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RESEARCH ARTICLE

Comparison of Improved Effect of Antibacterial and Antiviral Activity of Four Probiotic *Lactobacillus* Expressing Porcine Lactoferrin in Mice

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A B S T R A C T

The aim of this study was to evaluate the effects of four *Lactobacillus* transformant strains on mice, including recombinant *Lactobacillus casei* (*L. casei*), *Lactobacillus pentosus* (*L. pentosus*), *Lactobacillus plantarum* (*L. plantarum*), and *Lactobacillus paracasei* (*L. paracasei*) that expressed porcine lactoferrin with a level of 20.5, 23.6, 27.2 and 21.6 μg/ml, respectively. Treatment groups of mice were fed with 200 μl of 10^9 CFU of recombinant *Lactobacillus* strain in basal diet as a feed additive, respectively, until 14 days experimental period. Compared to the control group, feeding with recombinant *L. casei*, *L. pentosus*, *L. plantarum* or *L. paracasei* improved growth performance of mice, followed by the increasing of average daily gain (ADG) by 14% and average daily feed intake (ADFI) by 6%, and the decreasing of feed efficiency (F/G) by 7%. Supplementation with recombinant *Lactobacillus* augmented the production of total IgG and sIgA by 11.2 and 33% in mice, respectively. Moreover, the levels of IL-2 and TNF-α were respectively shown increasing by 27 and 46%, and the levels of IL-4 were shown a significant reduction by 39% in mice received recombinant *Lactobacillus* strains. In the intestinal tract of treatment groups of mice, the total viable counts of *E. coli* were decreased and the total viable counts of *Lactobacillus* and *Bifidobacterium* were increased by 5 and 2.7% compared to the control. After *E. coli* K88 and porcine pseudorabies virus (PRV) challenged, approximate 10 and 30%, respectively, of the control mice exhibited abnormal health with the symptoms that feeble body, relatively dark hair, loss of body weight, and death, which did not observed in the mice received the recombinant *Lactobacillus* expressing porcine lactoferrin. In contrast, the recombinant *L. pentosus* and *L. plantarum* treatment expressing porcine lactoferrin showed a better beneficial effect on the health of animal.

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INTRODUCTION

Lactic acid bacteria (LAB), a kind of Gram-positive bacteria that can metabolize carbohydrates into lactic acid, exist widely in the digestive tracts of human and animals and have important physiological functions. *Lactobacillus* has a wide range of biological functions, including resistance to pathogens, antitumor activity, and immunity enhancement (Saunier and Dore, 2002). Moreover, *Lactobacillus* not only survive in the animal gastrointestinal tract but are beneficial to maintain intestinal microflora balance (Kekkonen *et al*., 2008). In recent years, LAB have been generally recognized as safe and food-grade microorganism with local mucosa engraftment characteristics, which have been applied widely in the field of vaccine development, and the advantage of LAB used as vaccine carriers to deliver and express exogenous genes has been highlighted (Grangette *et al*., 2001; Corthésy *et al*., 2005).

Lactoferrin (LF) is a natural iron-binding glycoprotein that widely exists in biological fluids and secretions (García-Montoya *et al*., 2012). Studies have shown that the LF has many physiological functions, such as bacteriostatic activity (Rodriguez-Franco *et al*., 2005; Yin *et al*., 2014), antiviral activity (Weng *et al*., 2005; Francesca *et al*., 2011; Gualdi *et al*., 2013; Picard-Jean *et al*., 2014), iron homeostasis regulation through promotion of the transfer and absorption of iron, and immune function regulation (Legrand *et al*., 2010; Azmat *et al*., 2012).

In this study, four probiotic lactobacillus that *Lactobacillus pentosus* KLDS1.0413, *Lactobacillus plantarum* KLDS1.0344, and *Lactobacillus paracasei* KLDS1.0652 isolated by the Key Lab of Dairy Science, Ministry of education, Harbin, China, and *Lactobacillus casei* ATCC393 kept in our lab were used to construct transformant strains expressing porcine lactoferrin (pLF), which were orally administered to mice. A mouse model was established to study and compare the protective effects of these recombinant lactobacilli for animal. Interest has been given recently to the potential benefits of probiotic transformant as a bioreactor for the production of lactoferrin. *Lactobacillus* transformant producing lactoferrin used as an antimicrobial agent in diets for animals may be a cost-effective alternative due to its additional biological functions and wider applicability.

