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Effects of ultra-micro powder wuji wan on gut microbes and enzyme activities

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Abstract

To explore the effect of Chinese medicine ultra-micro powder Wuji Wan on gut microorganisms and enzyme activities, microbial culturing methods and enzymological methods were used to determine the amount of microbes and enzyme activities in gastrointestinal tract. Antibacterial activity of ultra-micro powder Wuji Wan were determined *in vitro*. Mice were randomly divided into 6 groups.The drugs were orally administered. The results showed that the amount of *Bifidobacterium* spp., *Lactobacillus* spp., and the activity of amylase and cellulase in gastrointestinal tract were not significantly affected by ultra-micro powder Wuji Wan ($P>0.05$). But the growth of bacteria, colibacillus and fungi, and the activity of the protease and xylanase in gastrointestinal tract were inhibited by the ultra-micro powder Wuji Wan ($P<0.05$). Ultra-micro Wuji Wan can significantly inhibit the growth of common opportunistic pathogens in gastrointestinal tract *in vitro*. The effects of ultra-micro powder Wuji Wan on microorganisms and enzyme activities in stomach were stronger than in intestine. The 25% of ultra-micro powder dose can produce the greatest effect in the stomach without injection. Ultra-micro technology can conserve Chinese herbal medicine.

Keywords: Wuji Wan, ultra-micro powder Wuji Wan, gut microbes, gut enzyme

Introduction

The Traditional Chinese Medicine (TCM) ultra-micro powder technology is the rising high-tech in recent years. The applications of the ultra-micro powder Chinese herbal pieces had a very important practical significance and value on both improving the effect and quality of Traditional Chinese Medicine and reducing the waste of Traditional Chinese Medicine resources. It's very useful for the modernization and internationalization of Chinese medicine [1]. But the appropriate clinical dosage of ultra-micro powder of TCM needed for the treatment is still not clear [2].

There are about10^{14} microorganisms in human gastrointestinal tract, a huge micro-ecological system [3-4]. It is important to parasis for a variety of physiological function (especially metabolic) as well as life activities for gastrointestinal flora [5]. At the same time, a number of investigations have shown that certain butyrate-producing firmicute bacteria are reduced in inflammatory bowel disease [6]. The human gastrointestinal microbes and enzymes dissolve and transform the Chinese medicines through hydrolysis and reduction reactions [7]. The metabolic function of intestinal microbes may play important roles on the effect of Chinese medicine on diseases [8-10].

Wuji Wan, a prescription of TCM, consists of three herbs: Rhizoma Coptidis, Fructus Evodiae Rutaecarpae and Radix Paeoniae Alba. It has excellent curative effects on gastrointestinal diseases [11]. Wuji Wan have been used to treat stomach pain, Tunsuan, abdominal pain and diarrhea due to incoordination between the liver and the spleen [12], and in particular the treatment of gastric ulcer. Like all other Chinese medicine, the curative mechanisms of Wuji Wan are not fully understood [13]. Previous studies showed that coptis, fructus evodiae and peony from Wuji Wan had apparent antibacterial effects [14-15]. Ultra-micro powder technology has been used for Chinese medicine compound preparation, this can increase the contents and the dissolution of effective ingredients. The ultra-micro powder Wuji Wan contain higher levels of berberine hydrochloride, paeoniflorin, evodianmine and rutaecarpine compared with traditional Wuji Wan. In this study, we aimed with investigate the impacts of ultra-micro powder Wuji Wan on gut microbes and enzyme activities in mice.

Materials and methods

Experimental animals

60 Kunming mice (SPF grade) were provided by Shanghai Experimental Animal Center of Chinese academy of Sciences.

The weight of each mouse was about 20±2g. Mouse food was provided by Experimental Animal Center of Hunan University of Traditional Chinese Medicine.

