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LACTOBACILLUS PENTOSUS EXPRESSING PORCINE LACTOFERRIN ELEVATES ANTIBACTERIAL ACTIVITY AND IMPROVES THE EFFICACY OF VACCINATION AGAINST AUJESZKY'S DISEASE

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In this study, *Lactobacillus pentosus* expressing porcine lactoferrin (pLF) was tested for *in vitro* antibacterial activity and for its ability to enhance immunity induced by an orally administered Aujeszky's disease virus (ADV) vaccine. The cDNA encoding N-terminus of pLF was cloned into a *Lactobacillus*-specific plasmid to produce *L. pentosus* pLF expressing transformants (pPG612.1-pLF-N/*L. pentosus*). The antimicrobial activity of the recombinant pLF protein inhibited bacterial growth *in vitro*. The supernatant of pPG612.1-pLF-N/*L. pentosus* had an inhibitory effect on *Staphylococcus aureus* strain CVCC26003, *Bacillus subtilis* strain CVCC63501, *Escherichia coli* strain CVCC10141 and *Salmonella enterica* ssp. *enterica* Choleraesuis strain CVCC79102, while it did not inhibit the growth of *Lactobacillus casei* strain ATCC393. A mouse model was established to test the effectiveness of the orally administered probiotic *L. pentosus* recombinant strain in the gastrointestinal tract. Mice were immunised with an attenuated porcine Aujeszky's disease virus (ADV) vaccine. Serum antibody levels determined using a mouse Aujeszky's disease IgG ELISA showed that IgG levels were significantly higher in the pPG612.1-pLF$_N$/*L. pentosus* group than in the PBS and *Lactobacillus pentosus* groups at days 7 and 21 ($P < 0.01$) and at day 14 ($P < 0.05$), indicating that this oral recombinant strain can improve the effectiveness of the vaccine and play a role in immune enhancement through humoral immunity. These results suggest that the recombinant *Lactobacillus pentosus* not only has the beneficial characteristics of lactic acid bacteria but also produces biologically functional lactoferrin.

Key words: Porcine lactoferrin, recombinant *Lactobacillus pentosus*, antibacterial activity, immune enhancement

Lactoferrin (LF) is an 80-kDa iron-binding glycoprotein. It is abundantly found in exocrine secretions of mammals and especially in milk and fluids of the digestive tract released by mucosal epithelia and neutrophils during inflamma-

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tion (Naot et al., 2005; Bhatia, 2011). It is well known that LF exerts a direct antimicrobial effect at the surface epithelia by killing microbes or inhibiting their proliferation and adhesion (Puddu et al., 2011). The antimicrobial properties of LF are mainly related to its ability to bind with iron in biological fluids to damage the membranes of microorganisms. However, iron-independent microbicidal activities require a direct interaction between LF and microbial surface components (Miller et al., 2008). In addition to the antimicrobial properties of LF, its ability to modulate the overall immune response and to elevate immunity against viral infections has been largely described (Wolf et al., 2007; Actor et al., 2009; Ishikawa et al., 2013). In this respect, it is noteworthy that LF can promote the recruitment and activation of antigen-presenting cells and the antigen-specific immune response (de la Rosa et al., 2008).

Due to its extensive biological activity, LF is widely used in high-efficiency iron supplements, feed additives, immune modulators, antioxidants and other agents. However, the traditional separation and purification of LF from the colostrum is a cumbersome process; therefore, the use of genetic engineering methods to produce recombinant LF has become a main focus of this technology (Wang et al., 1997; Wang et al., 2002; Pecorini et al., 2005; Chen et al., 2008). In genetically engineered cells, whether they are bacterial, yeast or animal cells, the expression and collection of exogenous protein usually require purification (Garcia-Montoya et al., 2013). The tedious purification process has been a barrier to applying genetically engineered cells in production.