MATERIALS AND METHODS

Construction of four recombinant *Lactobacillus* strains expressing pLF: The DNA encoding pLF (2016 bps, GenBank accession number KJ003838) was synthesized (Sangon Biotech, China), and cloned into the secreting shuttle expression plasmid pPG612.1 (a kind gift of Prof. Seegers), giving rise to pPG612.1-pLF (Fig.1). Then, the plasmid pPG612.1-pLF was electro-transformed into *L. casei*, *L. pentosus*, *L. plantarum*, and *L. paracasei* as the method previously described (Spath *et al*., 2012), generating four recombinant lactobacillus strains. The cell lysates of each recombinant strain and its culture supernatant were analyzed via 12% SDS-PAGE, followed by a fully wet transfer to a PVDF membrane, and was incubated with rabbit anti-pLF antibody (NEAU, Harbin, China), then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Qcbio Science & Technologies, China). The level of secretion of the pLF protein in the culture supernatant was quantitatively analyzed by ELISA (Chen *et al*., 2006).

Animals and experimental design: BALB/c mice (4-5 week-old, weighing 10-14 g) purchased from the Harbin Veterinary Research Institute were divided into 5 dietary treatments in average. Of which, four groups of mice (24 mice for each group) daily received 200 µl of 10^9 CFU of recombinant *L. casei*, *L. Pentosus*, *L. plantarum* and *L. Paracasei*, respectively; another group of 24 mice received PBS used as control. There were three replicate groups per treatment. Food and water were available to the mice ad libitum. Growth performance of each group of mice recorded as ADG, ADFI, and F/G was assessed at the end of 14-day experimental period. And then, the mice in each group were randomly sampled to prepare the samples of blood, intestinal mucus and proximal colonic contents (Blake *et al*., 2003).

Measurement of IL-2, IL-4, TNF-α, IgG in serum and sIgA in intestine mucosa: The concentrations of IgG, secretory immunoglobulin A (sIgA) and inflammatory factors including interleukin-2 (IL-2), interleukin-4 (IL-4) and tumor necrosis factor α (TNF-α) were determined by ELISA kit (Shanghai Meilian Biological Technology, China).

Intestinal microbial population: one gram of intestinal contents was fully mixed with 10 ml of anaerobic dilution solution (ADS). The total numbers of anaerobic bacteria were determined by standard plate count method using ADS as dilution solution (Meng and Gu, 2011). At the same time, aerobic bacterial populations were counted by plate count method using PBS as serial dilution solution (Wang *et al*., 2007).

Animal infections and evaluation: The mice in each dietary treatment group were divided into two groups in average, respectively. One group of mice received 200 µl of 2×10^7 CFU/ml (LD50) of *E. coli* K88 and another group of mice received $10^{-4.5}$ dilution ratio (LD50) of PRV. For infection and pretreatment inoculations, a gavage needle was used. Mice health was monitored daily until 7 days observation period. The incidence of clinical signs was determined by comparing the behavior of each infected mouse to the behavior of a healthy mouse for 15 min (Mosquito *et al*., 2010). A scoring system was established to define the severity of illness after challenge (Yen *et al*., 2009), which was designed to produce uniformity among observers. These mice were examined twice daily at 10:00 AM and 8:00 PM.

Statistical analysis: Repeated measures ANOVA models were used to test the statistical significance between groups. All statistical analysis was performed with SPSS Statistics17.

RESULTS

pLF protein expression in four *Lactobacillus* transformant strains: As shown in Fig. 2, an approximate 73 kDa interest protein was efficiently expressed in cell lysates and culture supernatants of four recombinant *Lactobacillus* strains cultured for 24 h. The expression levels of recombinant *L. casei*, recombinant *L. pentosus*, recombinant *L. plantarum*, recombinant *L. paracasei* was 20.5 µg/ml, 23.6 µg/ml, 27.2 µg/ml and 21.6 µg/ml, respectively.

Growth performance: The growth performance of mice was improved by supplementation with the recombinant *Lactobacillus* expressing pLF in basal diet as a feed additive (Table 1). Compared to the control, feeding these four recombinant *Lactobacillus* to mice could increase the average daily gain (ADG) and average daily feed intake (ADFI) of mice, and decreased the feed efficiency (F/G). There were no significant differences between these four recombinant *Lactobacillus* treatments.

