

Immunogenicity in chickens with orally administered recombinant chicken-borne *Lactobacillus saerimneri* expressing FimA and OmpC antigen of O78 avian pathogenic *Escherichia coli*

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Abstract

Purpose. Avian colibacillosis is responsible for economic losses to poultry producers worldwide. To combat this, we aimed to develop an effective oral vaccine for chicken against O78 avian pathogenic *Escherichia coli* (APEC) infection through a *Lactobacillus* delivery system.

Methodology. Eight *Lactobacillus* strains isolated from the intestines of broiler chickens were evaluated based on their *in vitro* adherence ability to assess their potential as a delivery vector. Fimbrial subunit A (FimA) and outer-membrane protein C (OmpC) of APEC with and without fusion to dendritic cell-targeting peptide (DCpep) and microfold cell-targeting peptide (Co1) were displayed on the surface of *Lactobacillus saerimneri* M-11 and yielded vaccine groups (pPG-ompC-fimA/M-11 and pPG-ompC-fimA-Co1-DCpep/M-11, respectively). The colonization of the recombinant strains *in vivo* was assessed and the immunogenicity and protective efficacy of orally administered recombinant strains in chickens were evaluated.

Results. The colonization of the recombinant strains *in vivo* revealed no significant differences between the recombinant and wild-type strains. Chickens orally administered with vaccine groups showed significantly higher levels of OmpC/FimA-specific IgG in serum and mucosal IgA in cecum lavage, nasal lavage and stool compared to the pPG/M-11 group. After challenge with APEC CVCC1553, better protective efficacy was observed in chickens orally immunized with pPG-ompC-fimA/M-11 and pPG-ompC-fimA-Co1-DCpep/M-11, but no significant differences were observed between the two groups.

Conclusions. Recombinant chicken-borne *L. saerimneri* M-11 showed good immunogenicity in chickens, suggesting that it may be a promising vaccine candidate against APEC infections. However, the activity of mammalian DCpep and Co1 was not significant in chickens.

INTRODUCTION

Avian colibacillosis is caused by avian pathogenic *Escherichia coli* (APEC). Infection of chickens with APEC serotype O78 strains can result in various diseases, including perihepatitis, airsacculitis, pericarditis and diarrhoea, leading to economic losses to poultry producers worldwide [1, 2]. Antibacterial drugs are commonly used to treat APEC infections; however, overuse of these drugs in animals has resulted in the emergence of drug-resistant bacterial strains and the presence of antibiotic residues in human foods derived from treated animals [3]. Therefore, safe and

efficient vaccines are critical for the prevention of APEC infections.

Research on virulence-related genes in APEC has shown that fimbriae on the bacterial surface mediate adherence to host cells during the initial step of infection, and FimA is the major subunit of the mannose-binding type 1 fimbriae that are commonly present in APEC [3, 4]. In addition, outer-membrane protein C (OmpC), a component of the outer membrane of *E. coli*, is an immunogen and is related to multidrug resistance [5–7]. Previous studies have demonstrated that intraperitoneal injection of FimA/OmpC-based

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Abbreviations: Cm⁺, chloramphenicol coding sequence; rep A, replicon A; Rep C, replicon C.

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Three supplementary tables and one supplementary figure are available with the online version of this article.

subunit vaccines can effectively induce systemic immune responses against colibacillosis [8], suggesting that they may be potential candidate immunogens for the development of an APEC vaccine.

Intestinal mucosal tissues are especially important in protection against pathogenic *E. coli* infections, making mucosal immunization an effective strategy against *E. coli* invasion. Induction of mucosal immunity should be focused on the interaction between the antigen and lymphocytes within the mucosa. One promising approach involves the use of live recombinant lactic acid bacteria. Probiotic *Lactobacillus* can enhance innate immune responses and epithelial barrier function, modulate the intestinal micro-environment, regulate immune cell behaviour, and elicit the release of cytokines in chickens [9, 10]. Further, several *Lactobacillus* strains that can survive gastric acid and digestive enzymes have been used as natural immune-stimulating adjuvants. Therefore, *Lactobacillus* spp. are attractive candidates for the delivery of immunogens to the mucosa due to their adhesion and colonization in the intestine, and superior safety, stability and immunogenicity [11–17].

Although a live recombinant *Lactobacillus* vaccine can propagate in the avian intestine, only a small amount of antigen can reach the effective immune sites. Reports have shown that microfold cell (M cells) are the major antigen sampling cells in the avian intestinal lympho-epithelium [18], while dendritic cells (DCs) are important in initiating immune responses [19]. However, inadequate research on avian M cells and DCs has hampered the development of avian mucosal vaccines. Therefore, it is necessary to initiate new attempts at generating avian vaccines against mucosal infections.