LF does not inhibit the low iron demand of lactic acid bacteria and it can also promote the growth of beneficial bacteria such as bifidobacteria and lactobacilli in the gut of animals. Many studies have shown that the use of the probiotic *Lactobacillus* as a vector delivery system has unmatched superiority compared to other known eukaryotes and prokaryotes (Wyszynska et al., 2015). In this research, using lactating porcine mammary tissue RNA as a template to obtain the N terminus pLF gene, we successfully obtained recombinant lactic acid bacteria expressing the pLF gene. Furthermore, we studied the antibacterial activity of *Lactobacillus pentosus* expressing porcine lactoferrin against different bacteria and its effects on immunity induced by ADV vaccine in a mouse model.

Materials and methods

*Construction of recombinant pPG612-pLFN/*Lactobacillus pentosus

The DNA insert containing codon-optimised pLF-N gene (1038 bp of the 5' terminus of porcine lactoferrin nucleotide) was synthesised by Sangon Biotech Co. Ltd. (Shanghai, China). The synthetic fragment was digested with BamHI and XhoI restriction enzymes and cloned into the plasmid vector pPG612.1. Cloning of the pPG612.1-pLF-N expression construct was performed as described in Mo-

lecular Cloning: A Laboratory Manual (Sambrook and Russell, 2001). A pair of primers was designed to identify the recombinant plasmid containing the pLF sequence: forward (5'-GGATCCAATGAAACTGTTTATTCCG-3') and reverse (5'-CTCGAGCAGGCCCTGAATGGCGGT-3'). The optimised pLF insert was subsequently cloned into the multiple cloning site (MCS) of a 5174-bp expression vector pPG612.1 (Lactrys, Denmark) that was linearised with BamHI and XhoI. The resultant construct known as pPG612.1-pLF-N (Fig. 1) contains the xylose operon promoter, a secretion signal peptide USP45, a repA replicon, and a chloramphenicol resistance (Cmr) gene for selection. The expression construct was then transformed into *Lactobacillus pentosus* (KLDS1.0413 strain, from Key Lab of Dairy Science, Ministry of Education, Harbin, China) competent cells via electroporation. DNA sequencing confirmed the identity of the plasmid. The original pPG612.1 vector without the pLF-N insert was also transformed into *L. pentosus* to serve as control. The operations described in this section were performed as described by Zong et al. (2011). The recombinant strains were named pPG612.1-pLF-N/*L. pentosus* and pPG612.1/*L. pentosus*, respectively.

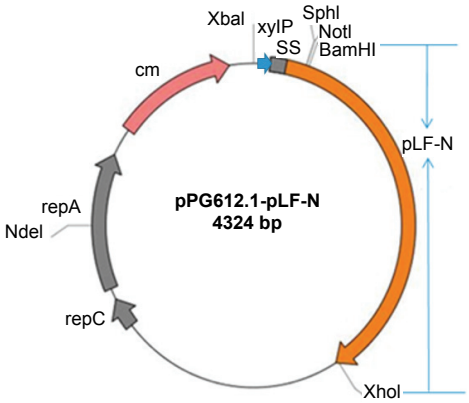

Fig. 1. Map of pPG612-pLF-N. Cm^r resistance determinant, repA and repC replication elements, xylP promoter and multiple cloning sites are shown. The signal sequence is translationally fused to the xylP promoter. The pLF-N gene was obtained from mammary gland tissue of a lactating sow on the third day of lactation by RT-PCR and then, according to the codon bias of *Lactobacillus*, the porcine lactoferrin N gene was codon-optimised and synthesised

Protein expression and localisation analysis

To analyse the expression and localisation of the pLF-N fusion protein by xylose induction, cultures of strains pPG612.1-pLF-N/*L. pentosus* and pPG612.1/*L. pentosus* were grown overnight in basal MRS medium supplemented with xylose to induce the expression of the recombinant protein and then collected by centrifugation at 12,000 *g* for 10 min. The pellets were washed twice with sterile 50 mM Tris-Cl, pH 8.0, and lysed in a Bead-Beater (Biospec, Bartlesville, OK)

