Screening probiotics from *Lactobacillus* strains according to their abilities to inhibit pathogen adhesion and induction of pro-inflammatory cytokine IL-8

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ABSTRACT

Probiotics can be screened according to their abilities to inhibit pathogen adhesion and inhibit the production of pro-inflammatory cytokines. Eleven *Lactobacillus* strains isolated from traditional fermented dairy foods in Xinjiang, China, were studied for their potential to inhibit adhesion of *Escherichia coli* to intestinal epithelial cells and to inhibit *E. coli*-induced production of interleukin (IL)-8 by intestinal epithelial cells. The results showed that the 11 strains could inhibit adhesion of *E. coli* to Caco-2 cell monolayers and inhibit the induction of IL-8 production by *E. coli* in HT-29 cells. The inhibiting activities of the *Lactobacillus* strains against *E. coli* adhesion and IL-8 induction were strain-specific and not positively correlated, whereas the excluding activity of the strains against *E. coli* adhesion and their coaggregation with *E. coli* were positively correlated. The effector molecules of the strains with probiotic potential should be identified to explain the mechanism behind these observations.

Key words: *Lactobacillus*, adhesion, inflammatory, IL-8

INTRODUCTION

Probiotics are live microorganisms that, when consumed in adequate amounts, produce beneficial effects on host, including restoring gastrointestinal tract flora, immunomodulation, and so on (FAO/WHO, 2001; Fukuda et al., 2011; Sánchez and Urdaci, 2012; Unno et al., 2015).

Specific probiotics should be selected based on their ability to inhibit adhesion or displace pathogens adhering to human intestinal mucosa (Collado et al., 2007). Adhesion and colonization of pathogenic bacteria on

the intestinal mucosa are important steps in pathogenic infection. Probiotics are able to adhere to the intestinal mucosa and thus inhibit the binding of pathogens and invasion of intestinal epithelial cells (Faghfoori et al., 2015). As probiotic strains, some *Lactobacillus* strains are reported to adhere to intestinal epithelium, inhibiting invasion by pathogens and improving epithelial barrier function (Chen et al., 2012; Saxami et al., 2016; Yu et al., 2017).

Immunomodulation capacity of *Lactobacillus* strains is considered a criterion for probiotic assessment (Hardy et al., 2013). Lactobacilli could affect pro-inflammatory responses [tumor necrosis factor- α (TNF- α), IL-8] and anti-inflammatory responses (IL-10) of eukaryotic cells (HT-29, Caco-2, or other eukaryotic cells) treated with pathogens, lipoprotein, or other factors (Preising et al., 2010; Stöber et al., 2010; Sun et al., 2012). Some *Lactobacillus* strains can lower secretion of the pro-inflammatory cytokine IL-8 and promote secretion of the anti-inflammatory IL-10 of intestinal epithelial cells (Butel, 2014; Belguesmia et al., 2016).

Some probiotics have been reported to inhibit pathogenic bacteria by adhering to intestinal epithelium and to decrease pro-inflammatory cytokine expression induced by pathogens (Vanderpool et al., 2008; Liu et al., 2010; Nueno-Palop and Narbad, 2011; Sun et al., 2012; Savino et al., 2015). However, the relationship between inhibiting ability of probiotics against pathogens adhering to epithelial cells and their inhibiting ability against pathogens that induce pro-inflammatory cytokine secretion from epithelial cells is not clear.

In our previous study (Tuo et al., 2013), we studied the adhesion ability of 22 strains of *Lactobacillus* isolated from traditional fermented dairy food in Nalati, Xinjiang, China, and some strains with higher adhering ability were considered potential probiotic strains. In this study, 11 *Lactobacillus* strains with the highest adhesion ability among the 22 *Lactobacillus* strains were selected to assess their ability to inhibit adhesion of *E. coli* to intestinal epithelial cells and inhibit induction by *E. coli* of IL-8 production by intestinal epithelial cells, and the relationship between the 2 abilities.

