Therapeutic effect of herb residue fermentation supernatant on spleen-deficient mice

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Abstract. To minimize the waste of active ingredients present in herb residues, in the present study, probiotics of *Bacillus subtilis*, *Aspergillus oryzae* and *Lactobacillus plantarum* M3 were selected to reuse herb residues from Jianweixiaoshi tablets, and the therapeutic effects of the herb residue fermentation supernatant were evaluated using a spleen-deficient mouse model. The results of the present study indicated that the fermentation supernatant may effectively improve the immunity of mice, as measured by body weight, spleen and thymus index, and inflammatory cytokines, including interleukin (IL)-2, IL-4 and interferon-γ. The viable cell count and denaturing gradient gel electrophoresis results indicated that the fermentation supernatant markedly enhanced bacterial diversity and the number of lactobacilli in mouse intestines. Therefore, the combination of the Jianweixiaoshi herb residue and probiotics provided a novel method to reuse herb residues and may in the future contribute to the treatment of spleen deficiency.

Introduction

According to the spleen-stomach theory in traditional Chinese medicine (TCM), the spleen is not anatomically, physiologically or pathophysiologically synonymous with one organ as it is in western medicine (1,2). In TCM, the term spleen is used to describe the digestive system, including its vegetative nervous system, immunity, hemopoiesis, muscle metabolism, endocrine function, hepatic metabolic function, and protein, nucleotide, energy, water and salt metabolism (1,2). Spleen deficiency is characterized by symptoms including epigastralgia, flatulence, lack of appetite, wilted complexion, loose stools, lassitude and fatigue. Spleen deficiency is one of the most common digestive diseases in China and is frequently associated with imbalances in the gastrointestinal microflora (3,4). Therefore, clinical studies are being conducted in order to identify an association between TCM and intestinal microbiota and to contribute to the treatment of spleen deficiency (1,5,6).

Jianweixiaoshi tablets were approved by the Chinese Ministry of Health as one of the national protected traditional medicines in 1995 and were listed as the first class of over-the-counter drugs in 1999. Jianweixiaoshi tablets, containing hawthorn, malt, tangerine peel, *Radix Pseudostellariae* and yam, are used for treatment of indigestion, anorexia and abdominal distension, via invigoration of the stomach and tonification the spleen. Jianweixiaoshi tablets have been demonstrated to promote gastrointestinal peristalsis and gastric secretion of digestive juices, and enhance the activity of pepsin, general physique and immune function (7). In China, sales of Jianweixiaoshi tablets generate >1.2 billion renminbi in income each year, although they also produce ~100,000 tons of herb residues which are primarily used in food additives (7,8).

Herb residues are by-products of TCM materials extracted by water or ethanol, and ~30-50% of the medically active ingredients remain inaccessible (9). The microorganism fermentation theory in TCM suggests that digestive enzymes, including cellulase, protease, pectinase, lignin and lipase, produced by microorganisms may effectively degrade plant cell walls, expand the intercellular region and improve the yield of extraction of active ingredients (10). In addition, microorganisms may degrade macromolecules to smaller molecules, making them accessible for direct absorption by the human body, reduce the side effects of drugs by degrading toxic substances and introduce novel therapeutic effects by biological modification. In addition, probiotics have been defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host', including anti-carcinogenic and anti-mutagenic properties, immune stimulation and lowering of serum cholesterol (11,12). Probiotics may be used for the prevention and treatment of certain pathological conditions (13,14).

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In the present study, herb residues of Jianweixiaoshi tablets were reused via probiotics, a spleen-deficient mouse model was established and the therapeutic effect of the herb residue fermentation supernatant on spleen deficiency was evaluated.