Drug treatment

Wuji Wan were prepared according to the Chinese Pharmacopoeia 2010. 300g of Rhizoma coptidis , 50g of Fructus evodiae and 300g of Radix paeoniae alba were ground into a fine powder. The ultra-micro powder Wuji Wan were made by Ultra-powder Chinese Medicine Engineering Research Center of Hunan Academy of Chinese Medicine. The Wuji Wan water decoction and the ultra-micro powder Wuji Wan water decoction(100% dose) were made into water decoction without filter and centrifuge, and the concentration of both water decoction were 0.069g/ml [16]. The Ultra-micro powder Wuji Wan water decoction was diluted to 50%, 25% and 12.5% dose. Mice were randomly divided into 6 groups (10 mice per group): normal control animals, Wuji Wan treated animals, ultra-micro Wuji Wan treated animals, 50% of ultra-micro Wuji Wan treated animals, 25% of ultra-micro Wuji Wan treated animals, and 12.5% of ultra-micro Wuji Wan treated animals. All the drugs were orally administered to the mice by gavage twice a day (0.3ml each time) [16]. The normal control mice were treated with boiled water.

Media

For bacteria: Beef extract-peptone medium [17], for colibacillus: EMB medium [17], for *Lactobacillus* spp.: MRS medium [18], for *Bifidobacteria* spp.: BBL medium [19], for fungi: martin Rose Bengal Medium [17].

Microbial strains

Standard microbial strains: *Candida albican, Staphyloccocus aureus, Salmonella* sp., *Escherichia coli* and *Pseudornonas aeruginosa* were provided by Microbiology Teaching and Research Section of Hunan University of Traditional Chinese Medicine.

Extraction of gastrointestinal contents

All the mice were sacrificed on the morning of the ninth day after treatment. Mouse intestinal (jejunum to the rectum) and gastric contents were collected in a steriled environment.

Determination of microorganisms from gastrointestinal contents

A certain amount of intestinal and gastric contents were weighed in a steriled environment and transferred into conical flask equipped with glass beads and steriled water. In order to release microorganisms from intestinal and gastric contents into steriled water completely, the conical flasks were put on a table concentrator for 30min with 120rpm shaking. The number of microorganisms of intestinal and gastric contents was determined with the method of plate culture counting. Total numbers of bacteria and colibacillus were counted after culturing 24 hours at 37°C. Total numbers of *Lactobacillus* spp., *Bifidobacteria* spp. and fungi were determined after culturing 48 hours at 37°C.

Analyze enzyme activities of mice stomach and intestinal contents

To dissolve enzyme from stomach and intestinal contents completely, the contents were diluted with steriled water and heated preservation for 30min in a 40°C water bath. The enzyme extracts were centrifuged for 10min at 2000rpm, following which the enzyme activities in the supernatant were analyzed. The activities of amylase, xylase and cellulase activities were determined by DNS colorimeter as previous described [20]. Cellulase activity was defined as a unit of the cellulase activity as generating 1mg reducing sugar of 1g contents at 46°C for 30min. Amylase activity was defined as a unit of the amylase activity as generating 1mg reducing sugar of 1g contents at 37°C for 60min. Xylase activity was defined as a unit of the xylase activity as generating 1mg reducing sugar of 1g contents at 46°C for 60min. Protease activity was determined by Folin-phend method [21]. Protease activity was defined as a unit of protease activity as generating 1g amino acid of 1g contents at 37°C for 40min.

The measurement of the antimicrobial cycle dimension

The antibacterial activities of Wuji Wan and ultra-micro Wuji Wan were tested using agar slant culturing medium. The Wuji Wan water decoction and the ultra-micro powder Wuji Wan water decoction were enriched to 1g/ml by filtering and centrifuging. Microbe suspension was made with a mixture of tested strains and steriled water at 10^6-10^7CFU/ml and applied onto plate culture. After static cool-down for 15min, 6-mm diameter paper disks soaked in 10μl Wuji Wan water decoction or ultra-micro powder Wuji Wan decoction were placed on the inoculums. Disks soaked in 10μl of steriled water were used as negative controls. The dimension of growth inhibition was measured using a calibrated ruler after the antimicrobial cycle was formed. The experiments were repeated three times.

Statistical analysis

Measurement data of every group were represented with mean-standard deviation ($\bar{X}$±s) and analyzed using DPS v7.05 statistical software.