In this study, genetically engineered *Lactobacillus saerimneri* M-11 (*L. saerimneri* M-11) strains derived from an isolate identified in the intestine of a chicken were constructed to express the OmpC and FimA proteins of O78 APEC. Dendritic cell-targeting petide (DCpep) [20], which to some extent binds to avian DCs *in vitro* [21], and microfold cell-targeting peptide (Co1) [22] were added to this oral *Lactobacillus* delivery system to increase the bioavailability of the immunogen of interest. The immunogenicity and protection efficacy of these recombinant strains against APEC were investigated following their oral administration.

METHODS

Bacterial strains and plasmids

Eight *Lactobacillus* strains (Table S1, available in the online version of this article) were isolated from 20-day-old chickens as described previously [23] and cultured in de Man, Rogosa and Sharpe medium (MRS; Hopebol, Qingdao, People's Republic of China) at 42 °C without shaking. The avian pathogenic *E. coli* CVCC1553 strain was obtained from the China Institute of Veterinary Drug Control and was cultured in nutrient broth (Solarbio, Beijing, People's Republic of China) at 37 °C. The constitutive expression plasmid,

pPG-T7g10-PPT (Fig. 1), contains a PgsA anchor from *Bacillus subtilis* for stabilizing the heterologous protein in the cell membrane (surface displaying), which was previously constructed by our laboratory [24]. pPG-T7g10-PPT was used to construct fimA/OmpC plasmids as described below.

In vitro adherence assay

The adherence ability of *Lactobacillus* strains isolated from chicken intestines was evaluated *in vitro* as described previously [25]. Basement membrane matrix (diluted 1:20; BD, Franklin Lakes, NJ, USA) was coated on coverslips inside a 24-well plate and quenched with 2 % BSA-PBS for 2 h at 23 °C, before being washed twice with sterile PBS. The *Lactobacillus* strains were cultured to the early stationary growth phase ($OD_{600} \approx 1$), collected by centrifugation and then washed twice with sterile PBS. The cells were counted by determining the viable counts (VC), and then added to the slides and incubated for 2 h. After washing, adherent bacteria were stained with Gram's crystal violet

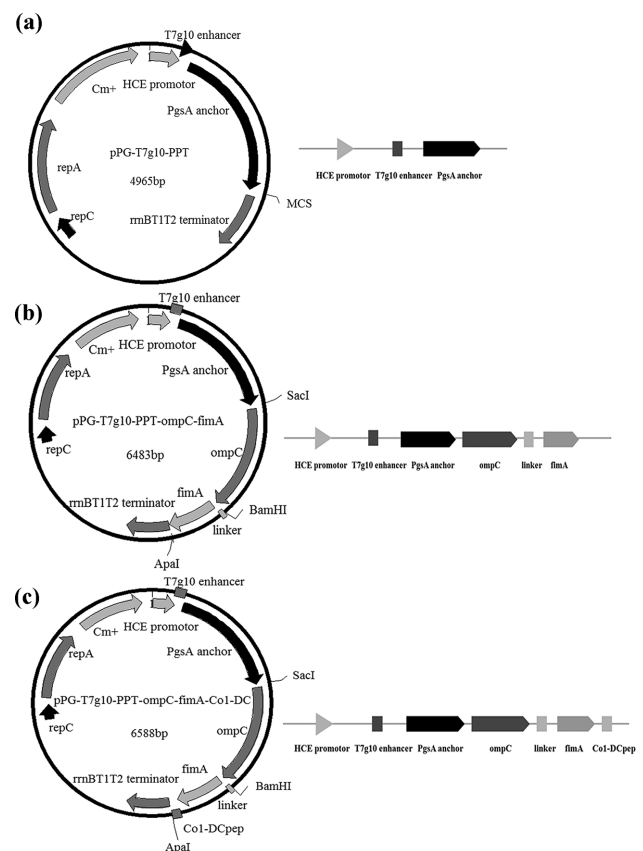


Fig. 1. Construction diagram for the recombinant plasmids. (a) pPG-T7g10-PPT, (b) pPG-T7g10-PPT-ompC-fimA and (c) pPG-T7g10-PPT-ompC-fimA-Co1-DCpep. HCE strong constitutive promoter; T7g10 transcriptional enhancer; PgsA anchor from *Bacillus subtilis*; *ompC* coding sequence; *fimA* coding sequence; M cell targeting peptide; DC targeting peptide; *rrnBT1T2* terminator; *repC*, replicon C; *repA*, replicon A; *Cm^r*, chloramphenicol coding sequence.

solution. Ten randomly chosen fields were photographed using a light microscope (ZYGD, Shanghai, People's Republic of China), and the number of bacteria in each field was counted (N). The adhesion parameter ($N \times 10^4 / VC$) was then calculated. Each strain was tested three times, and the results were expressed as mean $\pm$ standard error (SE).