Materials and methods

Preparation of herb residue extract and fermentation supernatant. The herb residue of Jianweixiaoshi tablets was obtained from Jiangsu Yangtze River Pharmaceutical Group Company, Ltd. (Taizhou, China) and homogenized for 2 h using a pulper. *Bacillus subtilis* (isolated from Douchi and stored in Professor Chen's laboratory at Nanchang University), *Aspergillus oryzae* (isolated from Douchi and stored in Professor Chen's laboratory as above) and *Lactobacillus plantarum* M3 (isolated from sourdough and stored in in Professor Chen's laboratory as above) bacteria (all 10^8 colony forming U/ml) were used as the inoculum for the preparation of the herb residue fermentation supernatant. *B. subtilis* and *A. oryzae* were added to the herb residue for 24 h, and *L. plantarum* M3 was added for 24-36 h at 37˚C. Fermented products were subsequently centrifuged for 30 min at 1,000 x g at 4˚C in a refrigerated centrifuge (Hunan Xiangyi Laboratory Instrument Development Co., Ltd., Changsha, China) to obtain the fermentation supernatant.

Spleen-deficient mouse model and treatment. The present study was approved by the Ethical Committee of the Second Affiliated Hospital of Nanchang University (Nanchang, China) and all methods were performed in accordance with the approved guidelines.

A total of 30 specific pathogen free 6-8-week-old male C57BL/6 mice weighting 20-30 g were housed and fed a commercial diet with water *ad libitum*. All mice were purchased from the Hebei Center for Disease Control and Prevention (Hebei, China), housed five per cage, on a 12-h light/dark cycle at 23±2˚C and at 50±10% relative humidity. To establish the spleen-deficient mice model, 20 ml/kg/day 100% rhubarb decoction (supplied by Jiangxi Provincial Hospital of Traditional Chinese Medicine, Jiangxi, China) was administered intragastrically to mice for 7 days. All control animals were treated with the equivalent volume of PBS. Subsequently, model mice were divided into 3 groups: i) The modeling group (n=10), in which mice were only administered PBS; ii) the probiotics + drug residues group (n=10), in which mice were administered herb residue fermentation supernatant (0.1 ml/20 g); and iii) the Jianweixiaoshi tablets group (n=10), in which mice were administered Jianweixiaoshi tablets (0.1 ml/20 g). Mouse feces were collected at four time-points: i) During the control stage, on day 0, prior to treatment; ii) during the modeling stage, on day 7, following treatment with rhubarb decoction; iii) during the treatment stage, on day 14, following drug treatment; and iv) during the recovery stage, on day 21. In each group, feces of three mice from the modeling, probiotics + drug residue and Jianweixiaoshi tablets groups were randomly selected and used for denaturing gradient gel electrophoresis (DGGE) analysis.

Determination of immune indices and inflammatory factors. A total of 24 h following the final drug administration, five animals in each group were sacrificed by decapitation. Tail vein method was used to obtain the whole blood, and serum was obtained by the centrifugation of clotted blood at 2,500 x g for 15 min at 4˚C, and interleukin (IL)-2, IL-4 and interferon-γ (IFN-γ) were determined using ELISA kits for IL-2 (88-7024-88; Mouse IL-2 ELISA Ready-SET-Go!), IL-4 (88-7044-22; Mouse IL-4 ELISA Ready-SET-Go!) and IFN-γ (BMS606; Mouse IFN-γ Platinum ELISA; all from eBioscience; Thermo Fisher Scientific, Inc., Waltham, MA, USA) (15). Spleen and thymus were harvested from mice and weighed immediately. Thymus and spleen indices were calculated according to the following formula: Thymus or spleen index = [(weight of thymus or spleen)/body weight] x 100.

Viable cell count. Fresh fecal samples were subjected to treatment within 2 h following collection. All samples were serially diluted 10-fold with saline solution and 300-μl solutions resulting from each dilution were separately plated on a brain-heart infusion agar supplemented with 10% sterile skimmed milk (Beijing Land Bridge Technology Co., Ltd., Beijing, China) for total anaerobic bacteria, and incubated anaerobically at 37˚C for 36 h. De Man, Rogosa and Sharpe agar (Oxoid; Thermo Fisher Scientific, Inc.) was used for anaerobic culture of *Lactobacilli* at 37˚C for 24 h. *Enterococci* were aerobically cultured at 37˚C for 24 h on Slanetz-Bartley medium agar and MacConkey agar (both from Oxoid; Thermo Fisher Scientific, Inc.) was used for aerobic culture of *Enterobacteria* at 37˚C for 24 h (16-18).