Immune indices: The levels of cytokines in the serum and intestinal mucosa of mice were determined. As shown in Table 2, the total levels of IgG and sIgA were increased in mice received recombinant *Lactobacillus* strains. Compared to the control group of mice that received PBS only, the levels of IL-2 ($P<0.05$) and TNF-α ($P<0.01$) were shown increasing in recombinant *Lactobacillus* treatment groups, and a significant reduction ($P<0.01$) of the IL-4 levels was shown in recombinant *Lactobacillus* treatment groups. The total cytokines in the serum and the intestine mucosa were no significant differences observed among the different recombinant *Lactobacillus* treatment groups.

Table 1: Effect of recombinant *Lactobacillus* expressing pLF on the growth performance of mice

Item	Control	*L. casei*	*L. pentosus*	*L. plantarum*	*L. paracasei*
ADG (g)	0.51±0.01	0.57±0.02[a]	0.57±0.02[a]	0.58±0.01[a]	0.57±0.02[a]
ADFI (g)	4.94±0.01	5.23±0.11[a]	5.21±0.16[a]	5.24±0.13[a]	5.23±0.02[a]
F/G	9.69±0.39	9.18±0.17[b]	9.14±0.29[b]	9.03±0.29[b]	9.18±0.07[b]

ADG=Average daily gain; ADFI=Average daily feed intake; F/G=Average daily feed intake/Average daily gain. Values (mean±SE) bearing [a](P<0.05) or [b](P<0.01) in a row differ significantly.

Table 2: Concentrations of IL-2, IL-4, TNF-α, IgG in serum and sIgA in intestine mucosa

Item	Control	*L. casei*	*L. pentosus*	*L. plantarum*	*L. paracasei*
IgG (μg/ml)	868.0±29.31	954.0±48.51[a]	963.0±52.72[a]	965.3±35.73[a]	954.0±42.04[a]
IL-2 (pg/ml)	28.34±0.87	33.04±1.55[a]	35.57±0.94[a]	35.91±2.45[a]	34.45±1.76[a]
IL-4 (pg/ml)	21.17±0.45	13.39±1.36[b]	12.80±1.11[b]	12.79±.49[b]	14.99±0.80[b]
TNF-α (pg/ml)	16.80±0.88	23.47±2.92[a]	24.55±1.94[a]	23.77±2.27[a]	23.55±2.38[a]
sIgA (μg/ml)	3.47±0.15	4.37±0.15[a]	4.56±0.25[a]	4.60±0.10[a]	4.20±0.36[a]

Values (mean±SE) bearing [a](P<0.05) or [b](P<0.01) in a row differ significantly.

Table 3: Effect of recombinant *Lactobacillus* expressing pLF on the intestinal microflora[1] of mice

Item	Control	*L. casei*	*L. pentosus*	*L. plantarum*	*L. paracasei*
Escherichia coli	5.21±0.081	4.47±0.37[b]	4.33±0.11[b]	4.23±0.07[b]	4.6±0.23[b]
Lactobacillus	6.30±0.11	6.55±0.17[a]	6.57±0.10[a]	6.58±0.11[a]	6.59±0.15[a]
Bifidobacterium	6.29±0.07	6.44±0.07[a]	6.46±0.09[a]	6.46±0.06[a]	6.45±0.09[a]

[1]Bacterial numbers are expressed as the $\log_{10}$ of colony-forming units per gram of dry matter of intestinal contents. Values (mean±SE) bearing [a](P<0.05) or [b](P<0.01) in a row differ significantly.

Table 4: The clinical illness scores of mice orally challenged with *E. coli* K88

Item	Control	*L. pentosus*	*L. casei*	*L. plantarum*	*L. paracasei*
0 (Normal)	3	6	5	6	5
1 (Slightly ill)	2	1	3	2	2
2 (Moderately ill)	1	2	1	2	2
3 (Dying)	2	1	1	0	1
4 (Dead)	2	0	0	0	0

Score 0: normal breathing, activity, color, and suckling; copious milk in the stomach; Score 1: pale, but perfusion acceptable, less activity, rapid breathing, gastric milk present (1 or more required); Score 2: pallor or gray color, abnormal breathing, reduced activity, decreased suckling and gastric milk, diminished skin turgor (2 or more required); Score 3: cyanosis and poor perfusion, labored breathing, marked lethargy, no righting response, shaking, no gastric milk, poor skin turgor, dehydration; Score 4: no signs of life, or rigor mortis. Same as below Table 5.

