Lactobacillus Strain with High Adhesion Stimulates Intestinal Mucin Expression in Broiler

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Lactobacillus fermentum 1.2029 strain with high adhesion was selected for studying the mucin expression of the small intestine epithelium of broilers after the strain was orally administrated. The strain was allowed to adhere to Caco-2 and to a hydrophobic solvent to evaluate its adhesive properties. Adhesion to intestinal mucin of chickens was also tested to further characterize the in vivo adhesion of the strain. Afterward, the acid and bile salt tolerance of the strain was also evaluated. L. fermentum 1.2029 showed good adhesive ability and was able to survive in low pH conditions and in the presence of bile salts. The in vivo study showed that the strain significantly increased goblet cell density in the jejunum and the level of MUC2 mRNA in both the jejunum and ileum (P < 0.05). L. fermentum 1.2029 treatment didn’t affect the thickness of the mucous layer. These results demonstrate that L. fermentum 1.2029 has protective functions, thereby regulating the goblet cell density and the expression of mucin in the small intestine of broilers. This work also suggests that L. fermentum 1.2029 is a potential probiotic feed additive for the improvement of the intestinal function of chickens.

Key words: broiler chickens, gastrointestinal tract, Lactobacillus, mucin


Introduction

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host.” (Yuan-kun, 2009). Specific members of the Lactobacillus genera have been widely studied as probiotics, the beneficial effects of Lactobacillus on hosts include anti-inflammatory effects against diseases like gastroenteritis, and inflammatory bowel disease, modulation of immune response, and retardation of carcinogenesis (Sullivan and Nord, 2002; Ventura et al., 2009; Lee et al., 2010). Increasing evidence has shown that Lactobacillus apecies are effective in preventing intestinal diseases in both humans and animals, due to their ability to maintain or restore normal micro-biota, inhibit pathogen adhesion to intestinal epithelium, and prevent inflammatory processes (Roller et al., 2004; Sgouras et al., 2005; Bernardau et al., 2006). Some studies have also shown the ability of the members of Lactobacillus to protect against pathogen-induced membrane barrier disruption (Roselli et al., 2007). However, to date, the exact mode of action of Lactobacillus species is still unknown.

The gastrointestinal mucus that, covers the mucosal surface, is an important factor in the physiological defense mechanism of the gastrointestinal tract (Iwai et al., 2009). As the main component of mucus, mucin may provide attachment sites for intestinal flora and pathogenic bacteria (Robbe et al., 2004), as well as simultaneously prevent bacterial and viral pathogens from adhering to intestinal cells, and toxins from being absorbed into the body (Robbe et al., 2004; Beyaz and Liman, 2009). Previous studies have reported that mucin dynamics in the intestines is altered by the ingredients of the diet, physiological state and posthatch development of chicken (Smirnov et al., 2004; Forder et al., 2007; Horn et al., 2009). Few researchers have studied the transcriptional response of Lactobacillus to mucin.

The ability to adhere to mucus and epithelial cells is an important property for probiotic lactic acid bacteria. The adhesive capability of intestinal bacteria is essential for colonization and growth in the intestinal tract (Namba et al., 2007). Hence, highly adhesive bacteria can reside for a longer period in intestinal tracts. The ability of some probiotic strains to inhibit pathogen colonization and invasion and modulate immune response (s) has also been related to their ability to adhere to intestinal mucus and / or epithelial cells (Laparra and Sanz, 2009). Detecting adhesive properties in vivo is difficult, so in vitro models, such as Caco-2 cell, have been developed for the study of bacterial adherence to intestinal epithelium (Lee et al., 2000; Botes et al., 2008;
Kaushik et al., 2009).

Although studies indicate that Lactobacillus species improve the intestinal health of their hosts and, particularly, enhancement of the barrier function, little is known whether Lactobacillus can enhance the intestinal mucosal barrier function of broiler chickens through modulation of mucin synthesis. In the present study, a Lactobacillus strain with high adhesive properties was screened, and the effect of the selected Lactobacillus strain on mucin dynamics and levels of mucin mRNA in the small intestine of chickens was investigated.