DGGE and statistical analysis. DNA was isolated using bead-beating method (19). Following phenol-chloroform extraction, DNA was precipitated with 75% ethanol and resuspended in 50 μl TE buffer (10 mM Tris-Cl, 1 mM EDTA; pH 7.6). Primers, including 357 forward (5'-TACGGGAGG CAGCAG-3') and 519 reverse (5'-ATTACCGCGGCTGCT GG-3'), were used to amplify total bacterial DNA, and Lac1 (5'-AGCAGTAGGGAATCTTCCA-3') and Lac2 (5'-ATT YCACCGCTACACATG-3') were used to amplify DNA from *Lactobacillus* species. GC clam-in primers were selected to generate GC-enriched polymerase chain reaction (PCR) products suitable for separation by DGGE. Subsequently, a PCR was performed using the Taq DNA Polymerase kit (Takara Biotechnology Co., Ltd., Dalian, China) in a Biosci PCR system, with 30 cycles of 94˚C for 30 sec, 56˚C for 30 sec, and 72˚C for 60 sec. Amplicons of 16S ribosomal RNA were used for sequence-separation by DGGE, as previously described (20,21). DGGE was performed on 8% polyacrylamide gels containing acrylamide, bisacrylamide, formamide and a gradient of 35-65% urea. Tris-HCl (40 mM; pH 8.0) was used as the electrophoresis buffer in a Bio-Rad DGGE system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Electrophoresis was initiated by a pre-run for 5 min at 220 V followed by run at a fixed voltage of 85 V for 16 h at 60˚C. Gels were stained with $AgNO_3$ and developed following electrophoresis. Subsequently, gels were covered with cellophane membranes and dried overnight at 4˚C. DGGE patterns were subsequently normalized and analyzed using Bionumeric software (version 2.0; Applied Maths, Sint-Martens-Latem, Belgium). During the processing, lanes were defined and the background was subtracted. In the process of normalization, differences in the intensity of the lanes were compensated

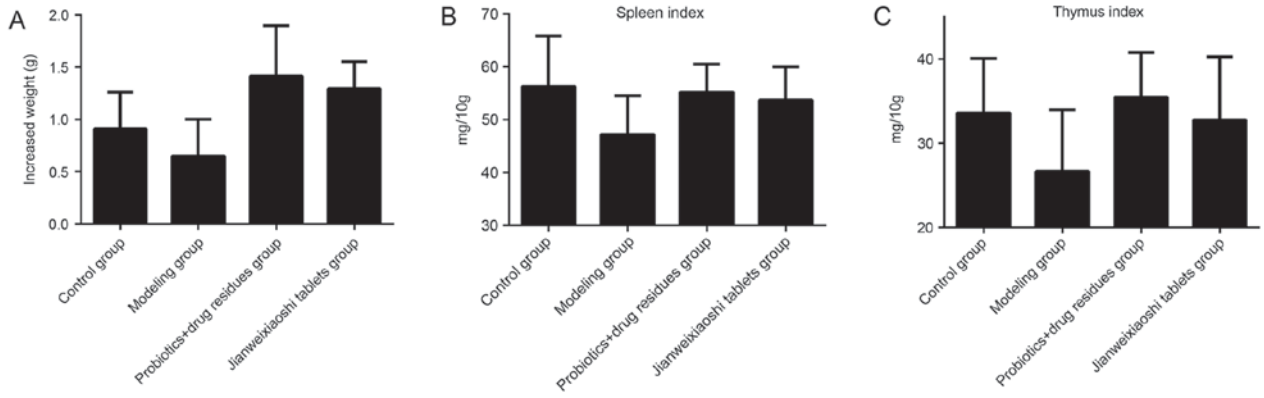

Figure 1. Effect of herb residue fermentation supernatant on mice. The effect on (A) weight, (B) spleen index and (C) thymus index. Data are expressed as the mean ± standard deviation.