Table 5: The clinical illness scores of mice infected with PRV

Item	Control	*L. pentosus*	*L. casei*	*L. plantarum*	*L. paracasei*
0 (Normal)	2	6	5	6	5
1 (Slightly ill)	4	2	2	2	2
2 (Moderately ill)	2	1	2	2	1
3 (Dying)	2	1	1	0	2
4 (Dead)	1	0	0	0	0

Effect of recombinant *Lactobacillus* on the intestinal microflora: Results showed that recombinant *Lactobacillus* strains expressing pLF displayed an effective antibacterial activity *in vivo* (Table 3). The *E. coli* flora was significantly suppressed (P<0.05) in the intestinal tract of the mice receiving the recombinant strains and there was a significant increase in the number of *Lactobacillus* and *Bifidobacterium* (P<0.05) compared to the control. There were no significant differences between recombinant *L. casei*, *L. pentosus*, *L. plantarum*, *L. paracasei* treatments.

Prevention of microbial toxicity by recombinant *Lactobacillus* expressing porcine lactoferrin based on illness score: The magnitude of illness after infection was defined by a scoring system that ranged from a score of 0 indicating healthy animals to a score of 4 indicating death. The scoring system was designed to produce uniformity among observers. Results showed that approximate 10% and 30% of the control groups of mice developed into , obvious clinical symptoms including a feeble body, relatively dark hair, loss of body weight and death post *E. coli* K88 and porcine pseudorabies virus (PRV) challenges (Table 4 and Table 5). None or few of the mice received recombinant *Lactobacillus* expressing pLF showed such symptoms.

DISCUSSION

In the present study, the results showed that the recombinant *L. plantarum* displayed a higher expression efficiency of interest protein pLF (27.2 μg/ml) than that of recombinant *L. casei*, *L. pentosus* and *L. paracasei* strains, while there was no significant difference observed among these three recombinant strains, which indicated that the replication, transcription and translation of exogenous genes in *L. plantarum* were different from the other three *Lactobacillus* strains. Moreover, the metabolic utilization degree of recombinant strains to each inducing agent was different, resulting in different expression efficiency.

Serum immunoglobulin IgG antibodies are the most important and stable antibodies in the mammalian primary immune response which exist only as monomers and are comprised of mostly antibacterial, antitoxic, and antiviral components, playing a central mediator to combat infections (Schwab and Nimmerjahn, 2013). Without the involvement of the systemic immunity, local immune system of the digestive tract can be stimulated by foreign antigens to result in the production and secretion of antibodies, known as sIgA, which can prevent pathogens from invading at local mucosa tissue. Therefore, the content of immunoglobulin in body could reflect the ability to fight against external pathogenic microorganisms and viruses. The function of cytokines in the regulation and modulation of the immune response has been highlighted. IL-2, as the indicator of predominantly cell-mediated responses, is recognized as a key cytokine in the Th1-type response. IL-4 secretion suggests a predominant humoral response, because it plays a role in Th2 polarization (Raymond and Wilkie, 2004; Duc,

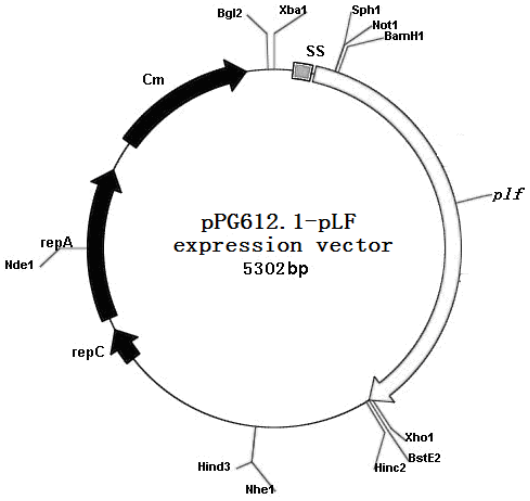

Fig. 1: Map of pPG612.1-pLF expression vector. Cmr resistance determinant; repA and repC replication elements; xylP-promoter and multiple cloning sites are shown. The signal sequence is translationally fused to the xylP-promoter. The pLF gene was cloned into the sites of *BamH-Xho*.