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Table 1. *Lactobacillus* strains evaluated in this study

Strain	Species	Origin ¹
22	<i>Lactobacillus plantarum</i>	Traditional fermented dairy food, Nalati, Xinjiang, China
23	<i>L. plantarum</i>	Traditional fermented dairy food, Nalati, Xinjiang, China
39	<i>Lactobacillus fermentum</i>	Traditional fermented dairy food, Nalati, Xinjiang, China
57	<i>L. plantarum</i>	Traditional fermented dairy food, Nalati, Xinjiang, China
66	<i>L. plantarum</i>	Traditional fermented dairy food, Nalati, Xinjiang, China
67	<i>L. plantarum</i>	Traditional fermented dairy food, Nalati, Xinjiang, China
89	<i>L. plantarum</i>	Traditional fermented dairy food, Nalati, Xinjiang, China
130	<i>L. plantarum</i>	Traditional fermented dairy food, Nalati, Xinjiang, China
196	<i>Lactobacillus casei</i>	Traditional fermented dairy food, Nalati, Xinjiang, China
m2.3	<i>L. casei</i>	Traditional fermented dairy food, Nalati, Xinjiang, China
f12	<i>L. plantarum</i>	Traditional fermented dairy food, Nalati, Xinjiang, China
LGG	<i>Lactobacillus rhamnosus</i> GG	Harbin Institute of Technology, Harbin, China

¹The fermented foods were made by local people using traditional methods under local climate and environmental conditions. Traditional fermented dairy foods from different pastures were sampled and strains were isolated from different pastures.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Lactobacillus strains tested in this study were isolated from traditional fermented dairy foods in Nalati, Xinjiang, China, stored in our stock culture collection (Tuo et al., 2013). Detailed information about the strains is listed in Table 1. *Escherichia coli* ATCC43889 was used from our stock culture collection. *Lactobacillus rhamnosus* GG (**LGG**) was a gift from L. Zhang from the Harbin Institute of Technology (Harbin, China). *Lactobacilli* were cultured anaerobically in de Man, Rogosa, and Sharpe (MRS) broth (Merck KGaA, Darmstadt, Germany) at 37°C for 18 h, and *E. coli* was cultured aerobically in brain-heart infusion (BHI) broth (Difco Laboratories Inc., Detroit, MI) at 37°C for 18 h.

Caco-2 and HT-29 Cell Culture

The human colonic cell line Caco-2 was provided by X. Chen (Ruijin Hospital, Shanghai, China). The Caco-2 cells were grown routinely in Dulbecco's modified Eagle medium (**DMEM**; Gibco/Life Technologies Corp., Grand Island, NY), supplemented with 10% (vol/vol) heat-inactivated (56°C for 30 min) fetal bovine serum (Sijiqing; Zhejiang Tianhang Biological Technology Co. Ltd., Hangzhou, China), penicillin (100 U/mL), and streptomycin (0.1 g/L; Gibco/Life Technologies Corp.) at 37°C in 5% CO₂ in 95% air. The culture medium was replaced every 48 h to maintain the cells. Caco-2 cells were used as intestinal epithelial cell model to study the adhering ability of *Lactobacillus* strains.

Human colonic cancer cell line HT-29 was obtained from the Chinese Academy of Science (Shanghai, China). The HT-29 cells were cultured in McCoy's medium (Gibco/Life Technologies Corp.) supplemented with 10% (vol/vol) inactivated (56°C, 30 min) fetal calf serum (Sijiqing Co. Ltd.) in a humidified atmosphere

of 5% CO₂ and 95% air at 37°C. The HT-29 cells were cultured until they were fully differentiated. The HT-29 cells were used as intestinal cell model to study IL-8 cytokine production induced by *E. coli*.

Effect of *Lactobacillus* Strains on Adhesion of *E. coli* to Caco-2 Monolayers

The effect of *Lactobacillus* strains on *E. coli* adhesion was assessed by using Caco-2 cells as an intestinal epithelial cell model according to Ramiah et al. (2008). Caco-2 cells were seeded on 12-well cell culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany) at 1×10^5 cells per well. The plates were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air until a confluent monolayer was obtained.