Construction of recombinant *Lactobacillus* strains

A recombinant expression plasmid was constructed as shown in Fig. 1. The primers used in this study are listed in Table S2. Genomic DNA was extracted from avian *E. coli* CVCC1553 with the Bacterial DNA Isolation kit (Takara, Dalian, Liaoning, People's Republic of China). A FLAG tag was fused to the N terminus of *ompC*, and a linker sequence (15 mer) was inserted between the *ompC* and *fimA* genes. Targeting peptides (DCpep and Co1) were fused to the C terminus of *fimA* by overlap extension PCR (the reverse primer A-M-DC contained the relevant gene of the targeting peptides). *ompC-fimA* and *ompC-fimA-Co1-DCpep* fusion genes were cloned into the expression plasmid pPG-T7g10-PPT (pPG) to generate pPG-T7g10-PPT-*ompC-fimA* (pPG-OF) and pPG-T7g10-PPT-*ompC-fimA-Co1-DCpep* (pPG-OF-Co1-DCpep), respectively (Fig. 1). The plasmids were then electrotransferred into *L. saerimneri* M-11 to construct the recombinant strains, as previously described [26].

Analysis of protein expression by Western blotting

Recombinant *L. saerimneri* M-11 was cultured in MRS broth containing chloramphenicol ($10 \mu\text{g ml}^{-1}$) at 42°C for 12 h, harvested by centrifugation, washed twice with sterile PBS and then incubated in SDS buffer. The bacterial proteins were then separated by SDS-PAGE and electrotransferred onto a PVDF membrane. The immunoblots were probed with a mouse anti-OmpC/FimA polyclonal antibody prepared by our laboratory (primary antibody, 1:200) and an HRP-conjugated goat anti-mouse IgG (secondary antibody, 1:4000). The results were visualized using a chemiluminescent substrate reagent according to the manufacturer's instructions.

Acid and bile salt tolerance and growth characteristics

Lactobacillus strains were cultured in MRS broth overnight. For the acid tolerance test, 1 ml of overnight culture was used to inoculate 10 ml of sterile PBS (at pH 1.0–6.0) and incubated for 2 h at 42°C without shaking. The culture was then transferred to MRS broth (1% inoculum) and incubated at 42°C for 8 h, after which the OD_{600} values were determined. For the bile salt tolerance test, 100 μl of overnight culture was used to inoculate 10 ml of MRS broth supplemented with bile salt (0.05, 0.1, 0.2, 0.3 and 0.4%) and incubated for 8 h at 42°C , after which the OD_{600} values were determined. To determine the growth characteristics, 100 μl of overnight culture was transferred to MRS broth and incubated at 42°C , and the OD_{600} values were determined every 2 hours using MRS broth as a control.

In vivo colonization ability of the recombinant *Lactobacillus* strains

As previously described [27, 28], recombinant *L. saerimneri* M-11 were labelled with CFDA-SE cell tracer dye (Invitrogen, Carlsbad, CA, USA). The bacteria were then washed twice with PBS, centrifuged and resuspended in 500 μl PBS. The staining efficiency was assessed by flow cytometry (BD Biosciences, San Jose, CA, USA). Sixty specific pathogen-free (SPF) chickens (1-day-old, obtained from the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences) were divided into 4 groups (15 chickens per group) as follows: chickens in group I, II and III were orally inoculated with CFDA-SE-labelled pPG-OF/M-11, CFDA-SE-labelled pPG-OF-Co1-DCpep/M-11 and CFDA-SE-labelled wild-type *L. saerimneri* M-11 (5×10^9 cells per chicken), respectively; chickens in group IV were inoculated with PBS as a control to exclude autofluorescence in the intestinal mixture. Water and feed without any antibiotics were provided *ad libitum* throughout the experiment. Three chickens in each group were sacrificed on days 1, 3, 5, 13 and 21 after oral administration, and the ileum, cecum and colon were extracted. Individual sections were cut longitudinally, and any visible residual food particles or faecal material was removed. The examination was performed by adding 200 μl of PBS to every 1.0 cm of intestine and this also dislodged microbes from the mucosal surface of the intestine. This was followed by flow cytometry analysis and the fluorescent percentage was calculated for groups I, II, or III. The fluorescent contents of the intestinal mucosal sample (sorted by flow cytometry) were amplified in MRS containing chloramphenicol ($10 \mu\text{g ml}^{-1}$) at 42°C for 12 h. Plasmids were extracted and used as a PCR template. Primers for *ompC-fimA* were used in PCR reactions to detect plasmid stability in *Lactobacilli*. All animal procedures in this study were approved by the Committee on the Ethics of Animal Experiments of Northeast Agricultural University.

