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Comparative evaluation of *Lactobacillus* strains with different adhesion ability on growth performance and immunomodulatory activity in broiler chickens

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ABSTRACT

The study was designed to assess the effect of 2 *Lactobacillus* strains (*Lactobacillus kefiri* 1.3207 and *Lactobacillus plantarum* 1.2567) with different adherence ability on growth performance and immunomodulatory activity in broiler. The BW and FCR were higher in *L. kefiri* 1.3207 and *L. plantarum* 1.2567 groups compared to control group at 42 days of age, and BW of broilers in *L. kefiri* 1.3207 group was significantly higher than that in *L. plantarum* 1.2567 group. IgA and IgG contents and the spleen and bursa of Fabricius indices in significantly increased in the *L. kefiri* 1.3207 group, but not in the *L. plantarum* 1.2567-treated group. *L. kefiri* 1.3207 had more significant effect on growth performance, plasma IgA and IgG levels and immune organs indices because it had better adhesion ability.

Key words: Adherence ability, Growth performance, Immune organs indices, *Lactobacillus*

Probiotics are defined as live microbiota, which when administered in adequate amounts confer a health benefit on the host (Reid and McCormick 2002). These microorganisms can improve the nutritional status of the host species by producing digestive enzymes and promote their growth and survival rates (Giri *et al*. 2013, Kritas *et al*. 2015).

The genera *Lactobacillus* are considered beneficial micro-organisms that have been used as a dietary supplement for improving animal health and performance (Pourgholam *et al*. 2016, Trabelsi *et al*. 2016). Numerous experiments have demonstrated that *Lactobacillus* can stimulate the immune system of host, by both enhancing maturation of the innate and adaptive immune systems (Kotzampassi and Giamarellos-Bourboulis 2012).

As potential probiotic strain, a key property of selected *Lactobacillus* is acid and bile tolerance to ensure their survival in intestine. To exert an effective probiotic function, bacterial maintenance in the gastrointestinal tract is necessary. Adhesiveness is also a significant criterium to choose a probiotic strain (Mishra and Prasad 2005). Adherence and colonization of probiotic are probably of crucial importance for their beneficial effect (Nielsen *et al*. 1994).

Most of the earlier studies showed that the individual and combined use of various *Lactobacillus* species increased the growth performance, feed utilization efficiency and the levels of immunity in the host (Peng *et al.* 2016, Pourgholam *et al*. 2016, Trabelsi *et al*. 2016). However, these reports did not address whether these effects of *Lactobacillus* are associated with its adherence ability.

L. plantarum 1.2567 and *L. kefiri* 1.3207 are the two acid and bile-tolerant strains. However, *L. kefiri* 1.3207 has more strong adhesion capacity compared with *L. plantarum* 1.2567. In this study, we hypothesized that *L. kefiri* 1.3207, which has more strong adhesion capacity, may have better efficacy. To address this hypothesis, we compared effect of the 2 *Lactobacillus* on growth performance and immunomodulatory activity in broilers.

MATERIALS AND METHODS

Preparation of bacteria: L. kefiri 1.3207 and *L. plantarum* 1.2567 were obtained from China General Microbiological Culture Collection Center (Beijing) and were cultured on de Man, Rogosa, and Sharp (MRS) broth in anaerobic conditions at 37°C to the stationary growth phase. The organisms were cultured anaerobically on peptone yeast agar for 18 h at 37°C, then aseptically inoculated into liquid medium and incubated anaerobically for 8 h at 37°C. Bacteria were harvested by centrifugation at 6,000 g for 10 min and resuspended in phosphate buffered saline (PBS) prior to use.

Acid and bile salt tolerance test: The 2 *Lactobacillus* were determined for their ability to resist low pH and bile salt. The bacteria were cultivated for overnight in MRS medium, then approximately 10^9 CFU/ml bacteria was inoculated into 10 ml sterile MRS broth of pH 2 (adjusted with 1 N HCl). The tubes were incubated at 37°C for 2 h

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and the number of viable cells 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:

$$\% \text{ survival} = Nf/Nb \times 100 \quad (1)$$

where, Nf, log CFU/ml after treatment; and Nb, log CFU/ml before treatment.