Caco-2 cell monolayers on the 12-well plates were washed twice with sterile PBS (pH 7.2) before the adhesion assay. Then, 18-h cultures of *Lactobacillus* strains and *E. coli* were harvested by centrifugation at $10,000 \times g$ for 10 min at 4°C (Avanti J30I, Beckman Coulter, Brea, CA), washed twice with sterile PBS (pH 7.2), and then resuspended in DMEM (without antibiotic and fetal bovine serum) and adjusted to 1×10^9 cfu/mL.

To study the ability of *Lactobacillus* strains to prevent *E. coli* from adhering to Caco-2 cells by exclusion, 0.5 mL of each *Lactobacillus* strain in DMEM suspension was added into wells with Caco-2 cell monolayers and supplemented with 0.5 mL of DMEM. After 1 h of incubation at 37°C, 0.5 mL of *E. coli*-DMEM suspension was added into the same wells. After 1 h of incubation, each well of the plates was washed 4 times with PBS (pH 7.2) to remove free, nonattached bacterial cells. Then, 1 mL of 1% (vol/vol) Triton X-100 (HFH10, Invitrogen, Carlsbad, CA) was added to each well, and the suspension was stirred to detach the bacterial cells attached to Caco-2 cell monolayers. Serial dilutions of the suspension in sterile PBS were plated

onto eosin-methylene blue agar (Beijing Aoboxing Biotech Co. Ltd., Beijing, China) and incubated at 37°C to determine the *E. coli* bacterial cell number adhering to Caco-2 cells. The inhibiting rate against *E. coli* adhesion to Caco-2 cells was calculated as follows:

$$\text{Inhibiting rate (\%)} = [1 - (N_1/N_0)] \times 100,$$

where N_1 = number of *E. coli* adhering to Caco-2 cells and N_0 = number of *E. coli* added into wells of 12-well plates initially.

Effect of *Lactobacillus* Strains on *E. coli*-Induced IL-8 Production of HT-29 Cells

The effect of *Lactobacillus* strains on *E. coli*-induced IL-8 production by intestinal epithelial cells was assessed by using HT-29 cells as an intestinal epithelial cell model according to Stöber et al. (2010).

The HT-29 cells were seeded in 12 well plates at 2×10^5 cells/well. The plates were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air for 48 h before infection. The 18-h cultures of each *Lactobacillus* strain and *E. coli* were harvested by centrifugation at $10,000 \times g$ for 10 min at 4°C, washed twice with PBS (pH 7.2), and then resuspended in McCoy's medium (antibiotic-free, fetal bovine serum-free) and adjusted to 1×10^9 cfu/mL.

We first studied whether the *Lactobacillus* strains induced IL-8 production by HT-29 cells. One milliliter of *Lactobacillus* strain suspension in McCoy's medium (hereafter, *Lactobacillus*-McCoy's) was added into the wells of a 12-well plate with HT-29 cell monolayers and incubated for 3 h (study wells); 1.0 mL of *E. coli* suspension in McCoy's medium (hereafter, *E. coli*-McCoy's) was inoculated into wells with HT-29 cells and incubated for 3 h (positive control wells), and McCoy's medium alone was the negative control.

To determine whether the *Lactobacillus* strains inhibited *E. coli* from inducing IL-8 production by HT-29 cells, 0.5 mL of suspensions of *Lactobacillus*-McCoy's and *E. coli*-McCoy's were added into the wells with HT-29 cell monolayers for 3 h of incubation. To determine the ability of lactobacilli to inhibit *E. coli*-induced IL-8 production by HT-29 cells, 0.5 mL of *Lactobacillus*-McCoy's was added into wells with HT-29 cell monolayers for 1 h of incubation, and then 0.5 mL of *E. coli*-McCoy's was added into the same wells for 2 h of incubation. To study the effect of different doses of live and heat-treated *Lactobacillus* strains on *E. coli*-induced IL-8 production, 0.5 mL of live (or heat-treated) *Lactobacillus*-McCoy's (at doses of bacteria: cell = 1,000:1, 100:1, and 10:1 respectively) and 0.5 mL

of *E. coli*-McCoy's (at a dose of bacteria:cell = 100:1) were added into the wells with HT-29 cell monolayers for a 3-h incubation.

