).

Discussion

In the present study, BYD, a classic Traditional Chinese medicine, was used. BYD has been

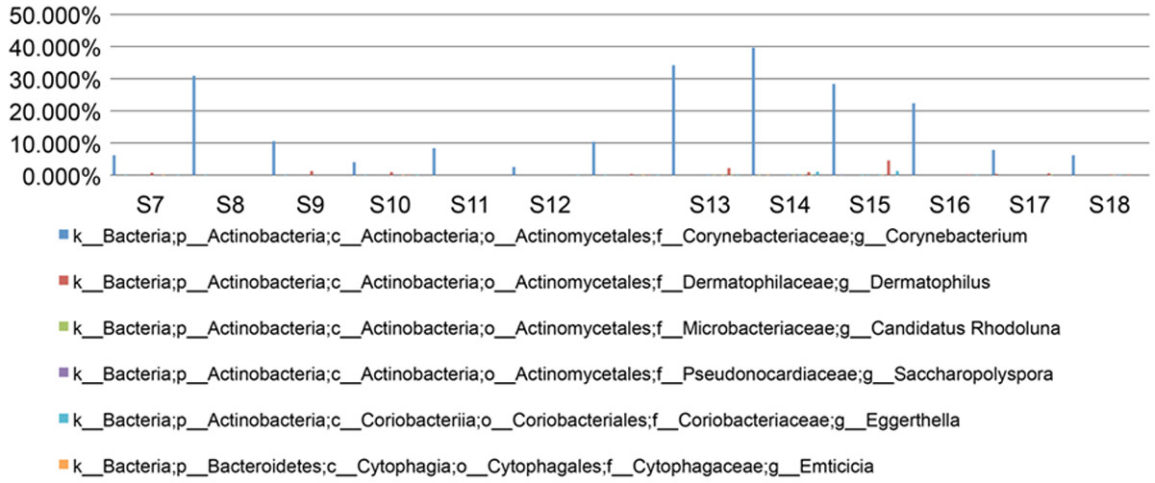


Figure 6. Distribution of flora in the wound of positive control group and negative control group at genus level. Significant differences were observed in 16 genuses (P<0.05). S1, S2, S3... indicated the number of original data.

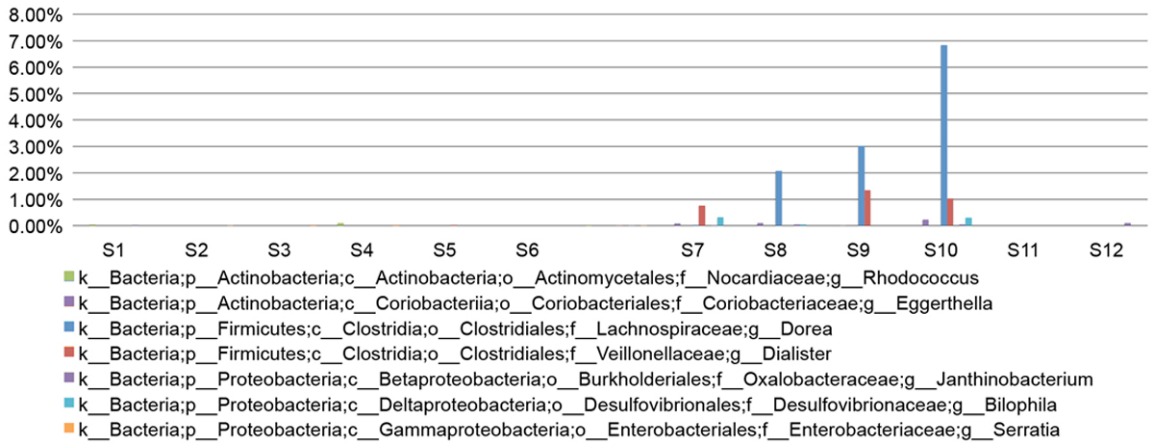


Figure 7. Distribution of flora in the wound of BYD group and positive control group at genus level. Significant differences were observed in 7 genuses between BYD group and positive control group (P<0.05). S1, S2, S3... indicated the number of original data.