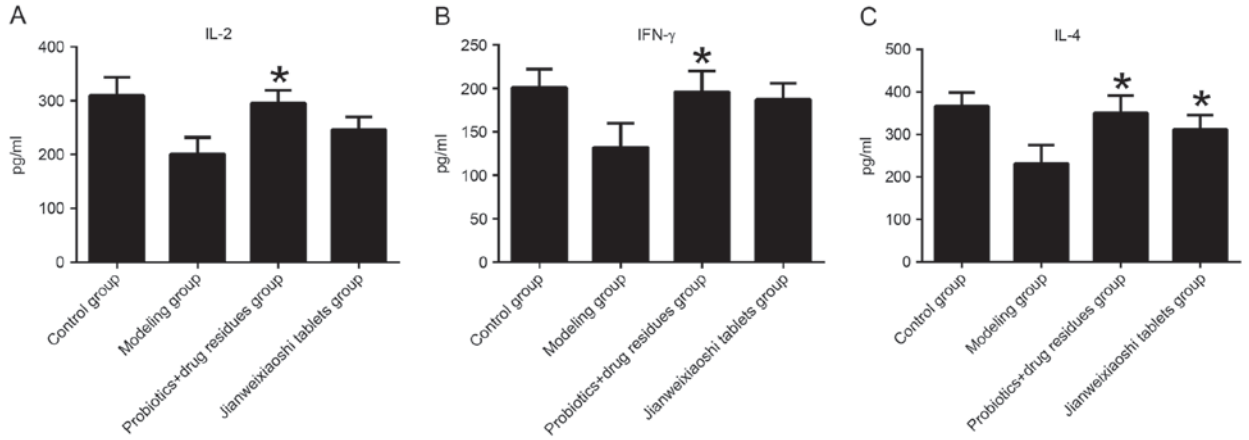

Figure 2. Effect of herb residue fermentation supernatant on immunity. The effect of herb residue fermentation supernatant on (A) IL-2, (B) IFN-γ and (C) IL-4 in mouse serum. Data are expressed as the mean ± standard deviation. *P<0.05 vs. respective modeling group. IL, interleukin; IFN-γ, interferon-γ.

and the association matrix was calculated. Clustering was performed using the Pearson correlation and unweighted pair group method with arithmetic mean (UPGMA) methods.

Sequencing of DGGE bands. Bands of interest were excised from the gel using a sterile blade and incubated overnight at 4˚C in Tris-EDTA buffer (pH 8.0) to allow for DNA diffusion out of the polyacrylamide matrix. The solution was used directly for further amplifications. For sequencing, eluted DNA was amplified using the same primer pairs and conditions as described above, without the GC clamp. PCR products for sequencing were purified using a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). PCR products were subcloned using the pMD18-T vector system I (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. *E. coli* DH5α cells (Beijing Tiangen Biochemical Technology Co., Ltd., Beijing, China) were subjected to electrotransformation with recombinant plasmids. Selection of transformants was performed on lysogeny broth agar (Oxoid; Thermo Fisher Scientific, Inc.) containing 100 mg/ml ampicillin. Transformants were randomly selected and sequenced by Invitrogen (Thermo Fisher Scientific, Inc.) (22-24).

Statistical analysis. Data are presented as the mean ± standard deviation. Data were analyzed using SPSS software (version 13.0; SPSS Inc., Chicago, IL, USA) using one-way analysis of variance with the least significant difference post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Treatment with fermentation supernatant increases body weight, thymus and spleen indices in mice. Compared with the control group, treatment with rhubarb reduced body weight, spleen index and thymus index (Fig. 1) in the modeling group, and presented depressive behavior, constipation, unkempt fur and poor appetite. Treatment with herb residue fermentation supernatant and Jianweixioashi tablets resulted in an increase in body weight, spleen index and thymus index, although these differences were not statistically significant.

Levels of IL-2, IL-4 and IFN-γ in serum. Treatment with rhubarb decreased the levels of IL-2, IL-4 and IFN-γ in the modeling group, compared with the control group. Administration of probiotics with herb residues significantly increased levels of IL-2, -4 and IFN-γ, compared with the modeling group (all P<0.05; Fig. 2). Jianweixioashi tablets significantly increased levels of IL-4, compared with the modeling group (P<0.05).

