

Effect of Dahuang Danpi Decoction on *Lactobacillus bulgaricus* growth and metabolism

In vitro study

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Abstract

Gut flora plays an essential role in disease and health. A traditional Chinese herb formula, Dahuang Danpi Decoction (DDD) can alleviate several gastrointestinal diseases.

In the present study, we assessed the effect of DDD on the growth and metabolism of *Lactobacillus bulgaricus*. *L. bulgaricus* was cultured in MRS with 40 mg/ml (high), 10 mg/ml (medium), and 2.5 mg/ml (low) of DDD, Ceftriaxone and blank (control). The growth of *L. bulgaricus* was measured by optical density. The levels of L-lactic acid and D-lactic acid were also measured.

Compared to the control group, the concentrations of *L. bulgaricus* in the medium and the high concentrations DDD groups were significantly higher ($P < .001$ for all), while the concentrations of *L. bulgaricus* in the ceftriaxone groups were significantly lower. In the 3 DDD groups, the L-lactic acid levels were significantly higher than those in the control group and the ceftriaxone groups ($P < .001$ for all), and the L-lactic acid level was the highest in the high DDD group. Similarly, the D-lactic acid level in the high concentration DDD group was significantly higher than those in the medium and low concentration DDD groups, the control group and the ceftriaxone groups. Both the L-lactic acid and D-lactic acid levels were lower than those in the control group and the DDD groups.

DDD could dose-dependently promote the growth of *L. bulgaricus* and enhance the secretion of L-lactic acid and D-lactic acid, which suggests DDD may be able to interact with the probiotics, improve the gut microbiota, and serve in the prevention and treatment of dysbiosis.

Abbreviation: DDD = Dahuang Danpi Decoction.

Keywords: bulgarian lactobacillus, Dahuang Danpi Decoction, growth curve, gut microbiota, lactic acid, metabolism

1. Introduction

The bacterial inhabitants of the human gastrointestinal tract constitute an enormously complex ecosystem that includes both aerobic and anaerobic microorganisms.^[1] The relevance and effect of resident bacteria on a host's physiology and pathology

has been well documented.^[2] Major functions of the gut microflora include metabolic activities that result in salvage of energy and absorbable nutrients, trophic effects on intestinal epithelia and immune structure and function, and protection of the colonized host against invasion by alien microbes.^[3] Gut flora may play an essential role in certain pathological disorders, including multisystem organ failure, colon cancer, and even cardiovascular disease.^[4] Studies have shown therapeutic manipulation of the enteric microflora would benefit patients with irritable bowel syndrome, inflammatory bowel disease, and *Clostridium difficile* infection.^[5-7]

Several attempts have been made to target the gut microbiota. Studies have shown that traditional Chinese herb could induce structural changes in gut microbiota, enrich the amounts of beneficial bacteria and benefit treatment of diabetes and prostate cancer.^[8,9] A standardized Chinese herbal formula containing rhubarb and moutan bark, Dahuang Danpi Decoction (DDD), has been used in traditional Chinese medicine for gastrointestinal disease for nearly 2000 years. WF Li et al revealed that DDD could help to lower the level of CRP and the Randson scores in acute severe pancreatitis,^[10,11] and also help to prevent and treat multiple organ dysfunction.^[12] However, the mechanism underlying DDD's impact on these status has barely been elucidated. A recent study revealed that rhubarb extract changed the microbial ecosystem, downregulated key markers of both inflammatory and oxidative stresses in acute-alcohol challenged mice.^[13] This study suggests that the gut microbiota might have a pivotal role in the effect of DDD on gastrointestinal disease.

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The authors declare that they have no competing interests.

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Lactobacillus bulgaricus is one of the dominant probiotic strains in the gastrointestinal tract, which binds closely to the intestinal mucosa to form the biological barrier of the intestine and helps to maintain the gut microflora balance.^[14] In this study, we aim to evaluate the effect of DDD on *L. bulgaricus* growth and lactic acid metabolite levels, to check DDD's impact on the gut microflora, which could be one mechanism for the effect of DDD on gastrointestinal disease.

2. Material and method

2.1. Materials

MRS medium: 63 g of solid MRS (Thermo Fisher Scientific, Cambridge, MA) was dissolved in 1000 ml of distilled water, and sterilized. *L. bulgaricus* lyophilized powder was purchased from Shandong Branch Ke Yi Biological Engineering Co., Ltd (Qingzhou, Shandong, China), and the total number of viable bacteria is about 1.28×10^{10} cfu/g.