After 3 h of incubation at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, cell culture supernatants were harvested by centrifugation ($10,000 \times g$ for 10 min at 4°C) and IL-8 levels were determined by using ELISA kits (Human IL-8 Valukine ELISA kit, R&D Company, Minneapolis, MN).

Statistical Analysis

All experiments were performed in triplicate. Statistical treatment of the data was conducted using ANOVA of IBM SPSS Statistics 22 (IBM Corp., Armonk, NY), and Duncan's test was used to compare the means when the overall *P*-value of the experiment was below the value of significance ($P < 0.05$). Mean values and standard errors were calculated and presented as coordinate pairs with error bars.

RESULTS AND DISCUSSION

Effects of *Lactobacillus* Strains on Adhesion of *E. coli* to Caco-2 Cells

We studied the ability of 11 *Lactobacillus* strains to prevent *E. coli* from binding to Caco-2 cell monolayers by exclusion. When the *Lactobacillus* strains were previously adhered to Caco-2 cell monolayers, adhesion of *E. coli* to Caco-2 cell monolayers was prevented, as shown in Figure 1. Probiotics with epithelial cell binding ability can competitively inhibit pathogen binding, thus hindering their colonization (Yu et al., 2017). The *Lactobacillus* strains occupied adhering sites on Caco-2 cell surfaces, preventing *E. coli* from adhering. Although strains 130 and LGG had lower adhesion ability than other strains, such as strain 57 (Tuo et al., 2013), they had higher inhibiting rates of inhibition against *E. coli* adhering to Caco-2 cell monolayers compared with other strains in this study.

In our previous study (Tuo et al., 2013), the ability of lactobacilli strains to adhere to Caco-2 monolayers was studied. In the current study, the strains with higher adhesion ability did not show higher inhibiting rate against *E. coli* adhering to Caco-2 monolayers, consistent with the report by Yu et al. (2017). It was reported that the inhibition of enterotoxigenic *E. coli* (ETEC) binding to IPEC-1 cells was due to coaggregation (Yu et al., 2017). We found a positive correlation ($P < 0.05$) between the excluding activities of *Lactobacillus* strains against *E. coli* adhesion and their coaggregation with *E. coli*, as shown in Figure 2.

Figure 1. Ability of *Lactobacillus* strains (see Table 1) to exclude *Escherichia coli* O157 from adhering to Caco-2 cells. Different lower-case letters (a–h) indicate significant differences ($P < 0.05$) among strains. Error bars represent SD.

Effect of *Lactobacillus* Strains on *E. coli*-Induced IL-8 Production by HT-29 Cells

In our previous study (Tuo, 2013), we found that IL-8 production of Caco-2 cells induced by *E. coli* and *Lactobacillus* strains was low and not significantly ($P > 0.05$) different from that of Caco-2 cells with no induction, whereas *E. coli* could dramatically induce IL-8 production in HT-29 cells. Therefore, HT-29 cells were selected to study IL-8 induction in this study.

The effects of different *Lactobacillus* strains on IL-8 production in HT-29 cells were assessed. As shown in Figure 3, the IL-8 levels of HT-29 cells induced by lactobacilli were significantly lower ($P < 0.05$) than those induced by *E. coli*. The IL-8 levels of HT-29 cells induced by strains 57 and LGG were not different ($P > 0.05$) from those of the negative control, indicating that these strains did not induce production of pro-inflammatory cytokine IL-8. It has been reported that the *Lactobacillus* strains not inducing IL-8 production by gastrointestinal epithelial cells could alleviate enteritis (Candela et al., 2008; Panpetch et al., 2016). The chemical difference in cell wall components between probiotic bacteria and pathogenic bacteria may account for the different pro-inflammatory cytokine expression of host cells (Wang et al., 2016).