by vigorous shaking. The lysates were centrifuged at 15,000 *g* for 10 min and the supernatants were examined using 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electro-transferred onto a nitrocellulose membrane and the immunoblots were developed using rabbit anti-pLF-N serum at a dilution of 1:500 with phosphate-buffered saline (PBS). Horseradish peroxidase- (HRP-) conjugated goat anti-rabbit IgG (Sigma-Aldrich Co, St. Louis, MO, USA) diluted at 1:2000 was used and the immunolabelled bands were then visualised using the Chemiluminescent Substrate reagent (Pierce™, Thermo Fisher Scientific Inc, Waltham, MA, USA) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA) was used to analyse the secreted protein from the recombinant *L. pentosus*. The induced (+) and non-induced (–) supernatants of pPG612.1-pLF-N/*L. pentosus* were collected by centrifugation. Polystyrene microtitre plates were coated overnight at 4 °C either with the supernatant of pPG612.1-pLF-N/*L. pentosus* or with supernatants harvested from pPG612.1/*L. pentosus* as negative control. ELISA plates were washed three times with PBS containing 1% Tween 20 and then blocked with PBS containing 5% skim milk for 2 h. Rabbit anti-pLF sera (produced and stored in our laboratory) were serially diluted in PBS 1% BSA and incubated at 37 °C for 1 h, washed three times and then incubated with a 1:2000 dilution (100 µL) of an HRP-conjugated goat anti-rabbit IgG (Sigma-Aldrich Co, St. Louis, MO, USA), washed and visualised following the addition of 100 µl of *o*-phenylenediamine dihydrochloride substrate (Sigma-Aldrich Co, St. Louis, MO, USA). The absorbance was measured at 490 nm.

In vitro *antibacterial activity assays*

Determination of the bacterial inhibition curve of the recombinant pLF protein produced from *L. pentosus* transformant was performed using a standard protocol with an inoculum of 1×10^3 bacteria colony-forming units per ml in MRS medium (for lactobacillus) or Luria-Bertani medium (for other tested bacteria), as described earlier (Chen et al., 2004). The tested bacteria include *Staphylococcus aureus* strain CVCC26003, *Bacillus subtilis* strain CVCC63501, *Escherichia coli* strain CVCC10141, *Salmonella enterica* ssp. *enterica* Choleraesuis strain CVCC79102 and *Lactobacillus casei* strain ATCC393.

Determination of the effect of pLF-N on the immune response

In order to assess the immune-modulating effects of recombinant pLF-N protein and to determine whether it can enhance the immunising effect of vaccines, we used a mouse model to study immunity against ADV virus infection.

A commercial attenuated porcine Aujeszky's disease virus vaccine (China Animal Husbandry Industry Company Ltd., China) was inoculated into 6–8

weeks old BALB/c mice (purchased from Harbin Veterinary Research Institute) housed in individual ventilation cages (IVCs; temperature, 32.70 ± 2.99 °C; relative humidity, 77.54 ± 2.34%; THI, temperature-humidity index, 87.27) and fed *ad libitum*. The mice (n = 120) were maintained under specific pathogen free conditions and received a sterile rodent food and water *ad libitum.* The experiment was approved by the Animal Research and Care Committee of Northeast Agricultural University, China. The mice were divided into three (PBS, *L. pentosus* and *L. pentosus*/pPG612.1-pLF-N) groups of 10 animals each. They were fed 2×10^9 cfu of the recombinant strain or *L. pentosus* for 21 days. Twelve randomly selected mice from each treatment (4 mice per treatment) were anaesthetised by isoflurane inhalation and blood samples were collected from their tail vein. Sera were collected at days 0, 7, 14, 21, 28, 35 and 42. Serum antibody levels were determined using an ADV IgG Elisa kit (The GreenSpring TM Porcine Pseudorabies ELISA Test Kit, Shenzhen Lvshiyuan Biotechnology Co. Ltd., China) according to the manufacturer's instructions.

Statistical analysis

The SPSS Statistics v17.0 software (International Business Machines Corporation, New York, USA) was used for single-factor analysis of variance. The differences between groups with probability values of less than 0.05 were considered to be statistically significant using Duncan's multiple comparisons test.