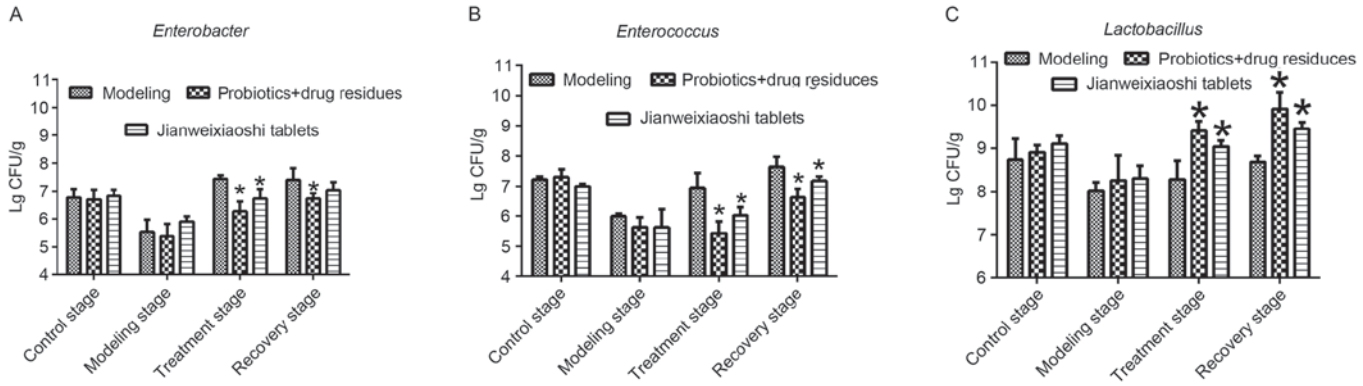

Figure 3. Effect of herb residue fermentation supernatant on the number of intestinal bacteria. The effect on (A) *Enterobacter*, (B) *Enterococcus* and (C) *Lactobacillus*. Data are expressed as the mean ± standard deviation. *P<0.05 vs. respective modeling group. Lg, Log; CFU, colony forming unit.