Results

Effect of Wuji Wan and ultra-micro Wuji Wan on treated mice

When compared with normal control mice treated with boiled water, all mice treated with Wuji Wan or ultra-micro Wuji Wan did not show any sign of sluggishness, aversion to cold, or diarrhea, and did not reduce food intake. The weight of mice in each treated group was similar to that of

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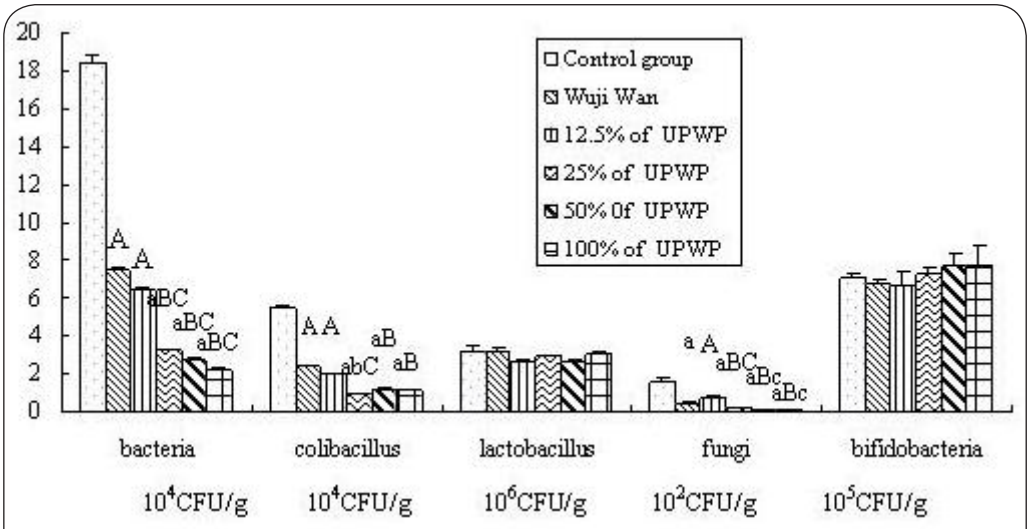

Figure 1. Effect of ultra-micro powder Wuji Wan on microbes in stomach(CFU/g).
a)CFU: Colony forming units.
b)UPWP: ultra-micro powder Wuji Wan.
c)Compared with control group: A:p<0.05, a:p<0.01; compared with traditional group: B:p<0.05, b:p<0.01; compared with 1/8 volume group: C:p<0.05, b:p<0.01; compared with 1/4 volume group: D:p<0.05, d:p<0.01; compared with 1/2 volume group: E:p<0.05, e:p<0.01.

control mice. It is suggested that Wuji Wan and Ultra-micro Wuji Wan had no effect on physiological activity in mice.

Effect of ultra-micro powder of Wuji Wan on on mice stomach microbes

The amount of flora in stomach is relatively very low because of the lower pH. The *Lactobacillus* spp., *Bifidobacteria* spp. and *Streptococcus faecalis* are beneficial bacteria in the gastrointestinal tract, other bacteria are generally opportunist pathogens and pathogenic.we could see from **Figure 1**, the amount of *Lactobacillus* spp. and *Bifidobacteria* spp. in stomach were significantly higher than the amount of other opportunist pathogens, but *Lactobacillus* spp. and *Bifidobacteria* spp. of each Chinese medicine treated group showed no significant difference (P>0.05), suggesting both Wuji Wan and ultra-micro powder Wuji Wan had no effects on the growth of *Lactobacillus* spp and *Bifidobacteria* spp. in stomach. The amount of bacteria, colibacillus and fungi in mice treated with Wuji Wan or 12.5% of ultra-micro powder Wuji Wan were significant lower than those in control group's mice (P<0.05). However, there was no significant difference between 12.5% of ultra-micro powder Wuji Wan and Wuji Wan group (P>0.05), suggesting 12.5% of ultra-micro powder Wuji Wan has a similar effect to the Wuji Wan. After treatment with 25% of ultra-micro powder Wuji Wan, 50% of ultra-micro powder Wuji Wan and whole ultra-micro powder Wuji Wan the amount of bacteria, colibacillus and fungi in the treated groups declined significantly(P<0.01). Compared with 12.5% of ultra-micro powder Wuji Wan and traditional Wuji Wan group, the amount of bacteria and fungi in 25%, 50% and whole ultra-micro powder Wuji Wan group declined significantly (P<0.05). Compared with traditional Wuji Wan group, the amount of colibacillus in 25%, 50% and whole ultra-micro powder Wuji Wan groups also declined significantly (P<0.05) (**Figure 1**). The results suggest that Wuji Wan and ultra-micro powder Wuji Wan can inhibit the growth of opportunist pathogens and pathogenic bacteria; the inhibiting effect of 25%, 50%, and whole ultra-micro Wuji Wan were superior to 12.5% of ultra-micro powder Wuji Wan and traditional Wuji Wan.

