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# Reclamation of Chinese herb residues using probiotics and evaluation of their beneficial effect on pathogen infection

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ABSTRACT

Environmental pollution caused by herb residues and the huge waste of medicinal ingredients contained in herb residues hinder the development of traditional Chinese medicine enterprises. To solve this problem, several probiotics were tested, and *Lactobacillus plantarum* (HM218749) was finally selected for the reuse of herb residues of Jianweixiaoshi tablets. A mouse model of *Helicobacter pylori* infection was developed to evaluate the anti-*H. pylori* infection activity of the herb residue fermentation supernatant using a urease activity test, histological imaging, an enzyme-linked immunosorbent assay (ELISA) and denaturing gel gradient electrophoresis (DGGE). The results demonstrated that the herb residue fermentation supernatant successfully inhibited urease activity, slowed cell infiltration in the gastric area and significantly reduced the production of interleukin-6 (IL-6), IL-8 and TNF-α in the treatment group ($p < 0.01$). In addition, the DGGE results indicated that the herb residue fermentation supernatant was beneficial for the recovery of the disturbed microbiota in the infected model to the normal condition, in which *L. gasseri* (GU417842.1) and *L. johnsonii* (HQ828141.1) were dominant in all groups. Therefore, the probiotics exhibited strong potential for the development of herb residues in this study, and the products showed strong potential in curing *H. pylori* infections.
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## Introduction

Chinese herbal medicine (TCHM) is among the important traditional industries in China and is a treasure of the Chinese nation. In recent years, the poor results of single-target drugs in the treatment of chronic diseases (e.g., diabetes and cardiovascular disease) and the urgent need for green and safe formulas have made TCHM a hot topic worldwide [1–3]. However, the huge amounts of herb residues produced by the continuous development of the Chinese herbal medicine industry have become a serious problem for large pharmaceutical companies.

In China, the annual yield of herb residues is approximately 30 million tons, mostly disposed of through stacking in the open, sanitary burial or burning, causing serious environmental pollution, especially in water quality. Although some studies have reused herb residues for feed additives, the preparation of activated carbon, raw material for papermaking, cultivation of edible fungi or preparation of ethanol, these methods consider only the nutritional benefits and texture of medical plants, ignoring their precious medicinal ingredients [4,5]. As is well known, herb residues are the by-products of traditional Chinese medicinal materials extracted by water or ethanol, and approximately 30%–50% of the medicinally active substances are still contained in them.

The microorganism fermentation theory in TCHM suggests that the digestive enzymes (e.g., cellulase, protease, pectinase and lignin enzymes and lipase) produced by microorganisms could effectively degrade plant cell walls, expand the intercellular region and improve the extraction yield of active ingredients [6]. In addition, microorganisms could degrade macromolecular material to small molecules for direct absorption by the human body, reducing the side effects of drugs by degrading toxic substances and introducing new medicinal effects by biological modification [7]. Probiotics are now accepted as useful in the prevention and/or treatment of certain pathological conditions [8]. At present, the most studied probiotics are lactic acid-producing bacteria, particularly *Lactobacillus* species [9], which have proven to be useful in the treatment of several gastrointestinal diseases such as acute infectious diar-

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**Table 1**
Concentrations of IL-6, IL-8 and TNF-α in the gastric mucosa (mean ± SD, pg/mg).

| Group | N | IL-6 | IL-8 | TNF-α |
|---|---|---|---|---|
| Control group | 6 | 63.52 ± 11.12 | 68.42 ± 16.12 | 87.39 ± 20.23 |
| Model group | 6 | 123.43 ± 20.52 | 110.83 ± 18.23 | 132.44 ± 25.65 |
| Treatment group | 6 | 85.87 ± 16.64[b] | 76.34 ± 16.31[b] | 97.45 ± 16.89[a] |

[a] $p < 0.05$.
[b] $p < 0.01$ (Compared with the Model group).

**Table 2**
Sequencing results of typical bands of DGGE patterns from Fig. 4.

| Strain No. | Closest relatives | Similarity (%) | GenBank No. |
|---|---|---|---|
| Bacterial DGGE | | | |
| a | *L. gasseri* | 99 | GU417842.1 |
| b | *L. johnsonii* | 100 | HQ828141.1 |
| c | *L. plantarum* | 100 | EF439684.1 |
| d | *L. casei* | 100 | JN974254.1 |
| Lactobacilli DGGE | | | |
| a | *L. gasseri* | 99 | GU417842.1 |
| b | *L. johnsonii* | 100 | HQ828141.1 |
| c | *L. lactis* | 100 | FJ171327.1 |

rhea or pouchitis. The intake of probiotics can also be beneficial in *Helicobacter pylori*-infected subjects [8].

*H. pylori*, first recognized in 1982, is now regarded as a major cause of gastritis and peptic ulcers and is a risk factor for peptic ulcers, chronic gastritis, and gastric malignancy. This organism can be found in 70%–90% of the population in developing countries and in 25%–50% of the population in developed countries [8,10–12]. At present, combination therapy consisting of 2 antibiotics and a proton pump inhibitor $(PPI)_6$ is regarded as the treatment of choice to eradicate *H. pylori* infection, and this regimen is 90% effective. However, it is expensive and subject to side effects and antibiotic resistance [13].