Materials and Methods

Bacterial Strains and Growth Conditions

Six strains, namely, L. rhamnosus 1.0120, L. acidophilus 1.1878, L. salivarius subsp. Salivarius 1.1881, L. brevis 1.2028, L. fermentum 1.2029 and L. animalis 1.2623, obtained from CGMCC (China General Microbiological Culture Collection Center), were used in the present study. Stock cultures were stored at -80°C, and the microorganisms were subcultured twice in liquid medium before the experiments.

The strain was grown in Man, Rogosa, and Sharp (MRS) broth under anaerobic conditions at 37°C for 16 h. The bacteria were harvested via centrifugation at 6,000 g for 10 min and resuspended in phosphate buffered saline (PBS) prior to use. The Escherichia coli EPEC was provided by Dr. Wang (College of Veterinary Medicine, NWSUAF), was grown overnight in LB broth at 37°C without shaking prior to use as the positive control strain for Caco-2 adhesion.

Caco-2 Cell Culture and Adhesion Assay

Caco-2 was kindly provided by Dr. Hou (Department of Cell Biology, School of Life Sciences, Shaanxi Normal University). The cells were grown in RPMI medium 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated (30 min, 56°C) fetal calf serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin (Sigma St Louis, MO, USA), and 100 U/ml streptomycin (Sigma). The cells were maintained at 37°C in 5% CO₂, 95% air, and the culture medium was changed every two days. For the adhesion assay, the Caco-2 cells were seeded at a concentration of 10⁵ cells / well into a 6-well tissue-culture plate (Nalge Nunc, Rochester, NY, USA), and were incubated up to confluence. The RPMI medium 1640 was replaced with the same medium without antibiotic one hour before the adhesion assay was performed.

For the adhesion assay, 3 ml bacterial suspension (culture medium) (total cell count, 10⁹ cells / ml) was added to washed Caco-2 monolayer on coverslips placed in a 6-well tissue culture dish. The dish was then incubated (1 h, 5% CO₂, 37°C). After 1.5 h of incubation, the monolayers were washed 5 times with sterile PBS, fixed with methanol, and stained with Gram-stain. The samples were then examined microscopically under an oil immersion lens. Each adhesion assay was performed in triplicate with cells from three successive passages. The number of bacteria per Caco-2 cell was recorded by examining 100 cells in 20 random microscopic fields.

Determination of Bacterial Cell Surface Hydrophobicity

Microbial cell surface hydrophobicity was determined by performing the microbial adhesion to hexadecane test, as described by Deepika et al. (2009). Briefly, the bacterial cells grown in MRS broth at 37°C for 16 h were centrifuged at 6,000 rpm at 4°C for 10 min. The cell pellet was washed twice with PBS buffer to remove the residual medium, and then resuspended in PBS. The absorbance of the suspension was adjusted at 600 nm (A₀). In a 10 ml syringe, 2 ml bacterial cell suspension was mixed with equal volumes of hexadecane (Sigma). The resulting mixture was mixed on a vortex mixer for 1 min and left to stand for 20 min to allow complete phase separation. After equilibration, the aqueous phase was gently taken out to measure its absorbance at 600 nm (A₁). Percent adhesion was calculated using the following equation:

\[ \% \text{Adhesion to hexadecane} = \frac{A₀ - A₁}{A₀} \times 100 \]  

Where A₀ is the initial absorbance of the bacterial suspension and A₁ is the absorbance after 20 min of incubation at 600 nm. Each experiment was performed in triplicate using cells from independent cultures.