Regulation of chicken bone marrow-derived DCs (chBM-DCs) by recombinant *L. saerimneri*

Marrow obtained from the femurs and tibias of 4–6-week-old broiler chicks was washed twice with sterile PBS, resuspended in PBS, loaded on an equal volume of Histopaque-1119 (Sigma-Aldrich, Missouri, SL, USA) and centrifuged at 1200 g for 30 min. Cells at the interface (chBM-DCs) were then collected as previously described [18] and seeded at 10^6 cells ml^{-1} in six-well plates containing RPMI-1640 supplemented with 1 U ml^{-1} penicillin and streptomycin, 10% foetal calf serum (Gibco, Grand Island, NY, USA), 50 ng ml^{-1} recombinant chicken granulocyte macrophage colony-stimulating factor (Abcam, Cambridge, UK) and 25 ng ml^{-1} interleukin-4 (Kingfisher, Saint Paul, MN, USA) at 37°C and 5% CO_2 for 6 days. Three-quarters of the medium was replaced with complete medium every 2 days [29].

Overnight bacterial cultures were inoculated (1:100) in MRS broth and cultured until the OD_{600} reached ~ 0.8 . After being washed twice with sterile PBS, the UV-inactivated bacteria were transferred to medium without antibiotics.

chBM-DCs that were cultured for 6 days were washed and resuspended in medium without antibiotics (10^6 cells ml^{-1}). The chBM-DCs were then infected by adding 100 μl of a bacterial suspension and incubated at 37°C in 5% CO_2 for 18 h (100 μl of medium was used as a negative control). After being washed three times with medium, the chBM-DCs were subjected to flow cytometry analysis. Post-stimulatory cells (1×10^6 cells ml^{-1}) were incubated with 25 μg ml^{-1} mouse anti-chicken CD40 and CD86 antibodies (Abd, Raleigh, NC, USA) for 30 min and then with an FITC-conjugated goat anti-mouse IgG (1:5000). After being washed with PBS containing 1% (v/v) foetal calf serum, the cells were resuspended in 200 μl of PBS for flow cytometric analysis using a FACS array flow cytometer [30]. In parallel, immature chBM-DCs were used as a control.

A mixed lymphocyte reaction (MLR) was used to test the primary lymphocyte stimulatory capacity of non-stimulated and bacteria-stimulated chBM-DCs. Allogeneic lymphocytes were isolated from the spleens of 6-week-old chickens and layered on an equal volume of Histopaque-1077 (Sigma-Aldrich, Missouri, SL, USA), and then centrifuged at 1200 g for 30 min. The cells at the interface were collected, washed, counted and used as responder cells. Lipopolysaccharide (LPS; 200 ng ml^{-1}) was used as a control. Graded numbers of stimulator cells were added to a 96-well plate, and then 10^5 responder cells were added to yield lymphocyte:DC ratios of 1:1, 10:1 and 100:1 in a 200 μl culture and incubated at 37°C in 5% CO_2 for 72 h. The control cultures contained only responder or stimulator cells. Cell proliferation was assessed using the WST-8 Cell Counting kit-8 (CKK-8; Qcbio, People's Republic of China). The stimulation index was calculated as follows: $\text{SI} = (\text{OD}_{450: \text{sample}} - \text{OD}_{450: \text{blank control}}) / (\text{OD}_{450: \text{cell control}} - \text{OD}_{450: \text{blank control}})$ [28]. All tests were performed in triplicate.

Immunization

Two-week-old SPF chicks were randomly divided into 4 groups of 30 chicks each: pPG-OF/M-11, pPG-OF-Co1-DCpep/M-11, pPG/M-11 and PBS groups. The chickens were housed under SPF conditions with *ad libitum* access to standard chow diet and water. The intragastric immunization dose per chicken was 2×10^9 c.f.u. of recombinant *Lactobacilli*, which was administered on three consecutive days (days 1, 2 and 3). Booster immunizations were administered on days 14, 15 and 16 at the same dose as the primary immunization. Detailed information about the feed intake, average daily gain and health status of the experimental SPF chickens in each group was recorded. Stool samples were collected at 0, 4, 7, 10, 14, 17, 21, 28 and 35 days after immunization. Ten chickens in each group were randomly selected and euthanized with excessive ether to collect sera, cecum lavage and nasal lavage, at 0, 7, 14, 21 and 35 days after immunization for the detection of specific IgG and IgA by ELISA. Cecums were washed with 200 μl of sterile PBS (pH 7.4), and nasal cavities were washed with 50 μl of sterile PBS (pH 7.4). The remaining chickens (20 in each group) were used for the challenge experiment.