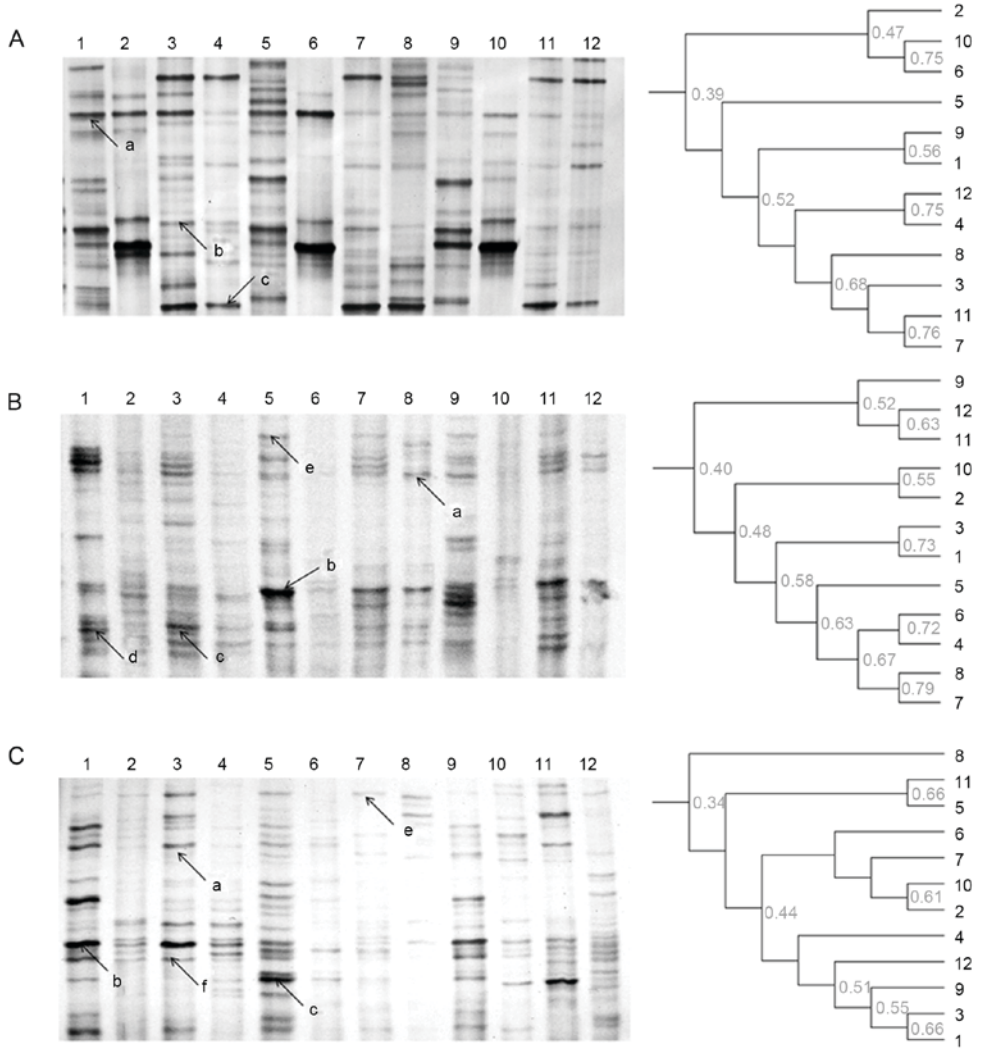

Figure 4. DGGE profile and unweighted pair group method with arithmetic mean analysis of fecal microbiota using bacterial primers. DGGE profile of (A) the modeling group, (B) the probiotics + drug residues group and (C) the Jianweixiaoshi tablets group. Lanes 1-3, control stage samples; lanes 4-6, modeling stage samples; lanes, 7-9 treatment stage samples; and lanes 10-12, recovery stage samples. a, uncultured bacterium; b, *Lactobacillus murinus*; c, *Enterococcus* sp.; d, *Clostridium* sp.; e, uncultured bacterium; f, uncultured bacterium. DGGE, denaturing gradient gel electrophoresis.

Effects of fermentation supernatant on microbial number in vivo. The effects of the fermentation supernatant on the viable number of *Enterococci*, *Enterobacteria* and *Lactobacilli* were determined in the present study. Viable cell counts indicated that, during the treatment stage, fermentation supernatant and Jianweixioashi tablets increased the number of *Lactobacilli*, and reduced the number of *Enterococci* and *Enterobacteria*, compared with the modeling group (all P<0.05; Fig. 3). During

Table I. Strains identified from mouse intestines by denaturing gradient gel electrophoresis using bacterial primers and *Bacillus* primers.

A, Bacterial primers

Strain no.	Closest relatives	Similarity, %	GenBank no.
a	Uncultured bacterium	100	HQ321493.1
b	*Lactobacillus murinus*	100	HQ668465.1
c	*Enterococcus sp.*	100	JF910016.1
d	*Clostridium sp.*	100	JF813180.1
e	Uncultured bacterium	100	GU606372.1
f	Uncultured bacterium	100	JF837882.1

B, *Bacillus* primers

Strain no.	Closest relatives	Similarity, %	GenBank no.
a	Uncultured bacterium	99	HM363549.1
b	Uncultured bacterium	100	EU491355.1
c	*Lactobacillus murinus*	100	HQ668465.1
d	Uncultured bacterium	100	HM363550.1
e	Uncultured bacterium	100	EU475615.1
f	Uncultured bacterium	100	EU006313.1
g	*Escherichia fergusonii*	100	HQ259962.1
h	Uncultured bacterium	100	FJ881122.1

the recovery stage, fermentation supernatant significantly inhibited the growth of *Enterococci* and *Enterobacteria*, and promoted the growth of *Lactobacilli*, compared with the modeling group ($P<0.05$).

Effects of the herb residue fermentation supernatant on bacterial diversity in vivo. The results of the DGGE analysis indicated that an uncultured bacterium, *L. murinus,* and *Enterococcus* sp. were the dominant bacteria in all groups throughout the experiments. In addition, the DGGE profile indicated that treatment with rhubarb markedly reduced the band number, while the administration of fermentation supernatant and Jianweixiaoshi tablets enhanced the bacterial diversity in the treatment and recovery stages (Fig. 4; Table IA). However, the results of the UPGMA analysis demonstrated low levels of similarity between bacteria in the control, treatment and recovery stages in the probiotics + drug residues and Jianweixiaoshi tablets treatment groups.