Effect of ultra-micro powder Wuji Wan on mice stomach enzyme activities

Gut enzymes come from dietary intake and secretion by gastrointestinal cells and microbial cells. Cellulase and xylase are secreted only by gastrointestinal microbes, not by the mouse itself. So the change of cellulase and xylase activities could reflect the change of microbes that secrete cellulase and xylase. We measured stomach enzyme activities in control and drug treated mice, cellulase activities were not significantly different in medincine treatment and control group(P>0.05). There was a remarked difference of xylase activities between Chinese medicine treatment group and control group(P<0.05), Xylase activities in groups treated with 25%, 50%, and whole ultra-micro powder Wuji Wan reduced more(P<0.05) (**Figure 2**). Amylase and protease are mainly secreted by the mouse itself. Amylase activities of all Chinese medicine treated groups showed no significant change compared with that of control group (P>0.05). However, the protease activities of all Chinese medicine treated group significantly reduced (P<0.05) (**Figure 2**). The protease activities of 25%, 50% and whole

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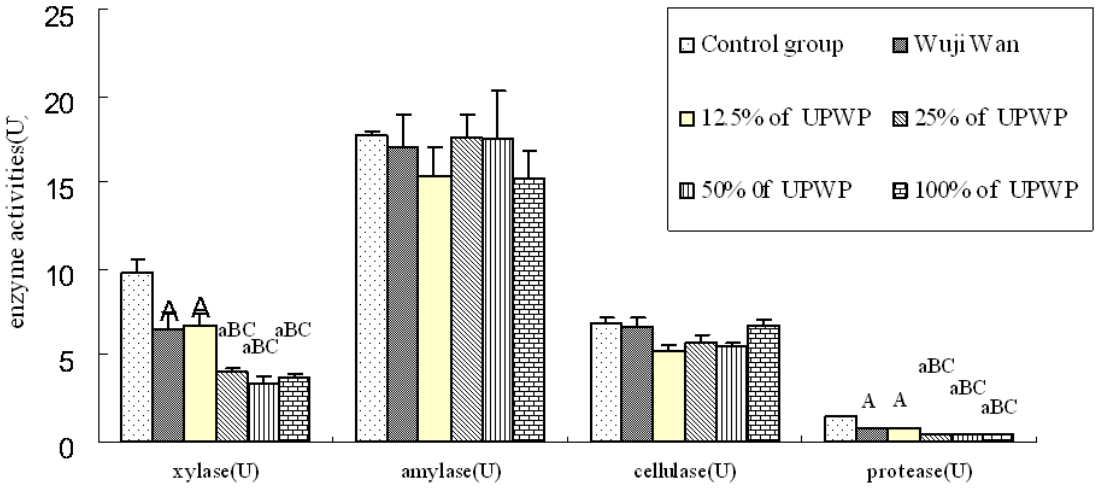

Figure 2. Effect of ultra-micro powder Wuji Wan on stomach enzyme activities.
The activities of xylase, amylase, cellulase and protease were measures as described in Materials and Methods.
a)Compared with control group: A:p<0.05, a:p<0.01; compared with traditional Wuji Wan treated group: B:p<0.05; compared with 12.5% of ultra-micro powder Wuji Wan treated group: C:p<0.05.
b)UPWP: ultra-micro powder Wuji Wan.