Results

pPG612-pLF-N/*L. pentosus* colonies were grown overnight in basal MRS medium (without glucose, and substituted by xylose). The cell lysates were analysed by SDS-PAGE and Western blot using rabbit anti-pLF serum. Coomassie blue gel staining showed an approximately 45 kDa fusion protein band in pPG612-pLF-N/*L. pentosus* (Fig. 2, lane 2) but not in *L. pentosus* KLDS1.0413 with pPG612.1 (Fig. 2, lane 1) or wild-type *L. pentosus* KLDS1.0413 (data not shown). Similarly, an immunoreactive band was detected (Fig. 3, lane 2) by Western blot in a similar position as observed in the SDS-PAGE in pPG612-pLF-N/*L. pentosus* (Fig. 2, lane 2). As negative control, *L. pentosus* KLDS1.0413 with pPG612.1 did not display the corresponding immunoreactive band (Fig. 3, lane 1). These results show that the xylP promoter from pPG612.1 could efficiently induce the expression of the pLF heterologous protein.

The indirect ELISA assay was developed with mouse anti-PLF serum and HRP-conjugated goat anti-mouse IgG to confirm the presence of cell-secreted pLF-N protein. The results indicated that pLF-N protein was present in the supernatant of overnight culture while it was absent from the control bacteria (Fig. 4). The results of localisation analysis suggested that the pLF-N protein could be se-

creted by the signal peptide of the expression vector plasmid. The recombinant protein could be detected in both the culture supernatant and intracellularly.

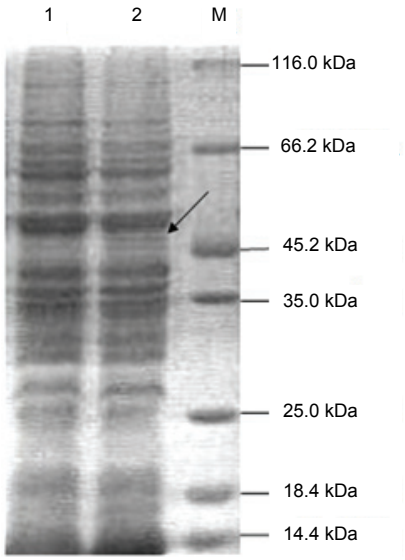

Fig. 2. Identification of the protein expressed in *Lactobacillus pentosus* by Coomassie blue gel staining. Lane 1: pPG612.1/*L. pentosus* induced by xylose; lane 2: recombinant strain pPG612.1-pLF-N/*L. pentosus* induced by xylose; M: low molecular weight protein marker

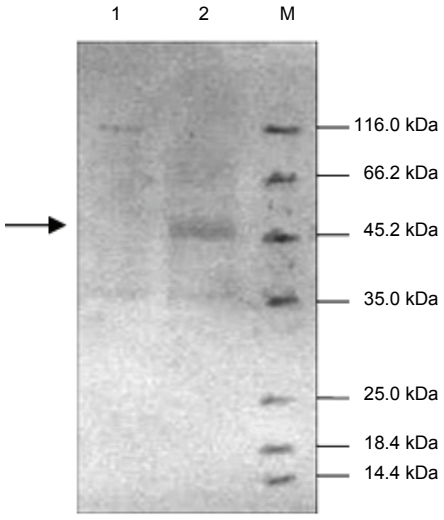

Fig. 3. Identification of the protein expressed in *Lactobacillus pentosus* by Western blot. Lane 1: pPG612.1/*L. pentosus* induced by xylose; lane 2: recombinant strain pPG612.1-pLF-N/*L. pentosus* induced by xylose; M: low molecular weight protein marker

