Effects of *Lactobacillus* on the inhibition of *Helicobacter pylori* growth

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**ABSTRACT**

This study was performed to screen *Lactobacillus* and evaluate its inhibiting effects on *Helicobacter pylori* (*Hp*). *Lactobacillus* was isolated from fermented food in northeastern China. After identification and physiological characterization, the identified *Lactobacillus* was used to interact with *Hp*. The aggregation ability of *Lactobacillus* and *Hp* was determined. Moreover, the inhibiting effects of *Lactobacillus* on *Hp* urease enzyme activity and antioxidant ability were assessed. The results showed that four *Lactobacillus* strains including *Lactobacillus sake*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus* and *Lactobacillus brevis* were isolated from fermented food in northeastern China. All four strains of *Lactobacillus* could inhibit *Hp* growth in different extent. The interaction with *Hp* was statistically analyzed with significant differences. The *Lactobacillus* isolated from fermented food in Northeast China, had an inhibitory effect on *Hp* growth. This study provided a foundation for development of probiotic preparations and exploring the inhibitory effect of *Lactobacillus* on *Hp*.

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**Introduction**

*Helicobacter pylori* (*Hp*) is a micro-aerobic, helical gram-negative bacillus that can colonize gastric mucosa. It is known as the only microorganism surviving in human stomach with an infection rate of up to 50% worldwide. In 1994, the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) listed *Hp* as a first class carcinogen, which has been identified to be associated with the development of some diseases such as chronic gastritis, peptic ulcer, gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma [1]. Therefore, eradication of *Hp* has become an important means for treatment and prevention of these diseases. With the development of microecology, the antagonistic effect of probiotics on *Hp* in vivo and in vitro has been investigated [2–5]. And a previous study has shown that addition of probiotics containing *Lactobacillus acidophilus* to standard triple therapy increased the eradication success rate [6]. In this study, we aimed to isolate new *lactobacilli* from fermented food in Northeast China and investigate their possible role in the inhibition of *Hp* growth.

**Materials and Methods**

**Bacterial isolates and materials**

The brine of Northeast pickle and spicy cabbage, yoghurt, MRS liquid and solid medium, calcium carbonate, and HBI *lactobacillus* biochemistry measuring band (GB) were purchased from Qingdao Haibo Company. Genomic DNA Extraction Kit was supplied by Tian’en Co., Ltd. DL2,000 DNA Marker and Premix Taq, pepsin, xylene, *Hp* standard strain (ATCC 43504), Colombian medium, and brain infusion were bought from Qingdao Haibo. Sterile sheep blood, fetal bovine serum (FBS) and diphenylpicrylhydrazyl (DPPH) were provided from Shanghai Jinsui Biotechnology Co., Ltd.

**Instruments and equipment**

Incubator GHP-9095 was purchased from Shanghai Heng Technology Co., Ltd. The biological safety cabinet BSC-1600IIIB2 was bought from Shanghai Su net Industrial Co., Ltd. Automatic autoclave pot LDZX-50KBS was provided from Shanghai Shen’an medical equipment factory. Magnetic microscope BA310-T was obtained from Mike Audi Chemical Industry Group Co., Ltd.
Lactobacillus and Hp were cultured and centrifuged at 2000 r/min. They were washed three times with PBS and adjusted to OD$_{600}$ = 0.40 ± 0.05 with the concentration of $10^7$–$10^8$ colony forming units (CFU)/mL. The absorbance value of Lactobacillus and Hp supernatant was measured, respectively, as $A_x$ and $A_y$. Then equal counts of Lactobacillus and Hp were mixed. The mixture was shaken for 5 min, and then cultured in a 37°C incubator. The OD$_{600}$ absorbance value of the upper liquid at different time points was determined and recorded as $A_{mix}$. Interactive Agglomeration Force (%) = \( \frac{A_x + A_y - A_{mix}}{A_x + A_y} \times 100\% \), where $A_x$ is the 0 h Lactobacillus absorbance value, $A_y$ is 0 h Hp absorbance value, $A_{mix}$ was the Lactobacillus and Hp mixed absorbance value [12].

**Oxford Cup antibacterial test**

Colombian medium blood plate and Hp bacteria solution were prepared using the previous method [13]. Then diluted Hp bacteria solution ($1 \times 10^8$ CFU/mL) was evenly coated on the fresh antibiotic-free Colombian culture plate three times. Four sterilized
Oxford Cups were put into the blood plate and each Oxford Cup was added 50 l L lactic acid bacteria liquid culture, 50 l L Lactobacillus supernatant, levofloxacin drug sensitive paper and amoxicillin drug sensitive paper. The plate was placed in an anaerobic culture bag at 37°C for 72 h, and then the size of the inhibition zone was measured.