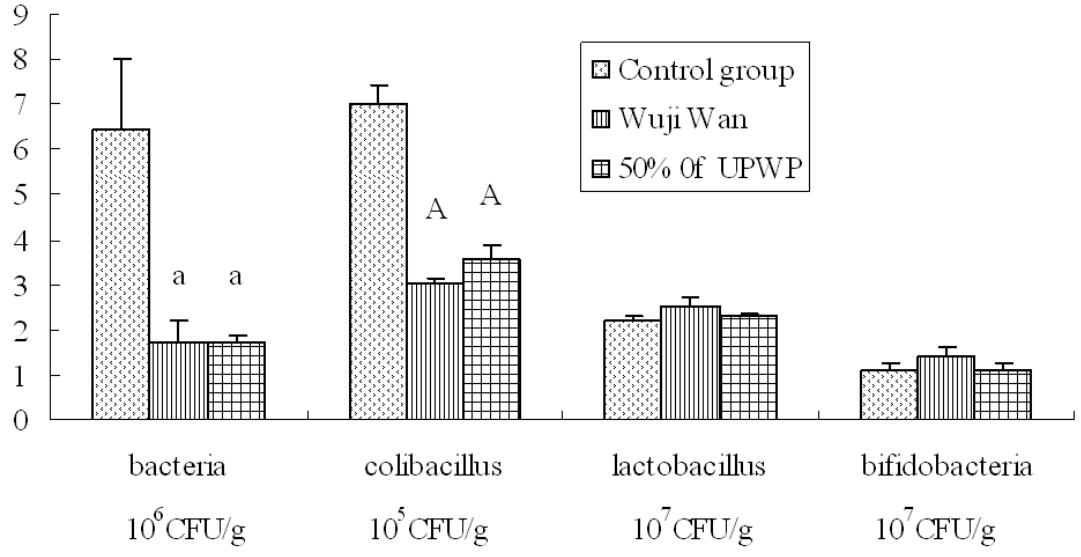

Figure 3. Effect of ultra-micro powder of Wuji Wan on intestinal microbes(CFU/g).
a)CFU: Colony forming units. **b**)UPWP: ultra-micro powder Wuji Wan.
c)Compared with control group: A:p<0.05, a:p<0.01.

ultra-micro powder Wuji Wan group were significant reduced compared with 12.5% ultra-micro powder Wuji Wan and traditional Wuji Wan group (P<0.05).

Effect of ultra-micro powder Wuji Wan on mice intestinal microbes

In our previous study 50% of ultra-micro powder Chinese medicine on intestinal microbes and enzyme activities was the best [22], so we adopted 50% of ultra-micro powder Wuji Wan as a working dose to investigate its effect on intestinal microbes and enzyme activities. The amount of intestinal microbes were significantly higher than in stomach because of elevated pH value (**Figure 3**). The amount of *Lactobacillus* spp. and *Bifidobacteria* spp. of Chinese medicine treated group were similar to the control group (P>0.05). Compared with control group, the amount of bacteria and colibacillus in 50% ultra-micro powder Wuji Wan and traditional Wuji Wan group

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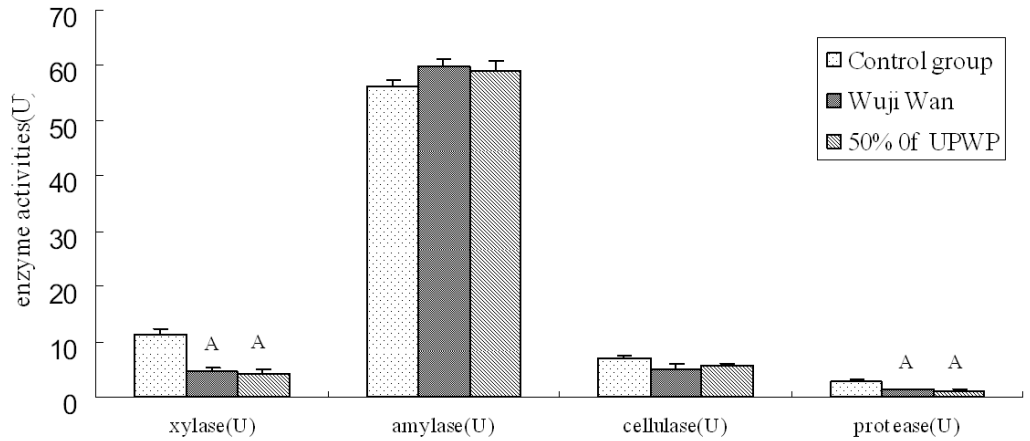

Figure 4. Effect of ultra-micro powder Wuji Wan on intestinal enzyme activities in mice(U).
The activities of xylase, amylase, cellulase and protease were measures as described in Materials and Methods.
a) Compared with control group: A:p<0.05.
b) UPWP: ultra-micro powder Wuji Wan.