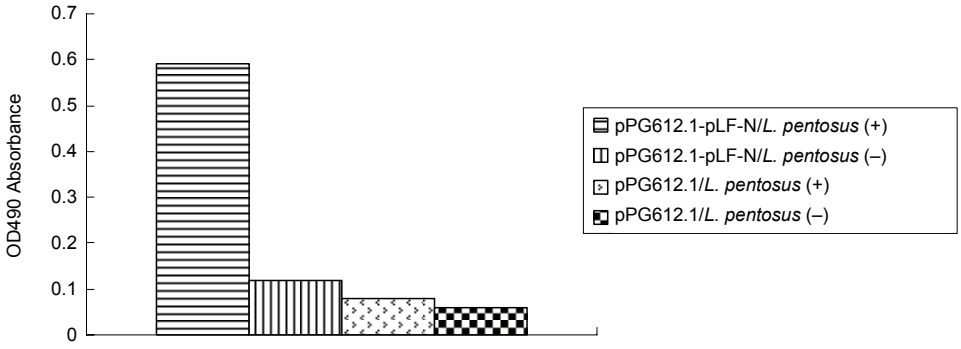

Fig. 4. Identification of the induced protein in supernatants by indirect ELISA. Mouse anti-pLF serum and HRP-conjugated goat anti-mouse IgG were used to confirm the pLF-N protein secreted from pPG612.1-pLF-N/*L. pentosus.* The results indicated that the pLF-N protein was present in the supernatant of overnight culture, while it was absent in the control bacteria pPG612.1/*L. pentosus*. '(+)' means MRS medium supplemented with xylose to induce the expression of recombinant protein, while '(–)' represents MRS medium without xylose

The expressed recombinant pLF-N protein showed antibacterial activity in the antibacterial activity assay. The time dynamic curve method indicated that the induced supernatant of pPG612.1-pLF-N/*Lactobacillus pentosus* had an growth-inhibiting effect on Gram-positive bacteria *Staphylococcus aureus* strain CVCC26003 and *Bacillus subtilis* strain CVCC63501 and on Gram-negative bacteria *Escherichia coli* strain CVCC10141*, Salmonella enterica* ssp. *enterica* Choleraesuis strain CVCC79102, while it did not inhibit the growth of *Lactobacillus casei* strain ATCC393 (Fig. 5).

Mouse sera were was collected on days 0, 7, 14, 21, 28, 35 and 42. Samples were stored at –20 °C until tested for specific anti-ADV antibodies by ELISA. IgG antibody levels to porcine ADV [expressed as optical density (OD) units] were significantly higher in the pPG612.1-pLF-N/*L. pentosus* group than in the control (PBS and *L. pentosus*) groups at 7 and 21 days ($P < 0.01$) and at day 14 ($P < 0.05$). Differences in antibody levels between the groups were not significant at days 28, 35 and 42, but IgG levels were somewhat higher in the pPG612.1-pLF-N/*L. pentosus* group than in the *L. pentosus* and PBS groups (Fig. 6), indicating that oral administration of pPG612.1-pLF-N/*L. pentosus* had an enhancing effect on humoral immunity induced in mice by a commercial attenuated porcine ADV vaccine.

Discussion

Lactoferrin has many biological functions, and it is particularly well known for its antimicrobial activity. LF is a broad-spectrum antimicrobial agent and has the ability to inhibit the growth of many Gram-positive and Gram-negative bacteria.