2.2. Drugs

DDD preparation: According to Zhang Zhongjing "Golden Chamber", the formula in our study was composed of five herbs, namely: rhubarb 18g, moutan bark 9g, peach seed 12g, waxgourd seeds 30g, and Glauber's salt 9g. Herbs were all provided and quality controlled by Guangzhou Xingyuanchun pharmacy (Guangzhou, Guangdong, China). Each unit of DDD formula yielded 1000 ml of decoction. The decoction was freeze-dried. Lyophilized samples were stored at -20°C . Ceftriaxone sodium was purchased from Wuhan Berkha Bio-medical Co., Ltd (Wuhan, Hubei, China).

2.3. Culture of *L. bulgaricus* and drug administration

We allocate MRS medium into 7 conical flasks. Each conical flask contained 100 ml MRS medium and 1 g *L. bulgaricus* freeze-dried powder (about 1.28×10^{10} cfu/g). The powder was then mixed to make a suspension of bacteria with concentration of about 10^8 cfu/ml. The 7 bottles were labeled and treated with the following drugs: 1 with blank; 2 to 4 with high, medium and low doses of

DDD respectively; 5 to 7 with high, medium and low doses ceftriaxone sodium respectively. The concentration for high, medium and low dose were 40, 10, and 2.5 mg/ml. The fermentation was carried out for 24 hours at 37°C with 5% CO_2 .

2.4. Strain growth stage determination and lactate metabolites measurement

Aliquots (100 μL) were taken every 2 hours for optical density (OD) measurement. Growth curve of *L. bulgaricus* was determined measuring the increase of OD at 600 nm with a UV-1202 UV-VIS spectrophotometer (Thermo Fisher Scientific, Cambridge, MA). The culture medium supernatant was collected at 0, 2, 6, 10, 14, 18, and 22 hours. L and D lactic acid levels were measured by ELISA commercial kit from BIOVISION (E4356, Milpitas, CA). Each measurement was repeated three times and the average was used for later analyses.

2.5. Analysis

Data were analyzed by SPSS 13.0 software (SPSS v13.0) (ISPP Inc., Chicago, IL). Continuous data were presented as mean \pm standard deviation. Shapiro-Wilk test was applied to test the normality of data. Repeated measures ANOVA test was used to analyze the levels of OD, L-lactic acid and D-lactic acid levels at different time points in different groups. Mauchly test was used to test the sphericity, and correction of the degree of freedom was applied if necessary. Multiple comparison between the groups was performed using S-L-D method. All tests were two-tailed, and statistical significance was considered to be $P < .05$.

3. Results

3.1. Effects of Dahuang Danpi Decoction and ceftriaxone on *L. bulgaricus* in vitro growth

The OD values of the *L. bulgaricus* in each MRS medium are shown in Table 1. As presented, the concentration of *Lactobacillus* at different time points and in different groups were significantly different ($P < .001$). No significant difference in the concentrations of *Lactobacillus* was observed in the low DDD

Table 1

Growth curve of *Lactobacillus* in different DDD and Ceftriaxone groups ($\lambda = 600\text{ nm}$).