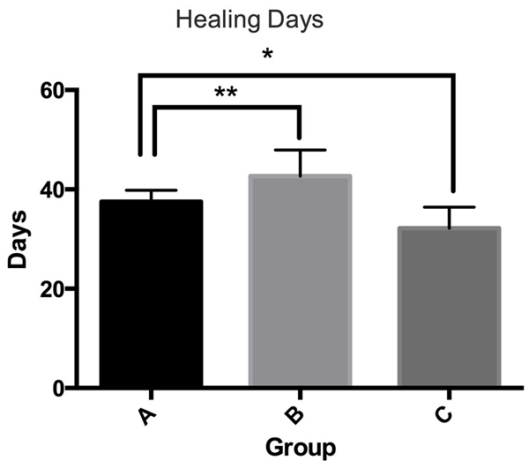


Figure 8. Wound healing time in different groups. There was significant difference in the wound healing time among three groups. A: BYD group; B: Positive control group; C: Negative control group.*P<0.05, BYD group compared with negative control group; **P<0.05, BYD group compared with positive control group.

widely used for the supplement the center and boost the energy as well as strengthening the spleen and replenishing blood in patients with deficient syndrome. In recent years, some studies have found that BYD has immunoregulatory activity. Some studies are conducted in animal models to confirm that BYD can aid Th1 lymphocytes to regulate immune response and resist intracellular pathogen infection [14, 15], enhance anti-tumor effect [16], increase NK cell activity [17] and stimulate IFN-γ secretion

[11, 18]. In addition, there is evidence showing that BYD is also able to inhibit the IgE secretion in the serum [19, 20]. In clinical studies, results have revealed that BYD has anti-inflammatory activity (inhibition of COX-2 expression), exerting long term therapeutic effect on allergic asthma at stable stage [21].

After surgery for anal fistula, the wound is special because it connects to the gut and is open and easy to contaminate. The types and compositions of flora in the wound will change with the change in wound environment, accompanied by change in secretions such as cytokines, proteins, and interferon. These factors have imunoregulatory activity, may induce angiogenesis, exert anti-apoptotic effect, induce chemical chemotaxis and inhibit the scarring, which may regulate the wound immunity and promote wound healing [21-23]. Thus, the imbalance of these factors and immune dysfunction may significantly affect the wound healing.

In healthy adults, more than 500 bacteria colonize in the gut, and 30-40 types of bacteria account for 99% of the gut flora [24-26]. There are mainly 6 phylums of bacteria in the gut flora: Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Proteobacteria and Verrucomicrobia. More than 98% of gut flora belong to Firmicutes (64%), Bacteroidetes (23%), Proteobacteria (18%) and Actinobacteria (3%) [27].

Traditional method used to investigate bacteria is bacterial culture, followed by bacterial counting. The gut has strict anaerobic environment and the growth of flora in the gut has special nutritional requirements. In the nature, no more than 1% of bacteria can be cultured, and the remaining bacteria can not be studied by cell culture. Thus, the traditional method does not help to study the unknown bacteria [28]. Introduction of metagenomics resolves this problem. Metagenome is also known as environmental microbial genome and refers to the sum of DNA of all the environmental microorganisms (currently including bacteria and fungus). The concept of metagenomics was first introduced by Handelsman et al in the Department of Plant Pathology of University of Wisconsin in 1998 [29]. It resolves the problem that some bacteria are difficult to culture. It can be used to screen useful genes and their products by cloning and heterologous expression to investigate the diversity and richness of microorganisms and explore the population relationship, evolutionary relationship, functional activity, mutual cooperation relation and relationship between microorganisms and environment. It may uncover the law of genetic evolution at a higher level [30] and help to identify common pathogens and new pathogenic microorganisms.

In the presence of stress, long term use of antibiotics and infection by exogenous pathogens, viruses or parasites, gut flora usually become abnormal, which is characterized by the alterations of types, amount, composition, localization and biological characteristics of microorganisms in the gut. The flora imbalance may in turn affect the absorption of nutrients and attenuate the intestinal barrier, deteriorating the existing disease, which forms a vicious cycle. Available studies have shown that gut flora imbalance may cause a lot of diseases such as type 1 diabetes mellitus [31], Crohn's disease [32], food allergy [33], obesity [34], and colon cancer [35].