**Urease activity of Hp by Lactobacillus**

After incubation of Hp with brain–heart leaching broth culture medium, Hp bacteria were washed with PBS buffer twice to adjust the concentration to 1 x 10^8 CFU/mL. Then three groups were added in a 96-well plate: 40 lL Hp bacteria and 10 lL MRS liquid medium; 40 lL Hp bacteria and 10 lL lactic acid bacteria cell-free supernatant; and 40 lL Hp bacteria and 10 lL Lactobacillus bacteria. The 96-well plates were added to the anaerobic culture bag and incubated at 37°C for 48 h. After that, 150 lL urease indicator was added and the absorbance at 540 nm was measured.

**Antioxidant capability of Lactobacillus**

Measurement of antioxidant capability was assigned to three groups: sample group: 1 mL bacterial solution and 1 mL DPPH ethanol solution (0.2 mmol/L); control group: 1 mL MRS liquid medium and 1 mL DPPH ethanol solution; blank group: 1 mL MRS liquid medium and 1 mL absolute ethanol. Then the mixtures were placed in darkness at room temperature for 30 min. The supernatant was centrifuged at 6000 r/min for 10 min and the absorbance at 517 nm was measured. The following formula was used: Scavenging rate (\%) = \left(1 - \frac{A_3}{A_2}\right) \times 100\% , where A_1 is the absorbance value of the sample group, A_2 is the absorbance value of the control group, and A_3 is the absorbance value of the blank control.

**Statistical analysis**

The data analysis was performed using SAS9.4 statistical analysis software (SAS Institute Inc., Cary, NC, USA). The tolerance of Lactobacillus to artificial gastric juice was analyzed using t test. Rank sum test was used for analyses of lactobacillus hydrophobicity and self-aggregation ability. The two pair comparison was performed by Bonferroni method. Determination of the aggregation ability of Lactobacillus and Hp was carried out by repeated measures analysis of variance. Urease activity and Lactobacillus scavenging DPPH used rank sum test.

**Results and discussion**

**Isolation of Lactobacillus**

Lactobacillus colonies were round uplift, smooth, moist, easy to pick, and neat edge colonies. They were milky white, opaque and shiny, with a diameter of 1–3 mm (Figure 1A–D). The colloidal bacteria were picked and Gram stained. Gram-positive bacilli were
arranged in a single, double or short chain shape. The screening four *Lactobacillus* stained by Gram is shown in Figure 1E–H. The results from the biochemical assays of the four screened strains for fermentation of aescinate, cellobiose, maltose, mannitol, salicylline, sorbitol, sucrose, raffinose, inulin and lactose are shown in Table 1.

**Table 1. Biochemical assays of screened *Lactobacillus* isolates.**

| No. | Aescinate | Cellobiose | Maltose | Mannitol | Salicylline | Sorbitol | Sucrose | Raffinose | Inulin | Lactose |
|-----|-----------|------------|---------|----------|-------------|----------|---------|-----------|--------|---------|
| 1   | –         | +          | –       | –        | –           | –        | +       | –         | +      | +       |
| 2   | +         | +          | +       | +        | +           | +        | +       | +         | +      | +       |
| 3   | +         | +          | +       | +        | +           | +        | +       | +         | +      | +       |
| 4   | –         | –          | –       | –        | –           | –        | –       | –         | –      | –       |

+, positive; −, negative.

Identification of 16SrDNA

In order to identify the species to which the isolates belong, genomic DNA of the *Lactobacillus* isolates was extracted. The molecular weight of the amplified bands of 16S rDNA was 1515 bp. Based on the sequencing data, the four strains of *Lactobacillus* were identified as: *Lactobacillus sakei* (No. 1; Figure 1A,E), *Lactobacillus plantarum* (No. 2; Figure 1B,F), *Lactobaellus rhamnosus* (No. 3; Figure 1C,G) and *Lactobacillus brevis* (No. 4; Figure 1D,H).

**Table 2. Tolerance of the four isolates to artificial gastric juice.**

| Isolate                  | Saline group | Gastric juice group | t       | P       |
|-------------------------|--------------|---------------------|---------|---------|
| *Lactobacillus sakei*   | 91.17 ± 5.19 | 89.67 ± 3.88        | 0.567   | >0.05   |
| *Lactobacillus plantarum* | 86.00 ± 3.80 | 85.83 ± 4.26        | 0.072   | >0.05   |
| *Lactobaeillus rhamnosus* | 87.83 ± 4.31 | 89.50 ± 2.67        | –0.806  | >0.05   |
| *Lactobacillus brevis*  | 87.83 ± 4.26 | 88.50 ± 2.59        | –0.327  | >0.05   |

Mean values with standard deviation the means (X ± S, n = 6).