In this study, the herb residues of Jianweixiaoshi tablets, which consist of hawthorn, malt, pseudostellaria root, Chinese yam, and orange peel and have been approved by the Ministry of Public Health as both a medicine and a food, were chosen for reuse by probiotics, and the anti-*H. pylori* infection effect was evaluated based on the invigoration of the stomach and promotion of digestion by the Jianweixiaoshi tablets in addition to the anti-*H. pylori* effects of the probiotics (Tables 1 and 2).

## Materials and methods

### *Acid, salt and antibacterial testing of the probiotics*

The *Lactobacillus plantarum* (HM218749), *L. reuteri* (EU547310), *L. rhamnosus* (RS05630), *L. johnsoni* (RS03965) and *L. paracasei* (*ATCC 334*) were grown in de Man Rogosa Sharpe (MRS) media at 37 °C overnight and were sub-cultured 3 times. The cultures were then centrifuged at 4500 × *g* for 10 min to obtain a pure culture. To test acid tolerance, each isolate was diluted 1:100 (v/v) in phosphate-buffered saline (PBS) at pH 1.5, 3.5, 4.5, 5.5 and 7.0 for 4 h. To test salt tolerance, freshly prepared cultures were inoculated into corresponding media containing 0.1–0.5% (w/w) bile salts and were incubated at 37 °C for another 4 h. All bacterial colonies were enumerated using the plate count method.

For antimicrobial activity, overnight (12–16 h) cultures of pathogenic micro-organisms, including *Shigella dysenteriae* 301, *Sh. dysenteriae* 2457, *Staphylococcus aureus* COWAN1, *S. aureus* CMC, *Enterobacter sakazakii* 45402, *Candida albicans* SC5314 and *H. pylori* SS1, were spread on the surfaces of their corresponding plates, and the culture supernatant (200 μL) of the probiotics was loaded into an Oxford cup (outer diameter: 7.8 ± 0.1 mm; inner diameter: 6.0 ± 0.1 mm; height: 10.0 ± 0.1 mm), which was placed on the surface of the agar. The size of the inhibition zone was measured until the formation of a clear zone around the Oxford cup. The experiment was conducted in duplicate [14].

### *Preparation of herb residue extract and fermentation supernatant*

The herb residue of Jianweixiaoshi tablets was obtained from River Pharmaceutical Co., Ltd. and was mashed using a pulper within 2 h. The probiotics *L. plantarum* (HM218749), *L. reuteri* (EU547310), *L. rhamnosus* (RS05630), *L. Johnsoni* (RS03965) and *L. paracasei* (*ATCC 334*)($10^8$ cfu/mL) were used as an inoculum to prepare the herb residue fermentation supernatant, which was incubated for 24–36 h at 37 °C, and the bacterial number was evaluated using the plate count method.

### H. pylori *infection model and treatment*

The study was approved by the Ethical Committee of the Second Affiliated Hospital of Nanchang University, and all methods were conducted in accordance with the approved guidelines.

The *H. pylori* SS1 strain was routinely cultured under microaerophilic conditions (85% $N_2$, 5% $H_2$, 10% $CO_2$) at 37 °C on Wilkins-Chalgren agar enriched with 7% (vol/vol) horse blood and 1% (vol/vol) VITOX (Oxoid, Basingstoke, United Kingdom). Specific-pathogen-free 6- to 8-week-old male C57BL/6 mice were housed and fed a commercial diet, with water ad libitum. *H. pylori* infections by the SS1 strain were conducted as previously described [15]. Briefly, freshly prepared aliquots (500 μl, $10^9$ CFU) of the *H. pylori* SS1 strain in brain heart infusion broth (Oxoid) were administered to mice via orogastric inoculation five times (days 1, 3, 5, 7 and 9). All noninfected control animals were inoculated with the same volume of plain brain heart infusion broth. Eight weeks after the last gavage of *H. pylori*, urease activity and histological imaging were examined to confirm successful modeling.

Next, the mice were divided into 3 groups: control group (N = 12): normal mice only given PBS; model group (N = 12): the infectious model, only given PBS; treatment group (N = 12): the infectious model, given herb residue fermentation supernatant. Ten weeks after the last gavage of *H. pylori* SS1, 0.5 ml of PBS (control group and model group) or herb residue fermentation supernatant (treatment group) was administered to mice for 3 weeks, and then the stomachs of 6 mice in each group were sterilely obtained and divided into 4 parts for the urease activity test, histological imaging, ELISA testing (IL-6, IL-8 and TNF-α), and DGGE analysis. Two weeks later, the stomachs of 5 mice in each group were obtained for DGGE analysis in the recovery stage.

### *Determination of urease activity and ELISA*

Urease activity was determined by a method based on the commercial rapid urease test (Sanqiang Biochemical Industry Corporation in Fujian, China) with a sensitivity of $10^2$ bacteria [16]. Following the manufacturer's instructions, each strip of the stomach antrum and body was homogenized and placed in 1 mL of reaction solution (1 g of urea/mL (wt/vol) containing 850 μg of phenol red/mL (wt/vol) as a pH indicator). The solution became pink red or dark red within 5 min as a positive result and remained yellow as a negative results.

The products of IL-1β, TNF-α and IL-6 in cell supernatants were determined using the ELISA kit for IL-1β (eBioscience), TNF-α (eBioscience) and IL-6 (eBioscience).

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