In clinical practice, we often found that the wound healing is usually delayed after surgery for anal fistula in patients with immune dysfunction. Especially in patients with food allergy, the wound healing is significantly delayed after surgery for anal fistula. We speculate that the delayed wound healing might be associated with the types and number of microorganisms in the wound of patients after surgery for anal fistula. Thus, this study was undertaken to investigate the diversity of gut microorganisms by analyzing the 16S rRNA sequence of gut bacteria. In this study, metagenome was employed to investigate the change in the flora of the wound after surgery for anal fistula in patients with and without Traditional Chinese Medicine treatment, which may provide evidence that Traditional Chinese Medicine may regulate the gut flora balance to promote wound healing in patients with food allergy.

In the present study, the time to wound healing was 37.50±2.34 days in BYD group, 42.6±5.27 days in positive control group and 32.16±4.26 days in negative control group, showing significant difference among three groups ($P<0.05$). This suggests that BYD is able to promote the wound healing after surgery for anal fistula, but the time to wound healing is still longer than in normal controls.

Flora analysis showed a high proportionof Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria and Proteobacteria in the wound of three groups, and especially Firmicutes had the highest proportion. These were consistent with the dominant flora in the gut of healthy subjects because the wound after surgery for anal fistula is connected to the gut, secretions from the gut may affect the wound, and thus the wound healing is closely related to the gut flora. Our results showed a higherproportion of Fusobacteria and Cyanobacteria at the phylum level in positive control group. At the genus level, the proportion of Corynebacterium, Dermatophilus, CandidatusRhodoluna, Saccharopolyspora, Emticicia, Bacillus, Clostridium, Bradyrhizobium, Rubellimicrobium, Alcaligenes, Comamonas, Morganella, Serratia and Stenotrophomonas in positive control group was significantly higher than in negative control group, but that of Eggerthella and Dialister in positive control group was significantly lower than in negative control group. In addition, the proportion of Eggerthella, Dorea, Dialister, Janthinobacterium and Bilophila in positive control group was significantly lower than in BYD group, but that of Rhodococcus and Serratia in positive control group was significantly lower than in BYD group. On the basis of alterations in gut flora, we speculate that the gut flora change in patients with food allergy, which may be one of causes of delayed wound healing after surgery for anal fistula.

After BYD treatment, the proportion of Fusobacteria and Cyanobacteria reduced ($P<0.05$), but that of Bacteroidetes and Proteobacteria increased ($P<0.05$). Proteobacteria is dominant in the gut of neonates and infants, but rare in the gut of adults [36]. Mirpuri et al [37] found that the γ-Proteobacteria-specific IgA response could induce the switch of gut flora from neonatal form to adult form. It is expected that this may induce the development and improvement of gut immunity. Administration of Bacteroides Fragilis in germ-free mice may avoid the occurrence of IBD [38]. Bacteroidetes in the gut may use fibers to synthesize polysaccharide and regulate the secretion of IL-10 in T cells [39] (IL-10 is a potent anti-inflammatory cytokine). In addition, Bacteroidetes may increase the activity of macrophages and T cells, inhibit the activation of pro-inflammatory pathways in Th17 cells. Thus, Bacteroidetes have been regarded as anti-inflammatory bacteria. The increase in Bacteroidetes and Proteobacteria may improve the wound immunity to inhibit the inflammation and promote wound healing. This implies that the capability of BYD to improve wound healing is related to the regulation of local flora.

In addition, our results also showed that Fibrobacteres had a low proportion in three groups, but their proportion in negative control group was significantly higher than in other two groups ($P<0.05$). The BYD treatment can increase the Fibrobacteres in the wound of patients with food allergy. Cellulose is indispensable in the wound healing. The Fibrobacter of Fibrobacteres has a large amount of cellulase in the periplasma. Cellulase can degrade cellulose and promote the absorption of cellulose. In patients with food allergy, the Fibrobacter reduced in the wound after surgery for anal fistula. Thus, the degradation and absorption of cellulose are impaired, thus leading to delayed wound healing. To increase Fibrobacteres is likely to be involved in the BYD induced wound healing.

Conclusions

BYD may regulate the immune function (especially the gut immunity) and change the compositions of gut flora to affect the flora in the wound after surgery for anal fistula, which alters the richness, distribution and metabolites of flora, thus leading to wound healing. Future studies with large sample size are required to investigate the metabolites of flora in wound by bioinformatics analysis.

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Disclosure of conflict of interest

None.

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