**Table 3. Measurements of the hydrophobicity (M ± Q) of the four *Lactobacillus* isolates.**

| Isolate                  | Hydrophobic Value | P         |
|-------------------------|-------------------|-----------|
| *Lactobacillus sakei*   | 0.131 ± 0.009     | <0.0001   |
| *Lactobacillus plantarum* | –0.144 ± 0.097   |           |
| *Lactobaeillus rhamnosus* | 0.234 ± 0.020     |           |
| *Lactobacillus brevis*  | 0.056 ± 0.025     |           |

Hydrophobicity of *Lactobacillus*

To explore the physiological characteristics of *lactobacillus*, the hydrophobicity of the *Lactobacillus* isolates was detected. The results showed that the hydrophobicity in the four strains of *lactobacilli* was statistically significant (P < 0.05). Among the four strains of *lactobacilli*, *L. rhamnosus* (0.234 ± 0.020) had the highest hydrophobicity, followed by *L. sakei* (0.131 ± 0.009), *L. brevis* (0.056 ± 0.025) and *L. plantarum* (−0.144 ± 0.097) (all P < 0.01; Table 3).

Self-aggregation ability of *Lactobacillus*

In order to unravel the potential probiotic properties of the identified bacteria, the self-aggregation ability of the *Lactobacillus* isolates at 37 °C was determined. The results showed that the aggregation abilities of the four strains of *lactobacilli* increased in a time-dependent manner and the aggregation ability was highest at 24 h (Table 4). At one time point, such as 24 h, *L. brevis* (0.494 ± 0.014) had the highest self-aggregation ability, followed by *L. rhamnosus* (0.477 ± 0.011), *L. plantarum* (0.452 ± 0.012) and *L. sake* (0.420 ± 0.00). The statistical analysis showed that the differences were statistically significant (all P < 0.05).

**Table 4. Measurements of the self-aggregation ability (M ± Q) of the four *Lactobacillus* isolates.**

| Isolate                | Self-aggregation ability | P       |
|------------------------|--------------------------|---------|
| *Lactobacillus sakei*  | 0.131 ± 0.009            | <0.0001 |
| *Lactobacillus plantarum* | –0.144 ± 0.097          |         |
| *Lactobaeillus rhamnosus* | 0.234 ± 0.020          |         |
| *Lactobacillus brevis* | 0.056 ± 0.025            |         |

Aggregation of *Lactobacillus and Hp*

To study the interaction of *Lactobacillus* and *Hp*, the aggregation ability was evaluated at 37 °C. The four strains of *lactobacilli* and *Hp* showed strong aggregation ability, and the aggregation ability was also elevated with the advancement of the incubation time. The aggregation of different *lactobacilli* was different.
Table 4. Self-aggregation ability (M ± Q) of the four Lactobacillus isolates at different points of times.

| Time (h) | L. sakei | L. plantarum | L. rhamnosus | L. brevis | Value | $P$  |
|---------|----------|--------------|--------------|-----------|-------|------|
| 2       | 0.365 ± 0.008 | 0.382 ± 0.019 | 0.410 ± 0.019 | 0.439 ± 0.011 | 53.15 | <0.0001 |
| 4       | 0.394 ± 0.02 | 0.414 ± 0.017 | 0.451 ± 0.015 | 0.475 ± 0.006 | 52.41 | <0.0001 |
| 16      | 0.350 ± 0.02 | 0.412 ± 0.023 | 0.432 ± 0.008 | 0.474 ± 0.015 | 53.61 | <0.0001 |
| 24      | 0.420 ± 0.00 | 0.452 ± 0.012 | 0.477 ± 0.011 | 0.494 ± 0.014 | 53.53 | <0.0001 |

Table 5. Anti-Hb activity. Inhibition zone (diameter, mm).

| Isolate                | Levofloxacin | Amoxicillin | Supernatant | Bacterial culture |
|------------------------|--------------|-------------|-------------|-------------------|
| Lactobacillus sakei    | 0            | 58          | 12          | 11                |
| Lactobacillus plantarum | 0            | 58         | 15          | 18                |
| Lactobacillus rhamnosus | 0            | 58          | 12          | 13                |
| Lactobacillus brevis   | 0            | 58          | 15          | 13                |

Table 6. Ability of the four Lactobacillus isolates to inhibit Helicobacter pylori urease activity (M ± Q).