Preparation of Intestinal Mucin from Broiler and Adherence of L. fermentum 1.2029 to Mucin

The isolation and purification mucin from the small intestine of chicken were performed as described previously (Alemka et al., 2010). The purified mucin was stained with periodic acid-Schiff (PAS) reagent to identify the presence of mucin and subsequently dissolved in PBS to a final concentration of 10 mg·mL⁻¹. The wells of a 96-well plate were coated with the purified mucin and incubated overnight at 4°C. Unbound mucin in each well was removed through washing with PBS twice. Controls consisted of PBS-treated wells and untreated wells. The results of at least four replicates were used to estimate the adhesion ability of a given strain. L. fermentum 1.2029 was then added at a concentration of 1×10⁸ CFU/ml into each well (100 μl/well) and incubated for 2 h at 37°C in MRS medium. The wells were washed twice with PBS to remove unbound bacteria. Bacteria bound to mucin were released and lysed using 200 μl 0.5% (v/v) Triton X-100 solution. The number of bacteria that adhere to mucin was determined using the plate count method. Prior to use, Triton X-100 concentration was tested to determine the influence of the reagent on bacterial viability.

Acid and Bile Salt Tolerance of L. fermentum 1.2029

To test tolerance of Lactobacillus fermentum 1.2029 at low pH according to Cukrowska et al. (2009) with minor modifications. The bacteria was cultivated for overnight in MRS medium, then grown in 10 ml sterile MRS broth of pH 2, 2.5 and 3 (adjusted with 1 N HCl). The concentration of bacteria was adjusted to 10⁶ CFU/ml (the initial bacterial concentration). After 2 h treatment, the number of viable cell was determined by the plate count method. The measurement was repeated at least three times in two independent experiments. The percentage of surviving bacteria was calculated using the following:
Where \( N_f \), Mucin Staining and Goblet Cell Counts

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liquid nitrogen. All procedures were approved by the Ani-

0.9% NaCl, and fixed in 4% (v:v) neutral-buffered formalin

Tissue samples (2 cm) were taken for histology, washed in

Meckel’s diverticulum and the ileo-cecal junction (ileum).

duodenum, from the midpoint between the bile duct entry

were inoculated with the same volume of PBS. At 21d,
orogastric inoculation. Accordingly, the control group birds

chickswerekilledandintestinalsegmentswereremovedand

were grown cultures of bacteria was inoculated in 10 ml sterile

MRS broth (pH 6.8) containing 0.3%, 0.5% and 1.0% (w/v)
bile salt. Growth was monitored by determining the number of

The thickness of intestinal mucin was measured by

Smirnov’s method (Smirnov et al., 2004). Intestinal tissues

measuring 1 cm² were removed and placed in 10 g/l Alcian

Blue dye solution in buffer containing 160 mmol/l sucrose

and 50 mmol/l sodium acetate, pH 5.8. After 2 h incubation,
excess dye was extracted with 250 mmol/l sucrose. The absorbed
dye was extracted from the tissue by incubation in 10 g/l docusate sodium salt solution overnight at room temperature. Samples were cleared by centrifugation at 700 ×g and their optical density was measured at 620 nm, Alcian

Blue solution as standard. The amount of absorbed dye was

reported as µg Alcian Blue/cm² of intestinal tissue.

Quantitative Real-Time PCR Analysis

Total RNA was extracted from the intestinal segments

using the Trizol isolation reagent (TaKaRa, Shiga, Japan)

according to the manufacturer’s protocol.

The quality of the isolated total RNA was determined by

the A260:A280 absorbance spectra (values of 1.9 to 2.1 in 10

mM Tris-HCl, pH 7.5, were considered acceptable). RNA

concentration was determined using a spectrophotometer.

cDNA was synthesized from 1 µg of total RNA using Oligo-
dT primer and PrimeScript™ RT enzyme (TaKaRa). Subse-
quenty, Quantitative real-time PCR for MUC2 was per-
formed using SYBR Premix Ex Taq™II (TaKaRa) in a Bio-
Rad iQ5 determination system (BIO-RAD, Hercules, CA, USA).

Each PCR was performed in triplicate and normalized with the housekeeping gene β-actin. The primer sequences used

were shown in Table 1. Relative gene expression data were

determined by the 2\textsuperscript{−ΔΔCT} method as previously described (Livak and Schmittgen, 2001).