ELISA of antibody levels and challenge experiment

The levels of IgG in sera and IgA in cecum lavage, nasal lavage and stool were measured by ELISA. Polystyrene microtitre plates were coated overnight at 4°C with OmpC and FimA protein ($10 \mu\text{g}$ ml^{-1}) expressed by an *E. coli* prokaryotic expression system. After blocking with 5% skim milk, the collected samples were added in triplicate and incubated at 37°C for 1 h. Either HRP-conjugated goat anti-chicken IgG (KPL, MD, USA) or mouse anti-chicken IgA (Southern Biotech, Birmingham, AL, USA), both diluted 1:3000, were then added as secondary antibodies and incubated at 37°C for 1 h. Colour was developed using o-phenylenediamine dihydrochloride (Sigma-Aldrich) as a substrate, and the absorbance at OD_{490} was measured.

O78 *E. coli* CVCC1553 was used to evaluate the protective efficacy of the oral immunization of chickens with the recombinant strains pPG-OF/M-11 and pPG-OF-Co1-DCpep/M-11. Four groups of SPF chickens (20 per group) were orally challenged with 5×10^{11} c.f.u. of O78 *E. coli* CVCC1553 at 35 days post-immunization. Chicken health was monitored daily for a 15-day observation period, and chickens that developed severe clinical symptoms were euthanized. The cumulative mortality of chickens in each group was recorded.

Statistical analysis

The experiments were repeated three times, and the results are shown as the mean $\pm$ SE of three replicates per condition. Tukey's multiple comparison tests were used to analyse the differences between groups. $P < 0.05$ was considered to be statistically significant, and $P < 0.01$ was considered to be highly significant.

RESULTS

L. saerimneri M-11 selected in *in vitro* adherence assay

The adherence ability of eight *Lactobacillus* strains isolated from the intestines of broiler chickens was evaluated using an *in vitro* adherence assay. The adhesion parameter showed that the *L. saerimneri* M-11 strain isolated from the cecum had a significantly better adhesion ability than other *Lactobacillus* strains (Fig. 2). *L. saerimneri* M-11 was selected as the antigen carrier for the development of a vaccine against APEC infection in chickens.

Recombinant strains express OmpC and FimA fusion protein

Recombinant *Lactobacillus* strains pPG-OF/M-11 and pPG-OF-Co1-DCpep/M-11 were cultured in MRS broth at 42°C for 12 h, and then the expression of OmpC and FimA was determined by Western blotting with a mouse anti-OmpC/FimA polyclonal antibody. The fusion protein (approximately 101 kDa) was expressed by both recombinant strains as shown by blotting with polyclonal anti-FimA antibody (Fig. 3a) and mouse anti-OmpC (Fig. 3b) antibodies, respectively. The negative control, pPG/M-11, did not display a corresponding immunoreactive band.

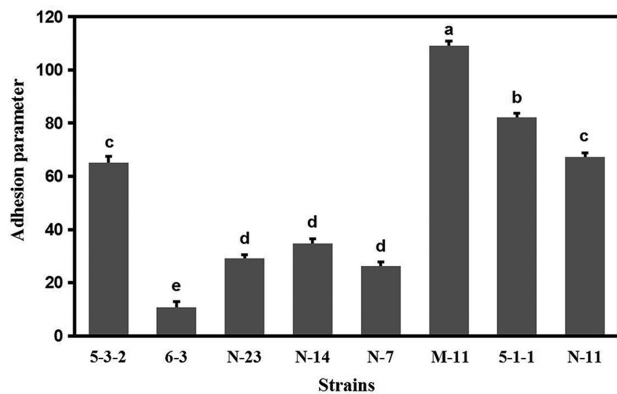


Fig. 2. Adhesion parameters of chicken-borne *Lactobacillus* strains evaluated by the basement membrane matrix. Data are expressed as mean $\pm$ SE and are from three independent experiments. The same superscript letter indicates no significant difference ($P>0.05$) and different superscript letters indicate a significant difference ($P<0.001$).

Acid and bile salt tolerance and growth characteristics of the recombinant *Lactobacillus* strains

Analysis of acid and bile salt tolerance of recombinant *Lactobacillus* strains and growth characteristics at 42 °C *in vitro* showed that the recombinant strains displayed good tolerance to high-acid environments (Fig. S1a) and could survive in MRS broth containing 0.1 % bile salt (Fig. S1b), like wild-type *L. saerimneri* M-11. The growth of recombinant strains, pPG-OF/M-11 and pPG-OF-Co1-DCpep/M-11 in MRS broth at 42 °C was similar to that of wild-type *L. saerimneri* M-11 (Fig. S1c).