| Time | Hp+MRS | Hp+Supernatant | Hp+ bacterial culture | $\chi^2$ value | $P$  |
|------|--------|---------------|-----------------------|----------------|------|
| L. sakei | 0.0740 ± 0.010 | 0.1600 ± 0.009$^a$ | 0.1540 ± 0.018$^a$ | 11.535 | <0.05 |
| L. plantarum | 0.0750 ± 0.009 | 0.0580 ± 0.002$^a$ | 0.0540 ± 0.006$^a$ | 15.494 | <0.05 |
| L. rhamnosus | 0.0740 ± 0.002 | 0.0720 ± 0.001 | 0.0750 ± 0.012$^a$ | 15.205 | <0.05 |
| L. brevis | 0.2005 ± 0.019 | 0.0910 ± 0.010$^a$ | 0.1050 ± 0.0170$^a$ | 13.528 | <0.05 |

$^a$ $P < 0.05$ compared with Hp + MRS group; $^P$ $P < 0.05$ compared with Hp + Supernatant.

Compared with the Bonferroni method, L. gasseri was used to compare the aggregation ability of four lactobacilli and Hp at different time points. The aggregation rate of L. brevis was higher than that of the other three lactobacilli ($P < 0.05$). The duration of the action could affect the aggregation of Lactobacillus, additionally, the aggregation of different lactobacilli varied with time.

Oxford Cup antibacterial test

In order to investigate the susceptibility of the identified bacteria against antibacterial agents, the Oxford Cup bacteriostatic test was performed. Levofloxacin was used as a negative control and amoxicillin was used as a positive control. The results in Table 5 showed that the four strains of Lactobacillus and their supernatant had inhibitory effects on Hp growth. The results showed that L. plantarum and L. sakei had the highest and the lowest inhibition, zone respectively, which indicated that L. plantarum had the strongest effect on Hp, and L. sakei had the weakest effect.

Urease activity of Hp by Lactobacillus

Hp urease activity was determined to further explore the effect of Lactobacillus on Hp. Ammonia can raise the pH value and then the colour of mixed bacterial liquid culture hence changes from yellow to red. The bacterial biomass and supernatant of L. sakei and L. plantarum could inhibit the activity of Hp urease, and the difference was statistically significant ($P < 0.05$; Table 6). Additionally, L. brevis and L. rhamnosus were more effective than the supernatant in the inhibition of Hp urease activity ($P < 0.05$).

Antioxidant capacity of Lactobacillus

The stronger the antioxidant ability of Lactobacillus, the longer it will survive in the environment of reactive oxygen free radicals, and consequently will be able to exert better probiotic functions. To understand the probiotic function of the Lactobacillus isolates, we assayed their antioxidant capacity. The results showed that the scavenging rate of DPPH by L. sakei (79.22 ± 11.69)% was highest compared with that of L. rhamnosus (73.28 ± 11.47)% and L. plantarum (69.87 ± 12.90)% and L. brevis (62.34 ± 11.69)% ($P < 0.05$; Table 7). According to the results, we concluded that the antioxidant ability of L. sakei was the strongest and that of L. brevis was the weakest.

Potential application

With the increase in drug resistance, long-term indiscriminate application of antibiotics can cause gastrointestinal disorders, imbalance of the gastrointestinal flora and other adverse reactions [14]. Micro-ecological therapy is a new point of view to solve a variety of problems in the traditional therapy [14]. Probiotics not only have anti-infective function, but also can regulate the immune function, balance the normal gastrointestinal tract flora, and reduce the side effects of antibiotics [15]. Therefore, the application of probiotics has...
important significance in the prevention of Hp related diseases. In this study, four Lactobacillus isolates from fermented food in Northeast China that were identified as L. sakei, L. plantarum, L. rhamnosus and L. brevis based on biochemical assays and 16SrDNA sequencing [16–18] were shown as potentially beneficial for the human body. The acidic environment in the stomach (pH 1.5–3.0) can kill bacteria unless they are sufficiently acid resistant to retain their viable state [19].

Currently, the ability to tolerate real gastric juice is commonly assessed by measuring the growth of Lactobacillus in simulated artificial gastric juice environment, which provides evidence of whether a studied Lactobacillus strain can play a health-promoting and probiotic role in the gastrointestinal tract. The four strains of Lactobacillus studied by us showed resistance to pH 3.0 artificial gastric juice, indicating that they have the potential to maintain their viable state in acidic environment.

