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Antagonistics against pathogenic *Bacillus cereus* in milk fermentation by *Lactobacillus plantarum* ZDY2013 and its anti-adhesion effect on Caco-2 cells against pathogens

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ABSTRACT

Lactobacillus plantarum ZDY2013 is a potential probiotic isolated from fermented bean acid. In this study, we aimed to evaluate the in vitro antimicrobial activity of this organism against *Bacillus cereus* in milk fermentation, the antiadhesion ability on intestinal epithelial cells, as well as its ability to abrogate the cytotoxic effect and expression levels of genes. We found no antimicrobial activity produced by *L. plantarum* once the pH was adjusted to 6.0 and 7.0. The pH decreased continuously when *L. plantarum* and *B. cereus* were co-incubated during milk fermentation, which caused a decrease in the *B. cereus* counts. Antiadhesion assays showed that *L. plantarum* can significantly inhibit the adhesion of enterotoxin-producing *B. cereus* ATCC14579 and pathogenic *B. cereus* HN001 by inhibition, competition, and displacement. The supernatants of *B. cereus*, either alone or in conjunction with *L. plantarum*, caused damage to the membrane integrity of Caco-2 cells to release lactate dehydrogenase. In addition, *L. plantarum* tended to attenuate proinflammatory cytokine and oxidative stress gene expression on Caco-2 cells, inducing with *B. cereus* HN001 supernatants. This study provided systematic insights into the antagonistic effect of *L. plantarum* ZDY2013, and the information may be helpful to explore potential control measures for preventing food poisoning by lactic acid bacteria.
Key words: *Lactobacillus plantarum* ZDY2013, *Bacillus cereus*, milk fermentation, antiadhesion, cytotoxic effect

INTRODUCTION

Probiotic bacteria are incorporated into foods and beverages and sold as nutritional supplements for improving human health. They are predominantly represented by the genera of *Lactobacillus* and *Bifidobacterium* (Marco and Tachon, 2013; García-Ruiz et al., 2014). The beneficial effects are mainly related to the maintenance of a healthy gut microbiota of the host, reduction in serum cholesterol levels, regulation of immune homeostasis, and prevention of diarrhea (Chouraqui et al., 2004; Leahy et al., 2005; Derrien and van Hylckama Vlieg, 2015).

Some lactic acid bacteria have been used widely as probiotics in dairy products, but it is still necessary to isolate more functional probiotic bacteria. To be a potential candidate of probiotics, the microorganism should resist extreme acid and bile salt in the gastrointestinal tract (Derrien and van Hylckama Vlieg, 2015), adhere to the intestinal mucosa (as adhesion is an important characteristic of their beneficial function; Collado et al., 2006, 2007), and produce antimicrobial substances in vitro and in vivo.

Recently, *Lactobacillus plantarum* has been isolated from many fermented foods. Generally, most of them are evaluated for their tolerance to extreme acid and bile salt environment, antioxidant activity (Li et al., 2012; Kuda et al., 2015), antibacterial activity (da Silva Sabo et al., 2014), lactic acid production (Zhang and Vadlani, 2015), cell envelope damage (Bravo-Ferrada et al., 2015), and for stimulating the immunomodulation (Zago et al., 2011; Górska et al., 2014). In our previous work, we isolated *L. plantarum* ZDY2013 from traditional acid bean fermented under natural conditions (Huang et al., 2015). This strain was able to tolerate an extreme pH of 2.0 for more than 6 h and remain alive under 0.45% bile salt for 3 h. In addition, oral administration of *L. plantarum* ZDY2013 could alter the composition of the microbiota in the intestine of mice and enhance the populations of *Lactobacillus* and *Bifidobacterium* group in either colon or cecum. However, the antagonistic activity of *L. plantarum* in fermented food or cell lines has not been assessed.

Bacillus cereus is a well-known food-borne pathogen that is ubiquitously distributed in nature and easily contaminates many kinds of foods, especially those of plant origin, as well as milk and dairy products (Hwang

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and Park, 2015; Zhang et al., 2016). The presence of *B. cereus* in processed dairy foods is responsible for a variety of food-borne infections and intoxications for its enterotoxin or vomitoxin production (Røssland et al., 2005). In the current study, we systematically studied the antagonistic effect of *L. plantarum* ZDY2013 on enterotoxin-producing *B. cereus* ATCC14579 and pathogenic *B. cereus* HN001 in vitro and during milk fermentation, as well as the antiadhesion of *B. cereus* on Caco-2 intestinal epithelial cells to determine whether the *B. cereus* can be inhibited by *L. plantarum* ZDY2013. Additionally, the ability of *L. plantarum* to abrogate the cytotoxic effect, and the mRNA expression levels in intestinal cells triggered by cell-free supernatants of *B. cereus* was also included.