	Control	High-DDD	Med-DDD	Low-DDD	High-Cef	Med-Cef	Low-Cef
0 h	0.5 \pm 0.04 [†]	0.49 \pm 0.01 ^{*,†}	0.48 \pm 0.03 ^{*,†}	0.51 \pm 0.01 [†]	0.49 \pm 0.03 [*]	0.51 \pm 0.004 [*]	0.54 \pm 0.01 [*]
2 h	0.65 \pm 0.01 [†]	4.02 \pm 0.17 ^{*,†}	2.04 \pm 0.28 ^{*,†}	0.80 \pm 0.03 [†]	0.64 \pm 0.03 [*]	0.73 \pm 0.05 [*]	0.73 \pm 0.03 [*]
4 h	1.01 \pm 0.03 [†]	4.01 \pm 0.33 ^{*,†}	2.06 \pm 0.11 [*]	1.6 \pm 0.1 [†]	0.58 \pm 0.02 [*]	0.63 \pm 0.03 [*]	0.65 \pm 0.01 [*]
6 h	2.64 \pm 0.25 [†]	4.34 \pm 0.29 ^{*,†}	3.45 \pm 0.11 ^{*,†}	2.82 \pm 0.05 [†]	0.59 \pm 0.01 [*]	0.72 \pm 0.04 [*]	0.73 \pm 0.02 [*]
8 h	2.98 \pm 0.21 [†]	4.98 \pm 0.16 ^{*,†}	3.91 \pm 0.59 ^{*,†}	2.97 \pm 0.33 [†]	0.47 \pm 0.05 [*]	0.51 \pm 0.13 [*]	0.57 \pm 0.15 [*]
10 h	3.56 \pm 0.23 [†]	5.64 \pm 0.72 ^{*,†}	4.81 \pm 0.47 ^{*,†}	3.82 \pm 0.3 [†]	0.67 \pm 0.03 [*]	0.65 \pm 0.19 [*]	0.66 \pm 0.1 [*]
12 h	3.62 \pm 0.02 [†]	5.51 \pm 0.6 [*]	4.76 \pm 0.05 ^{*,†}	3.78 \pm 0.07 [†]	0.54 \pm 0.06 [*]	0.52 \pm 0.07 [*]	0.59 \pm 0.07 [*]
14 h	3.93 \pm 0.04 [†]	6.32 \pm 0.81 ^{*,†}	5.18 \pm 0.59 ^{*,†}	3.77 \pm 0.59 [†]	0.58 \pm 0.07 [*]	0.63 \pm 0.04 [*]	0.67 \pm 0.05 [*]
16 h	3.72 \pm 0.13 [†]	6.47 \pm 0.1 ^{*,†}	5.18 \pm 0.25 ^{*,†}	3.7 \pm 0.22 [†]	0.66 \pm 0.03 [*]	0.56 \pm 0.17 [*]	0.59 \pm 0.06 [*]
18 h	3.82 \pm 0.05 [†]	6.67 \pm 0.31 ^{*,†}	5.52 \pm 0.22 ^{*,†}	4.16 \pm 0.31 [†]	0.75 \pm 0.03 [*]	0.74 \pm 0.05 [*]	0.66 \pm 0.02 [*]
20 h	3.7 \pm 0.1 [†]	6.7 \pm 0.19 ^{*,†}	4.94 \pm 0.39 ^{*,†}	3.48 \pm 0.43 [†]	0.63 \pm 0.13 [*]	0.64 \pm 0.08 [*]	0.61 \pm 0.03 [*]
22 h	3.73 \pm 0.21 [†]	6.93 \pm 0.12 ^{*,†}	5.44 \pm 0.12 ^{*,†}	4.02 \pm 0.17 [†]	0.71 \pm 0.06 [*]	0.74 \pm 0.02 [*]	0.68 \pm 0.05 [*]
24 h	3.52 \pm 0.1 [†]	6.15 \pm 0.04 ^{*,†}	4.82 \pm 0.33 ^{*,†}	3.45 \pm 0.25 [†]	0.73 \pm 0.03 [*]	0.7 \pm 0.01 [*]	0.58 \pm 0.03 [*]

Cef = Ceftriaxone sodium, DDD = Dahuang Danpi Decoction.

The concentration for high, medium and low dose were 40 mg/ml, 10 mg/ml, and 2.5 mg/ml.

Continuous variables were presented as Mean \pm standard deviation.

* $P < .001$ for comparisons between each group and control group, and $P < .001$ for comparisons between each group and low DDD group.

† $P < .001$ for comparisons between each group and high concentration Ceftriaxone group, $P < .001$ for comparisons between each group and medium concentration Ceftriaxone group, and $P < .001$ for comparisons between each group and low concentration Ceftriaxone group.

Table 2**Concentration of L-lactic acid in different concentrations of DDD and Ceftriaxone.**

	0 h	2 h	6 h	10 h	14 h	18 h	22 h
Control	147.59±7.79*	146.47±3.8*	202.92±11.6*	240.29±21.3*	273.65±30.3*	272.77±22.2*	204.45±19.1*
High-DDD	143.90±16.8*	224.81±16.*	273.49±28.6*	282.87±14.1*	332.67±18.9*	477.08±24.7*	526.08±36.4*
Med-DDD	176.86±7.73*	211.74±3.0*	223.85±19.6*	257.61±4.23*	290.33±20.3*	379.58±50.2*	380.14±42.8*
Low-DDD	149.6±6.28*	150.8±2.91*	205.89±5.2*	224.09±7.46*	276.45±12.8*	283.27±17.4*	266.59±7.69*
High-Cef	138.05±12.90	133.48±6.14	141.58±23.95	138.13±14.04	146.31±14.12	138.21±9.47	138.85±22.39
Med-Cef	130.91±6.96	148.88±5.72	136.85±6.15	140.14±8.07	175.18±41.52	143.1±11.04	140.14±6.31
Low-Cef	142.7±7.55*	147.19±2.1*	156.82±10.5*	146.39±9.57*	161.87±13.6*	154.81±5.3*	163.87±11.3*

Cef=Ceftriaxone sodium, DDD=Dahuang Danpi Decoction.