The inhibiting effect of the *Lactobacillus* strains against *E. coli*-induced IL-8 production of HT-29 was further studied. As shown in Figure 4, *Lactobacillus* strains significantly ($P < 0.05$) inhibited *E. coli* induction of IL-8 production. The results indicated the *Lac-*

Figure 2. Correlation between inhibiting rates of *Lactobacillus* strains against *Escherichia coli* adhesion to Caco-2 cells and their coaggregation with *E. coli*, significant at $P < 0.05$.

tobacillus strains could alleviate inflammatory symptoms. It was reported that *L. acidophilus* Bar13 and *Bifidobacterium longum* Bar33 alleviated inflammatory symptoms because of lower IL-8 levels in the intestinal tract by displacing the pathogenic bacteria adhering to intestinal epithelium (Candela et al., 2008). In this study, all 11 strains could alleviate *E. coli*-induced IL-8 production by HT-29 cell monolayers, which could be related to their ability to inhibit *E. coli* adhesion.

Figure 3. Induction of IL-8 production in HT-29 cells exposed to *Lactobacillus* strains (see Table 1) or *Escherichia coli*. *Significant difference ($P < 0.05$) between strains. Error bars represent SD.

Figure 4. Induction of IL-8 production in HT-29 cells exposed to mixtures of *Lactobacillus* strains (see Table 1) and *Escherichia coli*. *Significant difference ($P < 0.05$) between strains. Error bars represent SD.

Seven of the 11 *Lactobacillus* strains were selected for further study because of their ability to adhere to Caco-2 cells and their inhibitory activity against *E. coli*-induced IL-8 production by HT-29 cells. When *Lactobacillus* strain 57, 196, or LGG was cocultured with HT-29 cell monolayers for 1 h before *E. coli* was inoculated into the mixture, *E. coli*-induced IL-8 production of HT-29 cells was significantly ($P < 0.05$) inhibited, as shown in Figure 5. Strains 57, 196, and LGG could inhibit *E. coli*-induced IL-8 production by HT-29 cells and inhibit adhesion of *E. coli* to Caco-2 cells, indicating that these strains had the potential to alleviate intestinal inflammatory symptom induced by pathogens.

Inhibition of Pathogen Adhesion and Pathogen-Induced IL-8 Production

The inhibiting effect of the *Lactobacillus* strains against *E. coli*-induced IL-8 production by intestinal epithelial cells may be related to their ability to inhibit adhesion of *E. coli* to intestinal epithelial cells. *Bifidobacterium lactis* HN019 (Liu et al., 2010) and *Lactobacillus crispatus* K313 (Sun et al., 2012) were reported to inhibit the attachment of pathogenic bacteria to intestinal epithelial cells and to attenuate pathogen-induced IL-8 expression from intestinal epithelium. In the current study, *Lactobacillus* strains 57, 196, and LGG exhibited inhibiting activity against *E. coli*, inducing IL-8 production and adhering to Caco-2

cell monolayers. We wanted to determine whether these 2 activities—inhibiting IL-8 production and preventing adhesion—were positively correlated. We did not find a positive relationship between the inhibitory activities of the *Lactobacillus* strains against *E. coli* adhesion and against *E. coli*-induced IL-8 production, as shown in Figure 6A, or between the *Lactobacillus* strains' ability to adhere to Caco-2 cells and their inhibiting activity against *E. coli*-induced IL-8 production, as shown in Figure 6B.

The mechanism for the inhibition by *Lactobacillus* strains of *E. coli*-induced IL-8 production should be related to the cell surface-associated molecules. Probiotic functionality has strain specificity depending on the bioactive effector molecules (Bron et al., 2013). Ligands such as flagellin on the surface of pathogenic bacteria promote adhesion and invasion of host by pathogens. However, metabolites of some beneficial bacteria, such as polysaccharide, surface-bound proteins, or peptidoglycan, could protect the host from pathogen invasion, promoting Treg cell function through special ligand–receptor interactions (Belkaid and Hand, 2014). Perhaps special cell wall components and structure of the *Lactobacillus* strains account for their interaction with host cells. Surface-bound proteins and other macromolecules are known to play a role in the adhering abilities of 22 *Lactobacillus* strains (Tuo et al., 2013).