MATERIALS AND METHODS

Bacterial Strains and Growth Condition

Acid-resistant *L. plantarum* ZDY2013 was isolated from fermented bean acid and cultured anaerobically in de Man, Rogosa, Sharpe broth (**MRS**; Beijing Solarbio Science and Technology Co. Ltd., Beijing, China) at 37°C for 24 h. *Bacillus cereus* ATCC14579, obtained from the American Type Culture Collection (http://www.atcc.org/en.aspx), is an enterotoxin-positive (hemolysin BL, nonhemolytic enterotoxin and cytotoxin K) type strain (Ngamwongsatit et al., 2008). *Bacillus cereus* HN001 was isolated from vomitus of a patient involved in a food poisoning incidence in Henan province (China) and stored in Academy of Military Medical Science (Beijing, China). All the *B. cereus* strains were cultured in Luria-Bertani (**LB**) medium at 37°C.

Inhibiting the Growth of B. cereus by L. plantarum

Type strain *B. cereus* ATCC14579 and pathogenic *B. cereus* HN001 were used for measuring antibacterial activities of *L. plantarum*. *Lactobacillus plantarum* was activated in MRS for 24 h at 37°C. Each *B. cereus* was grown in LB for 12 h at 37°C in triplicate, and the final concentrations of each bacterium were adjusted to 10^6 cfu/mL.

The antibacterial activity was performed according to the previous study with some modifications (Huang et al., 2015). Briefly, aliquots of 200 μL of *B. cereus* suspension were spread on LB agar plates to give confluent colonies, dried at room temperature, and holes in the agar were made with oxford cup (stainless steel cylinder; diameter = 7.8 ± 0.1 mm) and aliquots of 200 μL of *L. plantarum* suspension were dropped into the holes. The antibacterial activity of *L. plantarum* suspension adjusted to pH 6.0 and 7.0 was also tested. Agar plates were incubated in an aerobic at 37°C for 10 h and then the diameter of the inhibition zone around each cup was measured. All experiments were performed in triplicate.

Preparation of Milk Samples

Milk powder (Wondersun, Inc., Heilongjiang, China) was purchased from the local supermarket. The empty flasks were autoclaved at 121°C for 21 min and 30 mL of 10% (wt/vol) reconstituted sterile milk was added. The sterile milk was inoculated with *B. cereus* (ATCC14579/HN001) at about 10^2 to 10^3 cfu/mL and *L. plantarum* at about 10^4 to 10^5 cfu/mL. Control groups were inoculated with *B. cereus* ATCC14579 or *B. cereus* HN001 alone. Samples were incubated anaerobically at 37°C, and samples for pH measurement and analyses were withdrawn at different fermentation time points (0, 24, 36, and 48 h). All experiments were repeated twice.

L. plantarum Adhesion Assay

Caco-2 cells were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (**DMEM**; Solarbio, Beijing, China) supplemented with 10% fetal calf serum and antibiotics (100 U/mL of penicillin, 100 μg/mL of streptomycin) at 37°C in an atmosphere of 5% CO_2 and 95% air at constant humidity. The adherence of *L. plantarum* to Caco-2 cells was carried out as described by Leite et al. (2015), with some modifications. A monolayer of cells was seeded at a concentration of 2.5×10^5 cells per well in 6-well tissue plates and incubated in an atmosphere of 5% CO_2 and 95% air at 37°C for 18 h. The monolayer was washed twice with 1.5 mL of PBS (pH 7.4) to remove antibiotics from the cells, then added with approximately 10^8 cfu of *L. plantarum*, which was suspended by 2 mL of DMEM medium without antibiotics. Subsequently, the cells were incubated for 2 h and gently washed 3 times with PBS to remove nonadhered *L. plantarum*. Caco-2 cells and adhered bacteria were disrupted using trypsin-EDTA solution (Solarbio) and the *L. plantarum* counts were performed in the MRS agar medium. Adhesion was expressed as the percentage of *L. plantarum* adhered with respect to total number of *L. plantarum* added initially.

Competition Between L. plantarum and B. cereus for Cell Adhesion

Competitiveness was assessed by adding 10^8 cfu/mL of *L. plantarum* and 10^8 cfu/mL of *B. cereus* (ATCC14579/HN001) simultaneously to Caco-2 monolayer cells in 6-well tissue plates; *L. plantarum* and *B.*