Effects of the herb residue fermentation supernatant on Bacillus diversity in vivo. As demonstrated by *Bacillus* DGGE profiles, treatment with rhubarb induced an effect on *L. murinus,* and uncultured bacteria d and f (Fig. 5). *L. murinus* was the dominant bacterium in all groups and during all stages. In addition, treatment with rhubarb eliminated uncultured bacterium d during the modeling stage in the probiotics + drug residue treatment group, while treatment with fermentation supernatant recovered the growth of uncultured bacterium d during the treatment and recovery stages (Fig. 5; Table IB).

Discussion

Large amounts of microbes have been identified in human intestines, particularly lactic acid bacteria, forming a complex ecological community that influences physiological homeostasis and serves a role in maintaining human health, including protection against entero-pathogens, extraction of nutrients and energy, and maintenance of normal immune functions (25-28). Jianweixiaoshi tablets have been demonstrated to alleviate various spleen-stomach diseases associated with intestinal bacteria, and the active ingredients identified in herb residues demonstrated a therapeutic effect on these diseases (29,30). Therefore, the combination of probiotics and herb residues may exert positive effects on spleen-stomach diseases.

In the present study, a spleen-deficient mouse model was established, which demonstrated symptoms comprising depressive behavior, constipation, unkempt fur and poor appetite (5,6). When treated with the herb residue fermentation supernatant, the mice exhibited a minor increase in body weight, spleen index and thymus index, suggesting that the fermentation supernatant was able to enhance growth and immunity in the mice.

Cytokines serve a role in immune responses. IL-2 and IFN-γ are classified as T helper 1 cytokines, and stimulate the proliferation of cytotoxic T lymphocytes, helper T lymphocytes, natural killer cells, lymphokine activated killer cells and macrophages (31-34). IL-4 is a T helper 2 cytokine, serving a role in the stimulation of activated B-cells, the proliferation of T-cells and the differentiation of B cells into plasma cells, and it has additionally been implicated in the regulation of humoral