In vivo colonization of the gastrointestinal tract by recombinant *Lactobacillus* strains

Flow cytometry analysis showed that staining efficiency of *Lactobacillus* strains with CFDA-SE dye was up to 100 % (Fig. 4a). The colonization ability of CFDA-SE-labelled recombinant strains in the intestinal tracts of chickens was

determined after oral administration. In the first 5 days after treatment, the average fluorescence in each group fluctuated, and then fluorescence increased, but by the last few days of the experiment the percentage of fluorescence declined (Fig. 4). Flow cytometry results indicated that the recombinant strains pPG-OF/M-11 (Fig. 4d) and pPG-OF-Co1-DCpep/M-11 (Fig. 4e) were able to survive and adhere to different regions of the chicken intestinal tract, and as with wild-type *L. saerimneri* M-11 (Fig. 4c), adhesion was more prominent in the cecum than in the ileum and colon. PCR was used to detect the stability of these plasmids in *Lactobacilli*. In agarose gel electrophoresis, the positive bands were consistent with expected results, proving that the respective plasmid was still present in these labelled bacteria (data not shown).

Regulation of chBM-DCs by recombinant *Lactobacillus* strains

In the present study, chBM-DCs were stimulated with the recombinant *Lactobacillus* strains, pPG-OF/M-11 and pPG-OF-Co1-DCpep/M-11, *in vitro*, and with pPG/M-11 as a control. To restrain bacterial growth in the medium without affecting proteins on the cellular surface, the bacteria were inactivated by UV radiation in advance. Flow cytometry analysis showed that after the DCs were stimulated by bacteria, CD40 (Fig. 5a) increased by 65.1 % (wild-type *L. saerimneri* M-11), 72 % (pPG-OF/M-11) or 78.4 % (pPG-OF-Co1-DCpep/M-11), while CD80 (Fig. 5b) increased by 56.7 % (wild-type *L. saerimneri* M-11), 69.9 % (pPG-OF/M-11) or 75.1 % (pPG-OF-Co1-DCpep/M-11). The percentage increase is relative to the levels in unstimulated chBM-DCs (with the absolute value corresponding to 100 %). The primary lymphocyte stimulatory capacity of non-stimulated and bacteria-stimulated chBM-DCs was tested using an MLR assay, and the results showed that the stimulation index of pPG-OF-Co1-DCpep/M-11 was higher than that of pPG-OF/M-11 and pPG/M-11 ($P<0.05$) by a ratio of 1 : 1 (DC:T cells; Fig. 5d).

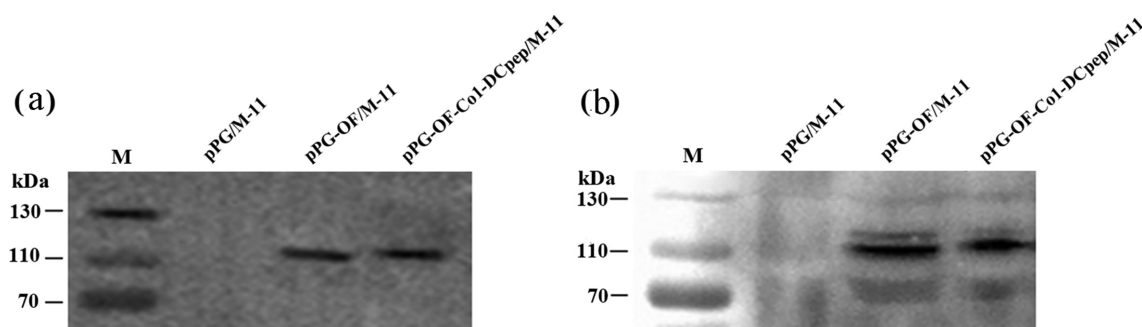


Fig. 3. Expression of the fusion protein from pPG-OF-Co1-DCpep/M-11 and pPG-OF/M-11 plasmids identified by Western blot. (a) Mouse anti-FimA antibody (primary antibody, 1 : 300). (b) Mouse anti-OmpC antibody (primary antibody, 1 : 300). The calculated molecular mass of the fused OmpC, FimA and anchor is about 101 kDa.