Tolerance to bile salts was verified as mentioned in the acid tolerance method. Overnight grown cultures of bacteria were inoculated in 10 ml sterile MRS broth (pH 6.8) containing 1.0% (w/v) bile salt. Growth was monitored by determining the number of the viable cells on MRS agar medium after 2 h incubation at 37°C by the plate count method. The percentage of surviving bacteria in the test samples was calculated according to Equation 1.

Caco-2 cell culture and adhesion assay: Caco-2 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% CO_2, 95% air, and the culture medium was changed every 2 days. For the adhesion assay, the Caco-2 cells were seeded at a concentration of 10^5 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 1 h before the adhesion assay was performed. For the adhesion assay, 3 ml bacterial suspension (culture medium) (total cell count, 10^8 cells/ml) was added to washed Caco-2 monolayer on coverslips placed in a 6-well tissue culture dish. The dish was incubated (1 h, 5% CO_2, 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.

Experimental birds and design: Animal experiments were approved by Animal Ethics Committee of the University. The experiment was carried out at the experimental farm of the University.

One-day-old ArborAcres broiler chickens (216; BW=40.27±0.21 g) were obtained from a local hatchery. All chicks were weighed individually, randomly assigned to 3 treatment groups with 6 replicates of 12 broilers and fed an antibiotic-free diet. The 3 experimental treatment groups included control group, *L. kefiri* 1.3207 group and *L. plantarum* 1.2567 group. During the course of experiment, daily the control group was treated orally with PBS, *L. kefiri* 1.3207 group birds were orally administered *L. kefiri* 1.3207 (1×10^9 CFU/bird) and *L. plantarum* 1.2567 group birds were orally administered *L. plantarum* 1.2567 (1×10^9 CFU/bird).

Table 1. Ingredient composition and nutritional composition of basal diets

Ingredient and composition (%)	1 to 21 days starter (%)	22 to 42 days finisher (%)
Corn	55.13	57.70
Soybean meal	38	36
Soybean oil	3.0	3.0
Limestone	1	1
Dicalcium phosphate	1.7	1.2
NaCl	0.3	0.3
L-LysineHL	0.25	0.3
DL-Methionine	0.25	0.16
Trace mineral premix[1]	0.1	0.1
Vitamin-mineral premix[2]	0.14	0.14
Choline chloride	0.13	0.10
Total	100	100
Calculated nutrient (%)		
CP	21.09	20.03
Ca	1.05	0.85
P	0.56	0.45
Available P	0.42	0.38
Lysine	1.28	1.27
Methionine	0.52	0.48
AME (kcal/kg)	3050	3010

[1]Trace mineral premix provided/kg of diet: manganese, 88 mg; zinc, 95 mg; iron, 100 mg; copper, 12.5 mg; selenium, 0.3 mg and iodine, 0.7 mg. [2]Vitamin premix provided per kg of diet: vitamin A, 8,000 IU; vitamin D_3, 2,000 IU; vitamin E, 30 IU; vitamin K, 2 mg; thiamine, 2 mg; riboflavin, 5 mg; niacin, 0.15 mg; vitamin B_6, 4 mg; vitamin B_{12}, 0.02 mg; pantothenic acid, 40 mg; folic acid, 10 mg; and biotin, 1.0 mg.

Experimental diets were fed in 2 periods: starter (day 1–21) and finisher (day 22–42). The composition and nutrient analysis results for the basal diet are shown in Table 1. All the birds were allowed to feed and drink water *ad lib*.

Growth performance and sample collections: Broilers in every replicate from each treatment group were weighed on day one, 21, and 42. Daily feed consumption was accurately recorded. Average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) were calculated.

On d 42, two chickens from each replicate were selected, fasted for 12 h, blood samples were collected in 10 mL sterile heparinized tubes via wing vein and centrifuged at 3,000×g and 4°C for 10 min to recover plasma. Plasma samples were immediately stored at –70°C until required for the IgA assays (described below). The chickens were euthanized, and spleen, thymus and bursa of Fabricius were excised and immediately weighed. The jejunum was also removed and immediately fixed in 10% neutral buffered formalin for morphology assay.