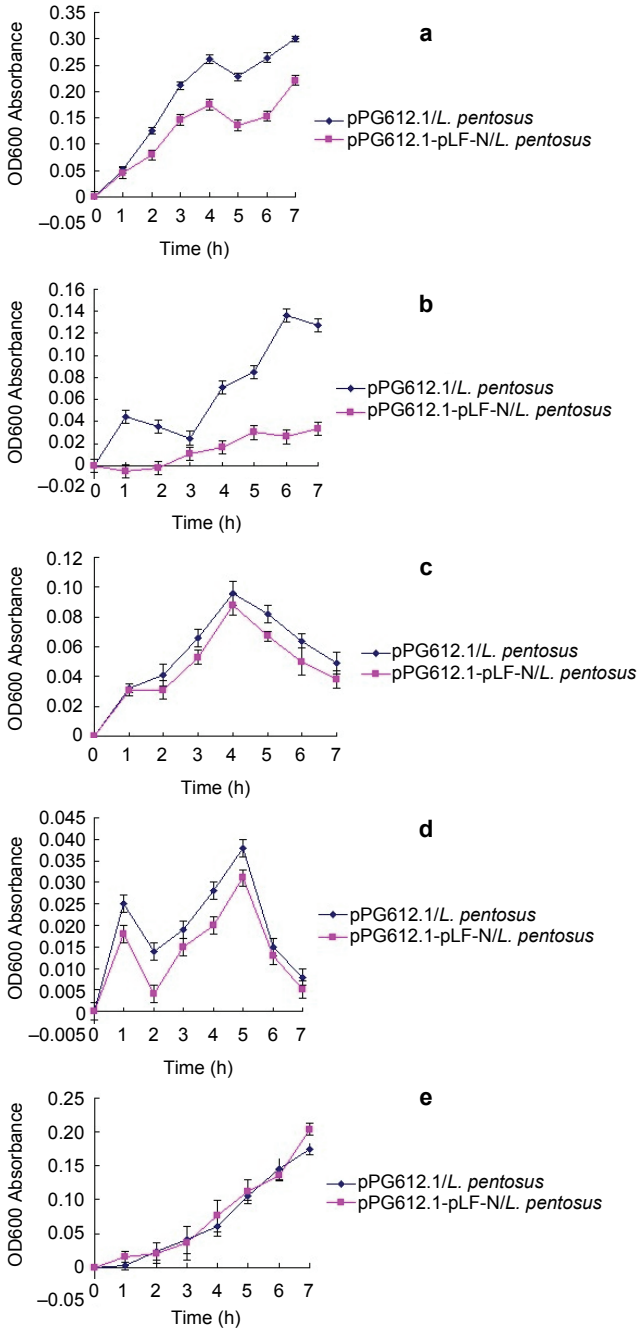

Fig. 5. The antimicrobial activity of recombinant porcine lactoferrin against the tested bacteria. a: *Bacillus subtilis* strain CVCC63501; b: *Staphylococcus aureus* strain CVCC26003; c: *Escherichia coli* strain CVCC10141; d: *Salmonella enterica* ssp. *enterica* Choleraesuis strain CVCC79102; e: *Lactobacillus casei* strain ATCC393

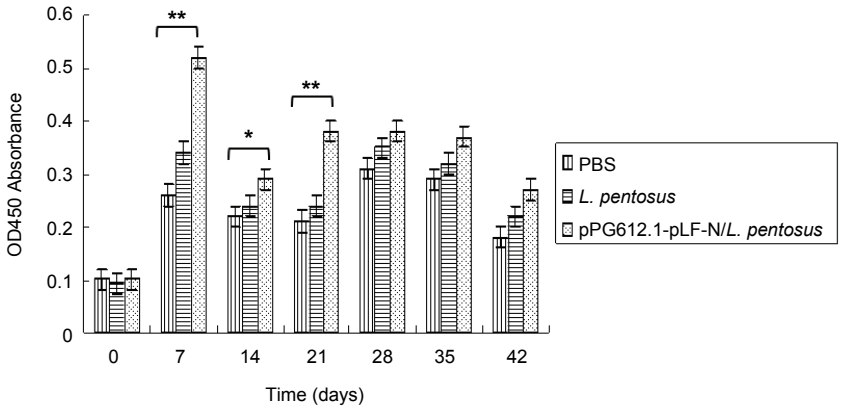

Fig. 6. IgG levels against Aujeszky's disease virus in the serum of mice. Mouse serum was collected on days 0, 7, 14, 21, 28, 35 and 42. Compared to the control PBS and *L. pentosus* groups, IgG antibody levels to Aujeszky's disease virus [expressed as optical density (OD) units] in the group orally treated with pPG612.1-pLF-N/*L. pentosus* were significantly higher on days 7 and 21 ($P < 0.01$) and on day 14 ($P < 0.05$). Although on days 28, 35 and 42 there was no significant difference in antibody levels between the groups, the IgG levels were somewhat higher in the pPG612.1-pLF-N/*L. pentosus* group than in the *L. pentosus* and PBS groups