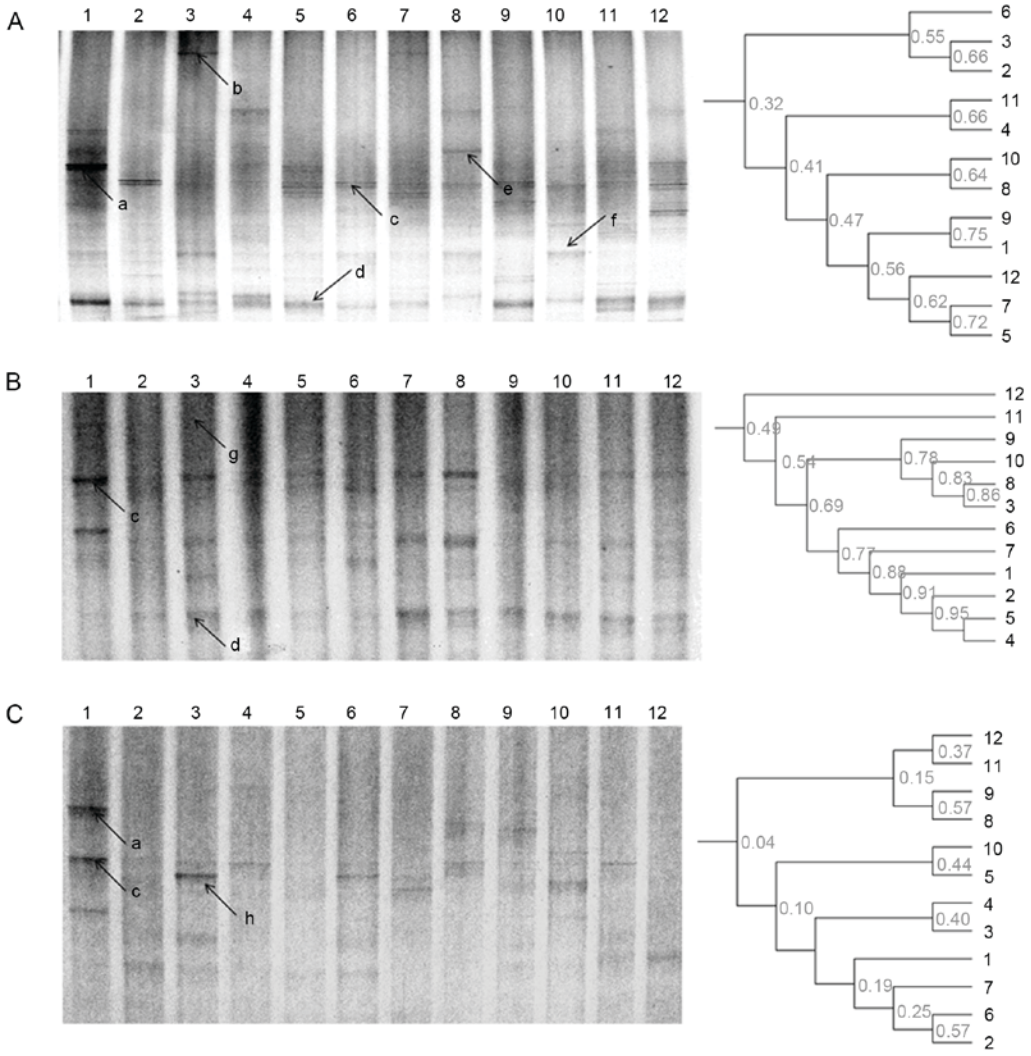

Figure 5. DGGE profile and unweighted pair group method with arithmetic mean analysis of fecal microbiota using *Bacillus* primers. DGGE profile of (A) the modeling group, (B) the probiotics + drug residues group and (C) the Jianweixiaoshi tablets group. Lanes 1-3, control stage samples; lanes 4-6, modeling stage samples; lanes, 7-9 treatment stage samples; and lanes 10-12, recovery stage samples. a, uncultured bacterium; b, uncultured bacterium; c, *Lactobacillus murinus;* d, uncultured bacterium; e, uncultured bacterium; f, uncultured bacterium; g, *Escherichia fergusonii*; h, uncultured bacterium. DGGE, denaturing gradient gel electrophoresis.

and adaptive immunity (31,32). Therefore, increased production of IL-2, IL-4 and IFN-γ indicated that the fermentation supernatant enhanced the immunity of the spleen-deficient mice.

Certain diseases, including obesity, malnutrition, inflammatory bowel disease, neurological disorders and cancer, may result from microbial imbalances (35-39). Considering the health-promoting effect of TCM and probiotics, fermentation supernatant may be hypothesized to enhance microbial diversity, promote the growth of probiotics and inhibit the growth of pathogens. The results of the viable cell counts in the present study indicated that the fermentation supernatant markedly enhanced the number of *Lactobacilli* and reduced the number of *Enterococci* and *Enterobacteria* during the treatment stage. The results of the DGGE analysis indicated that the fermentation supernatant enhanced bacterial diversity during the treatment and recovery stages. In the present study, low similarity levels between bacteria in the control, treatment and recovery stages indicated that a novel microbial balance was established by treatment with the fermentation supernatant. The diversity of bacteria in host intestines contributes to more efficient host defenses from external invasion and, therefore, the reduced number of bands in the modeling group indicated weak resistance to foreign pathogens, while enhanced diversity in the fermentation supernatant group indicated recovery of intestinal health (28,36,37,40).

In conclusion, in the present study, the therapeutic effects of herb residues from Jianweixiaoshi tablets were used for the treatment of spleen deficiency in mice *in vivo*. The results of the present study indicated that fermentation supernatant was able to enhance host immunity and inflammatory responses, by increasing the number and diversity of beneficial bacteria. Therefore, the present study demonstrated that the combination of Jianweixiaoshi herb residues and probiotics may, in the future, provide a novel method for the treatment of spleen deficiency.

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