Detection of concentration of plasma IgA and IgG in broilers: The concentration of the plasma IgA and IgG was determined using assay kits according to the manufacturer instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Detection of immune organs indices of broilers: The

spleen, thymus and bursa of Fabricius indices were expressed using the following equation:

The spleen, thymus or bursa of Fabricius index = weight of spleen, thymus or bursa of Fabricius (mg)/ body weight (g).

Statistical analysis: Statistical analyses of the data were performed using SPSS 16.0. Data are presented as means±standard deviation (SD). Differences between treatments were compared by one-way ANOVA. A P value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

In vitro *evaluation:* The results of the present study showed that both *L. kefiri* 1.3207 and *L. plantarum* 1.2567 could maintain higher survival rates in low pH and high concentration of bile salt (Table 2), suggesting that the 2 bacterial strains could be resistant to gastric stresses. Adhesion to intestinal epithelial cells is commonly evaluated by *in vitro* adhesion assays using Caco-2 cells as an intestinal cell model. In the present study, the 2 strains were able to adhere to Caco-2 (Fig. 1A, B), but *L. kefiri* 1.3207 had better adhesion characteristics than *L. plantarum* 1.2567 (Fig. 1C).

Table 2. Percentage survival of *Lactobacillus* in high bile salt and low pH

Strain	pH (pH=2.0)	Bile salt (1.0%)
L. kefiri 1.3207	81.33±3.52	75.04±3.01
L. plantarum 1.2567	81.97±3.16	74.65±2.28

Data are given as mean±SD. Difference nonsignificant.

Growth performance: The effects of different treatments on BW, ADG, ADFI, and FCR of broilers are shown in Table 3. There was no effect of treatment on the performance of broilers from day one to 21 (P<0.05). During the finisher period, broilers in the *L. kefiri* 1.3207 and *L. plantarum* 1.2567 treatments had higher BW (P < 0.05) and improved FCR (P<0.05) than broilers in the control group. Similar results were reported previously demonstrating that probiotic *Lactobacillus* supplemented to the broilers improved the body weight (Kalavathy *et al.* 2003, Liu *et al.* 2007).

Table 3. Effects of different treatments on broiler performance

Item	Experimental treatment		
	Control	*L. kefiri* 1.3207	*L. plantarum* 1.2567
d 1 to 21			
BW	781.34±12.41	835.66±14.55	812.87±16.42
ADG	34.67±0.71	35.73±1.34	36.12±1.09
ADFI	46.71±0.74	48.26±1.03	48.11±1.32
FCR	1.35±0.08	1.39±0.06	1.36±0.03
d 1 to 42			
BW/g	2361.57±50.38[a]	2546.43±37.22[b]	2429.51±41.53[c]
ADG/g	54.88±1.08	57.43±1.25	56.94±0.97
ADFI/g	95.67±2.33	96.18±1.52	96.03±1.07
FCR	1.84±0.05[a]	1.67±0.07[b]	1.71±0.02[b]

Data are given as mean±SD. Within the same row, means with different superscripts are significantly different (P<0.05).

At 42 d, BW of broilers in *L. kefiri* 1.3207 group was significantly higher than that in *L. plantarum* 1.2567 group (P<0.05). Therefore, we speculate that the better growth performance of *L. kefiri* 1.3207 might be their better adherence ability preventing their rapid removal by contraction of the gut.

Effect of *Lactobacillus on plasma IgA and IgG:* Fig.2 shows the levels of IgA and IgG measured in plasma. *L. kefiri* 1.3207 treatment influenced markedly the levels of IgA and IgG in plasma in comparison with the control group or *L. plantarum* 1.2567-treated group (P<0.05). IgA and IgG contents only were slightly increased in the *L. plantarum* 1.2567-treated group in comparison with the control group (P<0.05). IgA is the most abundant immunoglobulin in the body (Macpherson and Uhr 2004, Sun *et al.* 2012) and IgG is the main antibody isotype found in blood allowing it to control infection of body tissues. Some results showed that *Lactobacillus* could effectively stimulate the production of secretory antibodies such as IgA and IgG (Maassen *et al.* 2003), but others showed that *Lactobacillus* treatment did not increase IgA or IgG in blood (Huang et al. 2004, Mountzouris *et al.* 2010). No study