It can also maintain the balance of the gut flora and promote the growth of beneficial bacteria in the intestine of animals. Many studies have shown that the biological properties of LF are closely associated with the N-lobe (Mishra et al., 2013). In this study, the N-terminal of porcine lactoferrin gene was expressed using a *Lactobacillus* secretion expression vector and the resulting recombinant protein exhibited antibacterial activity against *Escherichia coli, Salmonella enterica* ssp. *enterica* Choleraesuis*, Bacillus subtilis* and *Staphylococcus aureus* but not against *Lactobacillus casei*. The study showed that the recombinant strain shows a clear inhibitory effect not only on Gram-negative bacteria such as *E. coli* and *Salmonella* but also on Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*. It inhibits Gram-positive bacteria slightly stronger than Gram-negative bacteria, but it has no inhibitory effect on *Lactobacillus casei*. In the pre-trial, a natural porcine lactoferrin (purchased from Sigma-Aldrich Co, St. Louis, MO, USA) was tested against *Lactobacillus casei*, *L. plantarum* and *L. pentosus.* The results indicated that lactoferrin did not influence the growth of these three strains. Earlier research (Casey et al., 2007) showed that *L. pentosus* inhibits the growth and reproduction of harmful bacteria and promotes the colonisation of the intestine by beneficial bacteria, thus contributing to the prevention of diarrhoea. As a result, *L. pentosus* was selected as the host strain to deliver the porcine lactoferrin.

Many studies have shown that lactoferrin has immunoregulatory function and acts as an immunological adjuvant (Hwang et al., 2007; Wilk et al., 2007;

Hwang et al., 2009; Welsh et al., 2010; Allaire et al., 2015; Sherman et al., 2015). Lactic acid bacteria are generally regarded as food-grade microorganisms, which can survive in the intestinal tract of animals and have the potential to express foreign genes. In this study, lactic acid bacteria were used as an expression system to produce porcine lactoferrin. The resulting product was orally administered to mice immunised with a porcine ADV vaccine to explore its ability to enhance the effect of the vaccine.

The results obtained by periodic determination of serum IgG antibody levels indicated that the orally administered recombinant *L. pentosus* elevated ADV antibody levels as compared to the *L. pentosus* control group and the PBS group, suggesting that lactoferrin can enhance vaccination-induced humoral immunity to a certain extent. A significant increase in antibody level was found in the group orally administered recombinant porcine lactoferrin; thus, the immunomodulatory function of lactoferrin was demonstrated. Lactoferrin possesses immune-enhancing effects, which may be related to its participation in immunoregulation. LF itself can induce a humoral immune response and affect many components of the immune system by influencing the maturation of T and B cells and the proliferation of lymphocytes and by regulating iron levels in monocytes and macrophages. It can also be used with single-core white blood cells, neutrophils, lymphocytes or macrophages to enhance the killing ability of natural killer cells (Moriuchi and Moriuchi, 2006; Chen et al., 2008; Chen et al., 2011). The administration of rPLF significantly increased the length of intestinal villi in chicken and enhanced the expression of IFN-gamma and IL-12 in chicken T lymphocytes. It has been reported that rPLF enhances cell-mediated immunity and augments the effectiveness of infectious bursal disease (IBD) vaccination in chicken (Hung et al., 2010). This suggests that lactoferrin impacts nonspecific and specific immunity by promoting both the humoral and the cell-mediated immune response.

In conclusion, we have successfully constructed a probiotic *L. pentosus* expression system producing porcine lactoferrin. The recombinant strain pPG612.1-pLF-N/*L. pentosus* has the ability to secrete porcine lactoferrin and is able to inhibit the tested bacteria. The oral administration of recombinant *L. pentosus* strain expressing porcine lactoferrin was found to elevate IgG antibody levels in mice immunised with an attenuated porcine Aujeszky's disease virus vaccine by enhancing the vaccine-induced humoral immune response.

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