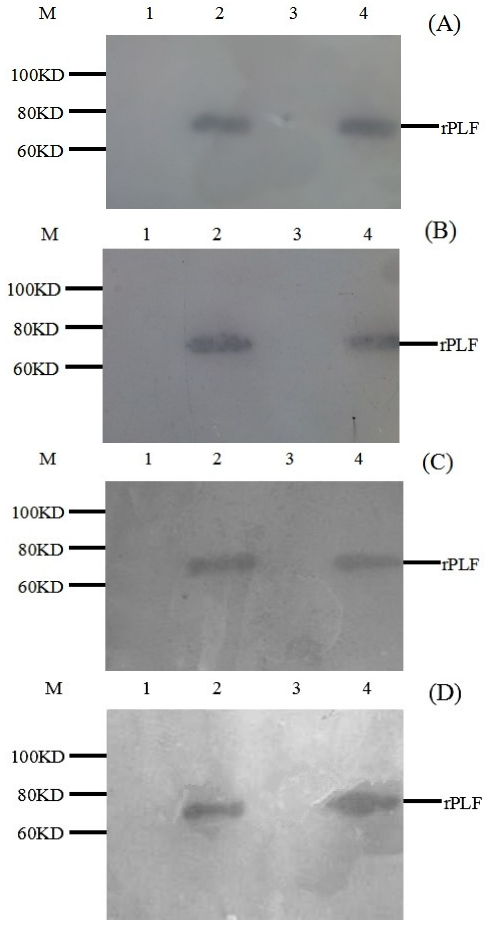

Fig. 2: Western blot analysis of rpLF expression by the four strains of *Lactobacillus* transformant. A: Recombinant *L. plantarum*; B: Recombinant *L. pentosus*; C: Recombinant *L. casei* ; D: Recombinant *L. paracasei.* The expression of rpLF with a molecular weight of approximate 73 kDa was determined in cell extracts of rpLF-expressing *Lactobacillus* transformant (lane 2) and bacterial culture supernatants (lane 4). Lane 1: Lysate of *Lactobacillus* harboring pPG612. 1. Lane 3: culture supernatant of *Lactobacillus* harboring pPG612.1.

2011). The up-regulated expression of Th2 mRNA is associated with an impaired Th1 profile, which suggests that a more significant Th2-type immune response may be induced by pLF colostrums supplementation. In this study, compared to the control group of mice, the ADG of mice receiving recombinant *Lactobacillus* treatment was significantly increased and the F/G was significantly decreased. Moreover, in the recombinant *Lactobacillus* treatment groups, the levels of total IgG, sIgA, IL-2, and TNF-α were significantly increased and the level of IL-4 was shown a significant reduction. The LF bound to the LF receptor expressed on T cells to stimulate the differentiation of T cells and promote the secretion of IL-2. Then, The IL-2 secretion further promoted the activation and proliferation of T cells. In macrophages, the LF bound to the LF receptor to stimulate the secretion of IL-1. Additionally, binding of recombinant LF to its receptor on B cells promoted the production of IgG and sIgA, and ultimately improved the overall immune function of the mice. Taken together, our results suggest that recombinant *Lactobacillus* expressing pLF has an immunomodulatory effect.

LF plays a prominent role in the host innate immune defense mechanism by inhibiting the growth of bacteria in the gut and helping the body sustain a gastric mucosal protective layer (Ramanathan *et al*., 2002; Flores-Villaseñor *et al*., 2010; Yeom, *et al*., 2011). Studies have shown that the LF protein has an inhibitory effect on other bacteria *in vitro* (Orsi, 2004). The bacteriostatic effect of LF depends on the degree of iron saturation, the microbial demand for iron, the exogenous bioavailability of iron, and other factors, such as the concentrations of salt, antibodies, and other immune substances (Chen *et al*., 2013; Toet *et al*., 2014). Therefore, LF has no inhibitory effect on lactic acid bacteria. In addition, LF could prevent bacterial biofilm development (Singh *et al*., 2002). Our results showed that supplementation with recombinant *Lactobacillus* expressing pLF in basal diet of mice as a feed additive can effectively inhibit pathogenic bacteria in the intestinal tract of mice, such as *E. coli*, and promote beneficial bacteria growth, such as *Lactobacillus* and *Bifidobacterium*, suggesting a promising alternative to antibiotics used for animals which reduce not only the pathogenic bacteria but also the beneficial bacteria (Blake *et al*., 2003). Moreover, the challenge experiments indicated that the recombinant *Lactobacillus* treatment can provide effective antiviral and antibacterial protection for animal *in vivo*. The LF and *Lactobacillus* together have been shown to contribute to additional biological functions (Chen *et al*., 2010), as the findings reported earlier (Wang *et al*., 2007). Additionally, the strategy using recombinant *Lactobacillus* to produce pLF protein may be a more convenient, effective, and beneficial approach.

Conclusion: Recombinant *Lactobacillus* expressing pLF used as a feed additive has a variety of biological activities due to the function of LF and beneficial effects of *Lactobacillus*, suggesting a promising application in animal husbandry.

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