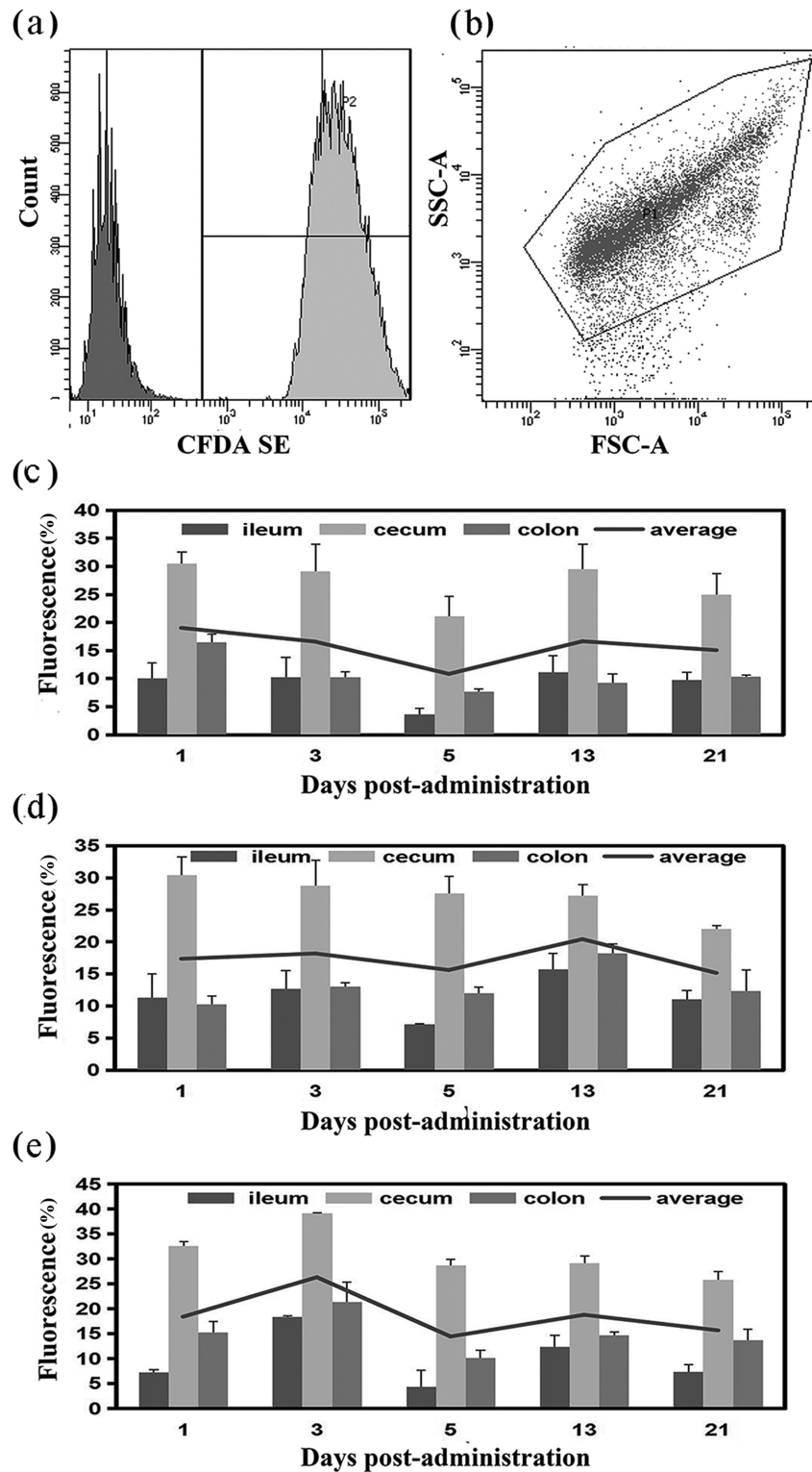


Fig. 4. Colonization ability of recombinant *Lactobacillus* strains. (a) Staining efficiency of *Lactobacillus* strains with CFDA-SE. (b) Cells were gated based on side scatter/forward scatter (SSC/FSC) parameters. (c) Wild-type *L. saerimneri* M-11, (d) pPG-OF/M-11 and (e) pPG-OF-Co1-DCpep/M-11 in the chicken intestinal tract. Data are expressed as mean $\pm$ SE. The mean was taken from three chickens in each group. The percentage fluorescence was taken from group I, II, or III (minus PBS control group IV). The average was the mean of the percentage fluorescence of the ileum, cecum and colon.