The concentration for high, medium and low dose were 40 mg/ml, 10 mg/ml, and 2.5 mg/ml.

Continuous variables were presented as Mean±standard deviation.

* $P=.000$ for comparisons between each group and high concentration Ceftriaxone group, and $P=.000$ for comparisons between each group and medium concentration of Ceftriaxone group.

group and the control group. The *Lactobacillus* concentration in the high DDD group and the medium DDD group was significantly higher than those in the control group and those in the three ceftriaxone groups ($P<.001$ for all). The concentration in the high DDD group was significantly higher than that in the medium DDD group ($P<.001$). DDD shortened the lag phase of *L. bulgaricus* (within 2 hours), prolonged the logarithmic phase (2–14 hours) and kept the stable period of high level reproduction (14–22 hours), especially in the 40 mg/ml DDD group. The *Lactobacillus* concentration in the ceftriaxone groups was significantly lower than that in the control group ($P<.001$ for all). In the 3 ceftriaxone groups, no significant difference in *Lactobacillus* concentration was detected ($P=.84$).

3.2. Effects of Dahuang Danpi Decoction and ceftriaxone on L-lactic acid levels

The L-lactic acid concentrations in different groups are shown in Table 2. As shown, the concentrations of L-lactic acid varied significantly at different time points and in different groups ($P<.001$). We noted no significant changes in the L-lactic acid concentrations in the three ceftriaxone groups over time, and no significant difference was detected in the high and the medium ceftriaxone group ($P=.15$). Compared to the control group, the concentrations of L-lactic acid were significantly higher in the three DDD groups, while significantly lower in the three ceftriaxone groups ($P<.001$ for all). The concentrations of L-lactic acid were highest in the high DDD group, followed by the medium DDD group and the low DDD group ($P<.001$ for all).

3.3. Effects of Dahuang Danpi Decoction and ceftriaxone on D-lactic acid levels

The D-lactic acid concentrations in different group are shown in Table 3.

The concentrations of D-lactic acid at different time points and in different groups were significantly different ($P<.001$). There was no significant difference of D-lactic acid levels between the control group and the medium DDD group ($P=.214$) and between the control group and the low DDD group ($P=.13$). The D-lactic acid levels in the high DDD group were significantly higher than those in the control group, the low and medium DDD group, respectively ($P<.001$ for all). In the three ceftriaxone groups, the levels of D-lactic acid were significantly lower than those in the control group and all three DDD groups ($P<.001$ for all). Compared to the low ceftriaxone group, the D-lactic acid levels in the medium and the high ceftriaxone group were significantly lower ($P<.001$ for both), while no significant differences were detected between the medium and the high ceftriaxone group ($P=.14$).

4. Discussion

In this pilot study, we found that Dahuang Danpi Decoction could dose-dependently promote the growth of *L. bulgaricus* and enhance the secretion of L-lactic acid and D-lactic acid, which are the metabolites of *L. bulgaricus*. Ceftriaxone could inhibit the growth and the metabolite secretion of *L. bulgaricus*.

The human gut is a natural reservoir for numerous species of microorganisms. A mutualistic relationship between beneficial

Table 3**Concentration of D-lactic acid in different concentrations of DDD and Ceftriaxone.**

	0 h	2 h	6 h	10 h	14 h	18 h	22 h
Control	11.57±0.08†	18.15±0.45†	28.71±0.11†	40.34±0.48†	37.74±0.34†	39.19±1.46†	37.46±3.52†
High-DDD	9.75±0.1*,†	14.61±0.08*	11.98±2.13*	31.24±0.19*	42.89±3.11*	40.52±0.81*	38.22±2.08*
Med-DDD	11.63±0.19†	20.35±0.23†	18.31±3.94†	40.15±1.78†	39.52±0.54†	39.09±0.15†	39.88±0.76†
Low-DDD	12.33±0.39†	20.46±0.66†	25.2±2.8†	39.2±2.68†	38.39±1.31†	39.38±0.55†	39.23±0.29†
High-Cef	12.65±0.21*	11.28±0.38*	10.51±2.59*	14.12±0.44*	13.88±0.14*	15.61±1.21*	16.47±0.39*
Med-Cef	11.73±0.22*	11.46±1.07*	8.43±1.21*	16.61±0.45*	16.15±0.22*	18.18±0.91*	17.01±0.12*
Low-Cef	13.18±0.05*,†	14.88±1.3*,†	11.04±5.1*,†	20.34±0.86*,†	19.69±0.15*,†	26.07±0.5*,†	25.72±0.43*,†

The concentration for high, medium and low dose were 40 mg/ml, 10 mg/ml, and 2.5 mg/ml.