Figure 5. Induction of IL-8 production in HT-29 cells pretreated with *Lactobacillus* strains (see Table 1) and exposed to *Escherichia coli*. *Significant difference ($P < 0.05$) between strains. Error bars represent SD.

Figure 6. Correlation between adhering ability of *Lactobacillus* strains (see Table 1) and their inhibiting activity against *Escherichia coli* invading HT-29 cells. (A) Correlation between inhibiting rates of *Lactobacillus* strains against *E. coli* adhesion to Caco-2 cells and inhibiting rates against *E. coli*-induced IL-8 production in HT-29 cells is not significant at $P < 0.05$. (B) Correlation between adhering ability of *Lactobacillus* strains to Caco-2 cells and their inhibiting activity against *E. coli*-induced IL-8 production in HT-29 cells is not significant at $P < 0.05$.

Effects of Live and Dead Cells of *Lactobacillus* Strains on *E. coli*-Induced IL-8 Production

We studied the effects of different doses of live and dead bacterial cells of *Lactobacillus* strains 57, 196, and LGG on *E. coli*-induced IL-8 production by HT-29 cell monolayers. The results showed that the IL-8 production decreased with increasing doses of live or

dead *Lactobacillus* strains (Figure 7). In Figure 7A, different doses of live *Lactobacillus* strains, except strain 57 at a bacteria:cell dose 10:1, could inhibit *E. coli*-induced IL-8 production of HT-29 cells; in Figure 7B, only heat-killed strains 57 and 196 at a bacteria:cell dose of 1,000:1 significantly ($P < 0.05$) inhibited IL-8 production. Live strains exerted a stronger inhibiting effect against *E. coli*-induced IL-8 secretion than dead cells. The different effects between live and dead lactobacilli could be due to the different bioactivities of cell surface-associated components. The extracellu-

Figure 7. Effect of different doses (bacteria:cell = 1,000:1, 100:1, or 10:1) of *Lactobacillus* strains (as listed in Table 1) on IL-8 secretion from HT-29 cells induced by *Escherichia coli*. (A) Live and (B) heat-treated *Lactobacillus* strains' inhibition of *E. coli*-induced IL-8 production in HT-29 cells. *Significant difference ($P < 0.05$) between bracketed columns. Error bars represent SD. Color version available online.

lar proteins secreted by *Lactobacillus* play important roles in preventing adhesion of pathogens to epithelial cells (Sánchez and Urdaci, 2012). It is possible that the heat treatment used in the current study inactivated the bacterial envelope effector molecules, weakening the inhibiting effect of heat-killed bacteria against IL-8 production.

The protective effects of viable lactic acid bacteria against pathogen-induced IL-8 secretion by HT-29 cells were shown to be strain specific, and the protective effects disappeared when viable lactic acid bacteria were disrupted by ultrasound and ball mill (Stöber et al., 2010). Both live and heat-treated cells of *Lactobacillus fermentum* CECT5716 were shown to attenuate the inflammatory process (Rodríguez-Nogales et al., 2015). Subtle structural differences in the effector molecules expressed by individual lactic acid bacteria are responsible for the strain specificity of probiotic functions (Lee et al., 2013), and structural properties determine the function. In our previous study, the autoaggregating and adhering abilities of 22 *Lactobacillus* strains decreased because of destruction of the surface-bound proteins by guanidine HCl (Tuo et al., 2013). In the current study, the bacterial cell structure of the *Lactobacillus* strains was disrupted by heat treatment, resulting in an alteration or loss of function.

CONCLUSIONS

In summary, *L. plantarum* 57 and *L. casei* 196 had potential to alleviate intestinal inflammatory symptoms induced by pathogens. We did not find a positive relationship between the inhibiting activity of the *Lactobacillus* strains against *E. coli* adhesion and their inhibiting activity against *E. coli*-induced IL-8 production. In this study, inhibiting activities of the *Lactobacillus* strains against *E. coli* adhesion and *E. coli*-induced IL-8 production is strain specific, and the effector molecules of *Lactobacillus plantarum* 57 and 196 should be identified and purified to explain their effects on *E. coli* adhesion and IL-8 induction.

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