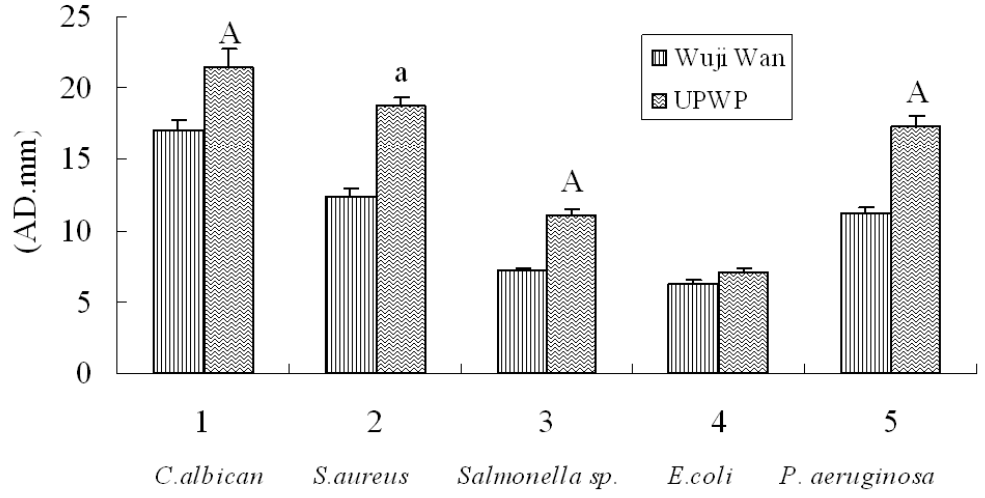

Figure 5. The anti-microbial test of Wuji Wan and ultramicro powder Wuji Wan *in vitro*(AD.mm).
a)AD: Inhibition zone diameter.
b)Compared with traditional decoction group: A:p<0.05, a:p<0.01.
c) UPWP: ultra-micro powder Wuji Wan.

significantly declined (**Figure 3**). There is no significant difference between 50% ultra-micro powder Wuji Wan and traditional Wuji Wan group (P>0.05).

Effect of ultra-micro powder Wuji Wan on intestinal enzyme activities in mice

We could see from the **Figure 2**,**4**. The activities of intestinal amylase was 3.5 times higher than amylase in stomach, while the activity of intestinal protease was two times higher than protease in stomach (P<0.01) (**Figure 2**,**4**). It is possible that the small intestine is the major organ for digestion and absorption. When compared with the control group, amylase activity in the 50% of ultra-micro powder Wuji Wan and traditional Wuji Wan group both slightly increased. Cellulase activity of both group slightly decreased (**Figure 4**), but there were no significant difference (P>0.05). The xylase and protease activities of Chinese medicine treated group significantly decreased compared with the control group (P<0.05) (**Figure 4**).

The anti-microbial test of Wuji Wan and ultramicro powder Wuji Wan *in vitro*

Candida albican, Staphyloccocus aureus, Salmonella sp., *Escherichia coli* and *Pseudornonas aeruginosa* are opportunistic pathogens and pathogenic bacteria in the grastrointesetinal tract. Four bacterial species

(*S. aureus*, *Salmonella* sp., *E. coli* and *P. aeruginosa*) used in the experiments were grown on beef extract-peptone medium, fungi *C. albican* was grown on martin rose bengal medium. **Figure 5** shows that both traditional Wuji Wan and ultra-micro powder Wuji Wan could inhibit the growth of all bacteria and fungi, with particular effectiveness for *C. albican*, *S. aureus, Salmonella* sp. and *P. aeruginosa*. The inhibition zones of ultra-micro powder Wuji Wan were significant bigger than the traditional Wuji Wan, suggesting ultra-micro powder Wuji Wan has higher effectiveness to inhibit growth of microbes.