Continuous variables were presented as Mean±standard deviation.

Cef=Ceftriaxone sodium, DDD=Dahuang Danpi Decoction.

* $P<.001$ for comparisons between each group and medium concentration DDD group, $P<.001$ for comparisons between each group and low concentration DDD group, and $P<.001$ for comparisons between each group and control group.

† $P<.001$ for comparisons between each group and high concentration Ceftriaxone group, and $P<.001$ for comparisons between each group and medium concentration Ceftriaxone group.

symbionts and commensals is important for the maintenance of health and wellbeing. Alterations in this balance can lead to dysbiosis and ultimately may result in clinical disease expression.^[15] Various disease states and treatments, especially antibiotics have profound influences on the presence and levels of various bacteria normally present in the human microbiome.^[16–19] In coeliac disease patients, level of IgA-coated bacteria is reduced and is associated with intestinal dysbiosis.^[16] The proportions of phylum Firmicutes and Clostridia were significantly reduced in the diabetic patients.^[17] Probiotics have been increasingly used in both prevention and treatment of a variety of diseases, including but not limited to inflammatory bowel disease, *C difficile* infection, and antibiotic-associated diarrhea.^[20] *L. bulgaricus* and other lactic-acid-producing lactobacilli are the main microorganisms classified as probiotic agents,^[20] and lactic acid is one of the underlying mechanism.^[21] By producing a high amount of lactic acid, lactic-acid-producing lactobacilli are able to inhibit the growth of other pathogenic microorganisms.^[21] In our work, DDD dose-dependently promoted the growth of *L. bulgaricus* in different periods and significantly enhanced the secretion of both L-lactic acid and D-lactic acid, which suggests DDD could exert a dose-dependent modulation on the gut microbiota. The effect of modulation of gut microbiota can be one of the mechanisms of DDD treatment effect of gastrointestinal disease.

Our findings, as well as prior findings, all suggest a modulation effect of rhubarb on gut microbiota.^[13,22] AM Neyrinck et al revealed that rhubarb extract could change the microbial ecosystem, and downregulated key markers of both inflammatory and oxidative stresses in rats.^[13] Peng Ying et al^[22] found that in the ileum of rhubarb-exposed rats, more bacterial diversity was observed. DDD formula, a form of polypharmacy, has been used in the treatment of gastrointestinal disease for over 2000 years in China. However, the underlying mechanism has barely been elucidated. Together with previous studies, our work suggested that gut microbiota might be involved. Moreover, we revealed that lactic-acid-producing lactobacilli in particular might be involved.

Our study has several limitations. One important limitation is that our study represents an in vitro study, and our results cannot be directly applied to humans. However, our study could be a useful preliminary study to investigate the effect of DDD on gut microbiota. Second, we only checked the effect of DDD on *L. bulgaricus*, while the effect of DDD on other microorganism remained unclear and the effect of DDD may be affected by other microorganisms. Third, our work is not able to further identify the possible effective ingredients of DDD, which could lead to a more specific treatment plan. Fourth, only 1 culture was done in each group. However, the *L. bulgaricus* culture and measurement in our laboratory were quality controlled. Further studies involving other main microorganisms, in vivo studies and exploration of possible effective gradient such as paeonol are required.

Although generally considered safe, some studies have highlighted that probiotics may be ill advised in specific patient populations. Bacteremia,^[23] sepsis^[24] and meningitis^[25] have been described on rare occasions in children treated with probiotics. Our preliminary work suggested DDD could be a new way for prevention and treatment of dysbiosis, and may be able to further regulate the immune and inflammatory response and other disease which are associated with gut microbiota.^[26]

In conclusion, our study suggests that Dahuang Danpi Decoction, the Chinese herbal formula could promote the

growth of *L. bulgaricus* and enhance metabolite secretion. This treatment revealed a beneficial effect on the import group of probiotics in the gut, which could be 1 mechanism of DDD treatment effect of gastrointestinal disease. Although further studies are in need to determine the in vivo effect, our work provides preliminary evidence that DDD might be able to interact with probiotics, and improve the gut microbiota, and DDD could be a new therapy for prevention and treatment of dysbiosis.

Author contributions

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