Statistical Analysis

Data from adhesion assay and test for bacterial cell surface

hydrophobicity were analyzed by One-way analysis of

variance (ANOVA) with D’Agostino’s multiple comparison test

to compare multiple experimental groups. The differences

between two groups were assessed by the student’s t test. All

values in this article are mean values ± standard deviation

(SD). A P value of <0.05 was considered significant.
Results

Adhesion to Caco-2

*Lactobacillus* strains were tested for their ability to adhere to Caco-2 cells. All the strains in this study were able to adhere to Caco-2 (Fig. 1). However, adhesion capacity was highly variable, depending on the bacterial strains. The most strong adhesion to Caco-2 was observed in *L. fermentum* 1.2029 compared with the positive control, an *E. coli* EPEC strain, *L. brevis* 1.2028, *L. rhamnosus* 1.0120 and *L. acidophilus* 1.1878 showed similar adhesion abilities, followed by *L. salivarius* 1.1881, and *L. animalis* 1.2623 showed the lowest adhesion ability.

Adhesion to Hydrophobic Solvent

The hydrophobic properties of the strains used in this study are shown in Fig. 2. A hydrophobicity of 70% was recorded for *L. fermentum* 1.2029, 61% for *L. brevis* 1.2028, and 44% for *L. rhamnosus* 1.0120, 51% for *L. acidophilus* 1.1878, 41% for *L. salivarius* 1.1881 and 33% for *L. animalis* 1.2623.

Adhesion of *L. fermentum* 1.2029 to Mucin

Adhesion of *L. fermentum* 1.2029 to mucin was tested. Results showed that the strain adhered to mucin-coated surface at a level of approximately $6.59 \times 10^5$ CFU per well. The number of bacteria that adhere to untreated-wells was less than $10^3$ CFU per well.

Tolerance of *L. fermentum* 1.2029 to Gastrointestinal Condition

*L. fermentum* 1.2029 showed good adhesive properties, but the strain must be able to survive the transit through the hosts’ gastrointestinal tract to exert their beneficial effect. Hence the strain’s tolerance to gastrointestinal conditions (acid and bile) was tested. *L. fermentum* 1.2029 was subjected to low pH for 2 h at 37°C. The percentage of bacteria that survived was 83%, 88% and 94% at pH 2.0, 2.5 and 3.0, respectively. The percentage of survival of the strain at low pH conditions indicated the good tolerance of *L. fermentum* 1.2029 to acidic conditions. After 2 h treatment at 37°C in MRS broth containing 0.3%, 0.5%, and 1.0% bile salt, the calculated percentage of survival of the strain was 93%, 91%, and 74%, respectively. These results indicated the survival potential of *L. fermentum* 1.2029 in the presence of bile salts (Table 2).

From these results, a promising strain, *L. fermentum* 1.2029 was selected for further studies in vivo.

Intestinal Morphometric Measurements

The goblet cell density and distribution of PAS-positive cells along the villi of the small intestine are presented in
Fig. 3. The effect of *lactobacillus* on goblet cell density in broilers. (A), PAS staining of small intestinal segments, light micrographs of the small intestine were visualized. Magnification ×400. The Photographs of a, c, e represent duodenum, jejunum, ileum of control group, respectively. The Photographs of b, d, f represent duodenum, jejunum, ileum of treatment group, respectively; (B), Quantification of goblet cell density in the broiler chicken small intestine. The error bars represent standard deviations. Statistically significant differences ($P<0.05$) were determined by Student’s t test and were indicated with asterisks.
Measurement of the Thickness of Adherent Mucus Layer

Small intestinal segments are: Duo-duodenum, Jej-jejunum, Ile-ileum. Error bars indicate standard deviations. Statistically significant differences ($P<0.05$) were determined by Student’s $t$ test and were indicated with asterisks.

Fig. 4. Effect of Lactobacillus on the thickness of the chicken small intestinal mucus layer. Small intestinal segments are: Duo-duodenum, Jej-jejunum, Ile-ileum. Error bars indicate standard deviations. Statistically significant differences ($P<0.05$) were determined by Student’s $t$ test and were indicated with asterisks.