Among the four strains, L. rhamnosus had the highest hydrophobicity, whereas L. plantarum had the weakest hydrophobicity. The results were in contrast to the study performed by Agaliya and Jeevaratnam [20], who reported that the hydrophobicity of L. plantarum was higher than that of L. rhamnosus isolated from fermented idli batter. The difference between the two studies may be due to the fact that the strains were isolated from different sources. Strong hydrophobic lactobacilli strains might be due to the presence of protein-like substances on their surface, and hence they had better self-protection. Hydrophobicity is a non-specific interaction between bacteria and gastric epithelial cells. Therefore, the stronger the hydrophobicity of the bacteria, the stronger the effect on the mucosal cells [20].

It is documented that adhesion in the gastrointestinal tract is more solid and the beneficial effect they play is more potent when Lactobacillus strains show some aggregation. The self-aggregation ability of a strain is positively correlated with its adhesion in the intestinal tract. The self-aggregation of probiotic strains might be one of the reasons for maintaining the vitality of the strain and inhibiting the invasion of the upper gastrointestinal resistant pathogens. In addition, self-aggregation can cause intestinal stomata to form. In this study, we found that the self-aggregation ability of Lactobacillus from strong to weak was L. brevis, L. rhamnosus, L. plantarum and L. sake. Interactive aggregation plays an important role in removing pathogens from the intestine. The self-aggregation ability of Lactobacillus can block the adhesion of pathogens and colonization of the intestine. The interaction of Lactobacillus with pathogens can make them more easily excreted from the gut [21]. Therefore, the aggregation of probiotics and intestinal pathogens is also a very important evaluation index. We found that the duration of action can affect the aggregation of Lactobacillus, and the aggregation of different lactobacilli varies with time. Additionally, the aggregation rate of L. brevis was higher than that of the other three lactobacilli, which indicated that L. brevis may have an important role in removing pathogens.

Hp is able to survive in the low acidity of the stomach as it can produce urease. Urea can decompose urea in the body to NH₃ and CO₂, and then the pH in the environment around Hp can rise, which may form a micro-environment where Hp can survive and proliferate [22]. Therefore, if probiotics can inhibit Hp urease activity, this will case Hp to lose its ability to create a favourable environment for its survival of; thereby the colonization and growth of Hp in the stomach will be inhibited. The four strains of Lactobacillus assayed in this study could inhibit Hp urease activity. And L. brevis and L. rhamnosus were more effective than the supernatant in the inhibition of Hp urease activity.

Oxidation is a necessary process for most living organisms, but excessive oxidation can cause damage to biological macromolecules. In the environment of reactive oxygen species, the survival time of Lactobacillus with antioxidant activity is longer than without antioxidant activity [23]. Most lactobacilli remove hydroxyl free radicals and hydrogen peroxide by producing antioxidant enzymes and glutathione [23, 24]. The results from the DPPH-scavenging assay showed that L. sake can scavenge the DPPH most effectively of all four strains.

We also found that all four strains of Lactobacillus could inhibit Hp growth to a different extent. The acid resistance, the interaction with Hp and its antioxidant ability were the strongest for L. sake. L. plantarum had the strongest inhibitory effect on Hp and Hp urease activity by Oxford Cup antibacterial test. The hydrophobicity of the L. rhamnosus was relatively the strongest and the self-aggregation of L. brevis was the

Table 7. DPPH scavenging rate (M ± Q) of the four Lactobacillus isolates.

| Time                  | Scavenging rate (%) | χ² value | P       |
|-----------------------|---------------------|----------|---------|
| Lactobacillus sakei   | 79.22 ± 11.69       | 36.85    | <0.001  |
| Lactobacillus plantarum | 69.87 ± 12.90   |          |         |
| Lactobacillus rhamnosus | 73.28 ± 11.47# |          |         |
| Lactobacillus brevis  | 62.34 ± 11.69#      |          |         |

* Compared with L. sakei; #, compared with L. planta; ▲, compared with L. rhamnosus.
strongest. Taken together, the obtained data suggest that the four strains isolated from fermented food in Northeast China have potential as probiotics in the food industry.

Conclusions
In this study, four Lactobacillus strains identified as Lactobacillus sake, Lactobacillus plantarum, Lactobacillus rhamnosus and Lactobacillus brevis were isolated from fermented food in northeastern China. We found that the self-aggregation ability of Lactobacillus from strong to weak was L. brevis, L. rhamnosus, L. plantarum and L. sake. All these four strains of Lactobacillus could inhibit Hp growth to a different extent. Thus, the Lactobacillus isolated from fermented food in Northeast China has an inhibitory effect on Hp growth and may have the potential to be used as probiotic.

Disclosure statement
All authors declare no financial competing interests and no non-financial competing interests.

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