Discussion

Under the circumstances of increasing antibiotic resistance in bacteria and antibiotic adverse reactions, a detailed study and accurate understanding of the function of natural plant ingredients will enable us to develop Chinese medicine as novel antimicrobial drugs. The present study showed that Wuji Wan and ultra-micro powder Wuji Wan can inhibit the growth of opportunistic pathogens and pathogenic bacteria in the grastrointesetinal tracts but have no effect on probiotics. There are at least six bioactive alkaloids: berberine, coptisine, jatrorrhizine, palmatine, evodiamine and rutacarpine identified in Wuji Wan [**23**]. most of the alkaloids have antibacterial activities (such as anti *H.pylori* and *E. coli*) [**24**-**25**]. Pepsin has been considered to be the attack factor for stomach diseases (such as gastritis, gastric ulcer and gastric cancer), our results showed that both traditional Wuji Wan and ultra-micro powder Wuji Wan can restrain xylase activities. It is possible that berberine embeds pepsin and trypsin molecules through hydrophobic interaction to form a static quenching caused by a particular structure of the association complex, and can function for a long time in the gastrointestinal tract because of the relatively poor fat solubility [**26**]. The mechanism for the treatment of gastrointestinal diseases is that Wuji Wan can inhibit the growth of opportunist pathogens and pathogenic bacteria, and can inhibit protease activity in stomach.

Wuji Wan are the classical recipe for the clinical treatment of peptic ulcer and gastritis, but the residence time of traditional Wuji Wan in the stomach is very short, usually for about two hours. It is difficult to form the effective concentration in the stomach. However, ultra-micro powder Wuji Wan could solve this problem as the bioactive ingredients of wall-breaking ultra-micro powder Wuji Wan can be rapidly dissolved in the stomach where it could form the highest concentrations of its active ingredients. But in terms of traditional Chinese medicine, the adhesion to intestinal walls is poor and traditional Wuji Wan still contain intact cell walls. When traditional Chinese medicine are orally administrated, the bioactive ingredients are released through wall-breaking by microbes and enzymes in the gastrointestinal tract [**7**]. Ultra-micro Chinese medicine has the surface, volume, quantum and macro tunnel effects which can strengthen the adhesion to the intestinal walls [**27**-**28**]. Our results showed the ultra-micro powder Wuji Wan are more effective than traditional Wuji Wan in inhibiting the growth of bacteria, *colibacillus* and fungi. Previous studies have shown that fungi and bacillus can excrete xylase and cellulase [**29**-**31**]. The xylase activities of ultra-micro powder Wuji Wan group significantly decreased in stomach, but the cellulose activities only slightly decreased, suggesting there are possibly other unknown reason causing for the decline of enzyme activities. Both traditional Wuji Wan and ultra-micro powder Wuji Wan also could inhibit the growth of intestinal bacteria and *colibacillus*, and lead to the decline of xylase and protease activities. Overall our study suggests ultra-micro powder Wuji Wan are more effective than traditional Wuji Wan in modulating the gut microbial system, and provides insights for understanding the mechanisms of Wuji Wan in treating gastrointestinal diseases.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ZhouJin Tan conceived of the study, and participated in its design and coordination and helped to draft and revise the manuscript. Ao Zeng participated in its design and acquisition of data and helped to draft and revise the manuscript. XinHua Shu participated in its interpretation of data and helped to draft and revise the manuscript. NenQun Xiao made substantial contributions to analysis and interpretation of data. HuaLing Zhang made substantial contributions to interpretation of data. KangXiao Guo participated in the detection of enzyme activies. GuangXian Cai made substantial contributions to conception and design and coordination. All authors read and approved the final manuscript. There won't be any further changes in the authorship which includes either addition or removal of author's details and ZhouJin Tan will be sole responsible person for all the communications and proceedings that are needed to be done with the publisher (According to the necessity of the publisher) on behalf of all the authors.

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