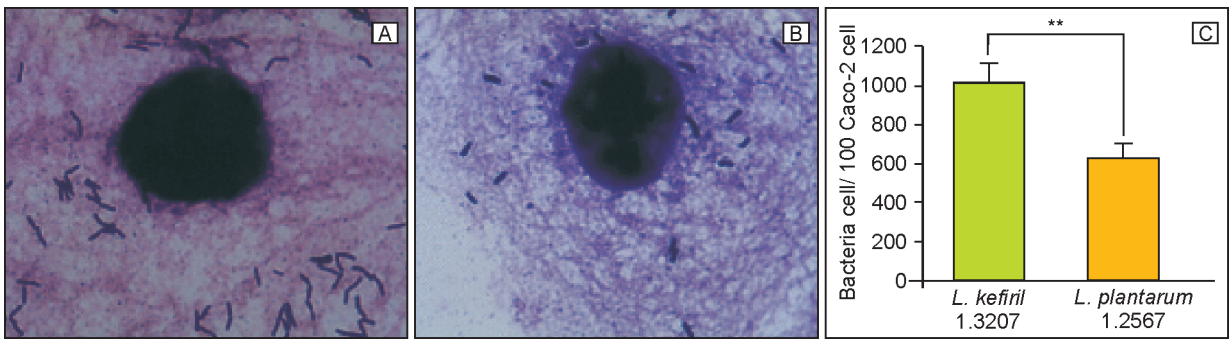

Fig. 1. Adhesion of the two *Lactobacillus* to Caco-2. **A**. Image of *L. kefiri* 1.3207 adhesion to Caco-2; **B**. Image of *L. plantarum* 1.2567 adhesion to Caco-2; **C**. adhesion capacity of the two *Lactobacillus* to Caco-2. All images are at 1,000× magnification (Original). Error bars represent standard deviations. Two asterisks (**) represents a significant difference (P<0.01).

Table 4. Effects of different treatments on immune organs indices of broilers

Item	Experimental treatment		
	Control	*L. kefiri* 1.3207	*L. plantarum* 1.2567
Spleen index	1.14±0.11a	2.11±0.13b	1.39±0.15a
Thymus index	4.47±0.12	4.60±0.17	4.28±0.16
Bursa of Fabricius index	2.47±0.10a	3.74±0.16b	2.82±0.14a

Data are given as mean±SD. Within the same row, means with different superscripts are significantly different (P<0.05).

reported whether the discrepancy among these studies stem from differences of adhesion capacity of selected strains. So, the two *Lactobacillus* with different adhesion capacity were used for further studies in the present experiment. The result showed that IgA and IgG contents were significantly increased in the *L. kefiri* 1.3207 group, but not the *L. plantarum* 1.2567-treated group. Previous work demonstrated that gut colonization of probiotics resulted in the enhancement of the antibody-mediated immune response (Rhee *et al.* 2004, Tlaskalova-Hogenova *et al.* 2004), indicating that *L. plantarum* 1.2567 could not firmly colonize the intestinal mucosa, therefore could not influence the immune-modulating activity via IgA and IgG. The results presented here support the view that mucosal bacteria make closer contact with the host than luminal ones, and therefore have a stronger health influence (Van den Abbeele *et al.* 2009).

Effect of Lactobacillus *on immune organs indices of broilers:* The immune organs indices for experiment treatments are shown in Table 4. No statistical differences were observed in the thymus index among three treatment groups (P<0.05). The spleen index and the bursa of Fabricius index were higher for birds given *L. kefiri* 1.3207 compared with *L. plantarum* 1.2567 treatment groups and control groups (P<0.05). But *L. plantarum* 1.2567 treatment had no effect on immune organs indices. The relative weights of immune organs have been considered as important indices for monitoring whole body immune response (Heckert *et al.* 2002). The previous study showed that *Lactobacillus* could stimulate immune organ development (Li *et al.* 2009, Slawinska *et al.* 2014). *L. kefiri* 1.3207 possessed stronger adhesion ability and might have stimulated and promoted growth of spleen and the bursa of Fabricius.

According to the results in the present study and the above-mentioned discussion, it can be concluded that *L. kefiri* 1.3207 had more significant effect on growth performance, plasma IgA and IgG levels and immune organs indices because it had better adhesion ability.

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