Mucin mRNA Expression

The expression of the mucin gene in the small intestinal of the chickens was measured by quantitative real time-PCR. The treatment group with administration of lactobacillus induce a 3.89-fold and 3.32-fold increase in MUC2 mRNA level in the jejunum and in the ileum compared with the controls ($P<0.05$), respectively. In the duodenum, mucin mRNA expression tended to increase after lactobacillus treatment, although the increase was nonsignificant (Fig. 5).

Fig. 5. The effect of lactobacillus on chicken intestinal mucin mRNA expression. Changes in mucin mRNA expression were measured by quantitative real time-PCR. Small intestinal segments are: Duo-duodenum, Jej-jejunum, Ile-ileum. Error bars indicate standard deviations. Statistically significant differences ($P<0.05$) were determined by Student’s $t$ test and were indicated with asterisks.

Based on the results above, L. fermentum 1.2029 as a successful candidate was fed to broiler chicken by oral gavage to study its effects on MUC2 expression in the small intestine in vivo.

Discussion

Probiotic supplementation has been recommended for the treatment or prevention of various stress conditions and diseases in different animals (Bernardeau et al., 2006). Members of the genus Lactobacillus are a kind of probiotic bacteria commonly used for the supplementation of human and animal diets and have a variety of proposed beneficial effects, including promotion of gut health. Several studies have highlighted the value of Lactobacillus in poultry (Hofacre et al., 2003; Higgins et al., 2008; Panda et al., 2008). The application of probiotics in poultry feeding could be enhanced by a preliminary in vitro screening. The adhesion of probiotics to mucosa and/or intestinal epithelial cells is one of the main criteria considered in the selection of probiotics. Bacterial adhesion to epithelial cells or mucosa is considered the first and key step in the colonization of host intestine. Van den Abbeele et al. posited that mucosal bacteria could make closer contact with the hosts than luminal ones and can therefore have stronger health effects (Van den Abbeele et al., 2009). According to Lee’s report, prolonged adhesion and colonization of probiotics in the intestinal tract would favor their potential beneficial health effects (Lee et al., 2004). In the present study, the assessment of adhesion of six Lactobacillus strains to Caco-2 cells in vitro was performed by Gram staining method. L. fermentum 1.2029 exhibited high adhesion ability to Caco-2 cells. Hydrophobicity also plays a key role in the initial contact of bacterial cell and mucus or epithelial cells (Schillinger, et al., 2005). Hydrophobicity has been used to predict bacterial adhesion and colonization in the gut (Starostina et al., 1997; Pasteris et al., 2009). A correlation between hydrophobicity of Lactobacillus strains and their adhesion to porcine enterocytes has also been found (Wadstrom et al., 1987). Moreover, L. fermentum 1.2029 showed high cell surface hydrophobicity in this study. To further identify the adhesion of L. fermentum 1.2029 in vivo, mucin from small intestine of chickens was isolated, and the ability of the strain to adhere to mucin-coated surfaces in vitro was tested. Results confirmed the good adhesion ability L. fermentum 1.2029. The results of in vitro adhesion assays are believed to reflect the ability of Lactobacillus strains to persist in the gut. Hence, in this study L. fermentum 1.2029 is proposed as a probiotic candidate. However, bacteria must be capable of withstanding the harsh conditions often encountered during the passage of gastrointestinal tract. Therefore, further tests were done to assess the acid and bile salt tolerance of L. fermentum 1.2029. As mentioned, the results of the tests suggest that the strain can survive in low pH (pH 2.0) conditions, as well as in the toxicity of bile salts, indicating that L. fermentum 1.2029 possesses the potential to survive under harsh conditions present in the GI tract.

Based on the results above, L. fermentum 1.2029 as a successful candidate was fed to broiler chicken by oral gavage to study its effects on MUC2 expression in the small intestine in vivo.
The mucus layer which covers the epithelial surface of GI tract possesses lubricative and protective functions against damaging mechanical and other stresses. Mucins are the main component of mucus. There are two types of mucins: membrane-bound and secreted. In humans, nine membrane-bound (MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC16 and MUC17) and seven secreted (MUC2, MUC5B, MUC5AC, MUC6, MUC7, MUC19 and MUC20) mucins have been identified (Lang et al., 2006). Intestinal mucins are mainly produced by goblet cells which are highly polarized secretory cells present throughout the intestinal tract (Specian and Oliver, 1991). These specialized epithelial cells can synthesize and secrete mucins and other mediators such as trefoil factor 3 (Taupin and Podolsky, 2003). Thus, they play an important role in the formation of the intestinal mucosal barrier. In the present study, the oral administration of L. fermentum 1.2029 enhanced the goblet cell density in the jejunum of the broilers. Changes in goblet cell density indicate that L. fermentum 1.2029 can induce the proliferation of the epithelium, which differentiates into goblet cells, which could exert protective function.

The intestinal mucous layer is a vital component of the gut barrier. Some evidences have shown that in a pathological intestine, the thickness of the mucus layer is reduced, resulting in a closer contact between bacteria in the intestinal lumen and the intestinal epithelium, which may induce inflammatory processes in the bowel (Fyderek et al., 2009). In our study, no changes between the thickness of the mucus layer of the control and treatment groups were observed. The thickness of the mucus layer is related to the dynamic equilibrium between mucous secretion and subsequent mucus removal. Our results indicate that the oral administration of L. fermentum 1.2029 sustains the dynamic balance of the degradation and renewal of the mucus layer, and that the strain exerts protective properties in the small intestine.

MUC2 mRNA has been localized in the small intestine and colon, particularly in goblet cells, of a variety of animals (Chang et al., 1994). The protective function of MUC2 in the intestine is actually proven. In mice, MUC2 can build a mucus barrier that separates bacteria from epithelia. Bacteria in MUC2-deficient mice have direct contact with epithelial cells and more easily trigger an inflammatory response (Johansson et al., 2008). MUC2-dependent mucus production is critical for the effective management of both pathogenic and non-pathogenic bacteria during infections caused by EPEC/EHEC-like pathogens (Bergstrom et al., 2010).

In vitro and in vivo studies have shown increased production of mucin after treatments with several strains of Lactobacillus, which indicates that probiotics can stimulate the up-regulation of MUC2 gene expression in Caco-2 cell culture models, and induce MUC2 gene expression and secretion by colonic epithelial cells (Mack et al., 1999; Mattar et al., 2002; Caballero-Franco et al., 2007). Mucin synthesis depends on mucin mRNA expression. In the current study, the administration of L. fermentum 1.2029 significantly increased the synthesis of mucin in the jejunum and ileum of broilers according to the results of quantification of MUC2 mRNA expression. These results showed that L. fermentum 1.2029 could enhance the maintenance and function of the small intestinal barrier of broilers. Intestinal epithelial cells are the most important target cells of probiotic action (Lebeer, et al., 2008). An increase in the level of MUC2 mRNA may directly influence the interaction between Lactobacillus and hosts, and initiate host response. Considering the observed adhesive properties of L. fermentum 1.2029, L. fermentum 1.2029 possibly binds to specific receptor sites on enterocytes and stimulates the expression of mucin, although L. fermentum effector molecules and their corresponding receptors are unknown from this study. Intestinal microbiota and their products can also influence the composition and production of mucin (Bry, et al., 1996; Sharma and Schumacher, 1995), and the alteration in the expression of MUC2 in the intestines of broilers is due to L. fermentum 1.2029 may change the microbial environment of the intestine and the metabolic activity of microbiota.

In conclusion, the results of the current study show that L. fermentum 1.2029 could protect the mucosa of the small intestinal of broilers by stimulating mucin production. This work also suggests that the strain has the potential to be a probiotic feed additive for improving the intestinal function of chickens.

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