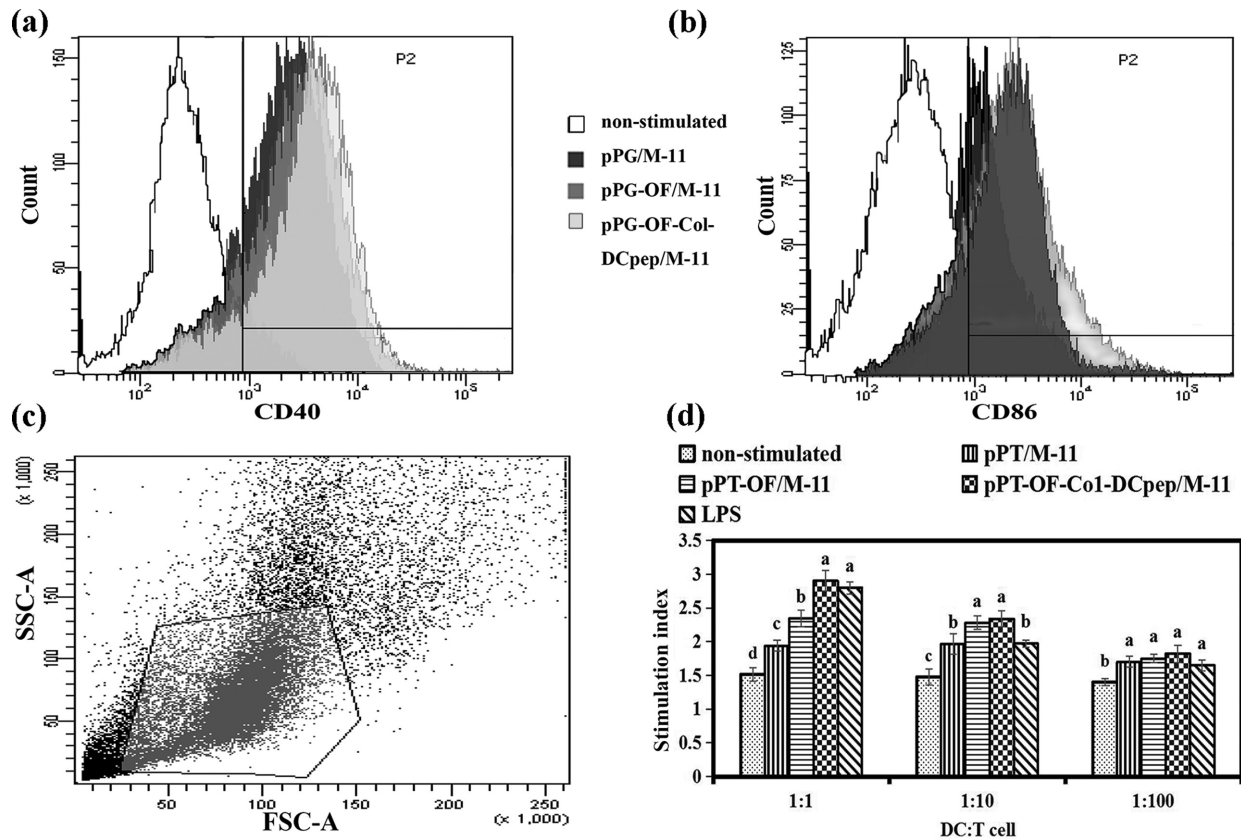


Fig. 5. Regulation of chBM-DCs by recombinant *Lactobacillus* strains. chBM-DCs were stimulated by pPG-OF/M-11 and pPG-OF-Co1-DCpep/M-11 *in vitro*. The regulation of chBM-DCs CD40 (a) and CD86 (b) by recombinant *L. saerimneri* was analysed by flow cytometry. (c) Cells were gated based on side scatter/forward scatter (SSC/FSC) parameters. (d) Mixed lymphocyte reaction (MLR) was assessed by the CCK-8 assay in duplicate after the co-culture of DCs was activated *in vitro* with recombinant *L. saerimneri* and allogeneic lymphocytes. Data are expressed as mean $\pm$ SE of three independent experiments. The same superscript letter indicates no significant difference ($P>0.05$) and different superscript letters indicate a significant difference ($P<0.05$).

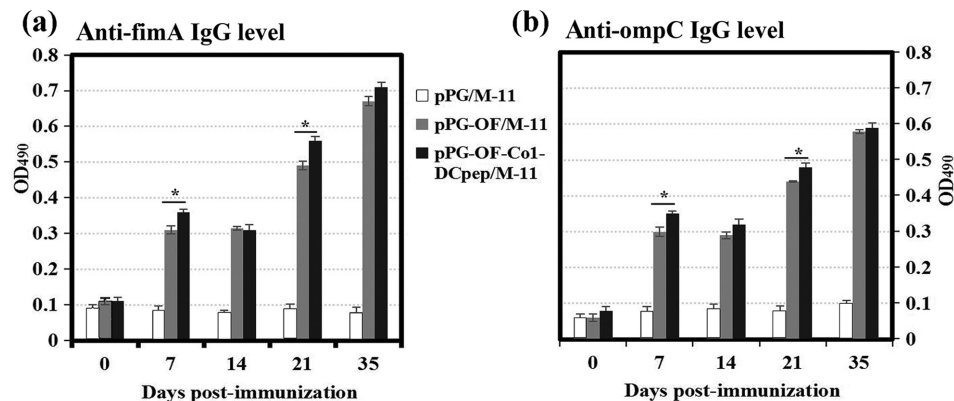


Fig. 6. Anti-fimA-specific IgG levels (a) and anti-OmpC-specific IgG levels (b) in chickens post-immunization. Bars represent the mean $\pm$ SE of each group ($n=3$, $*P<0.05$). The mean was taken from three chickens.

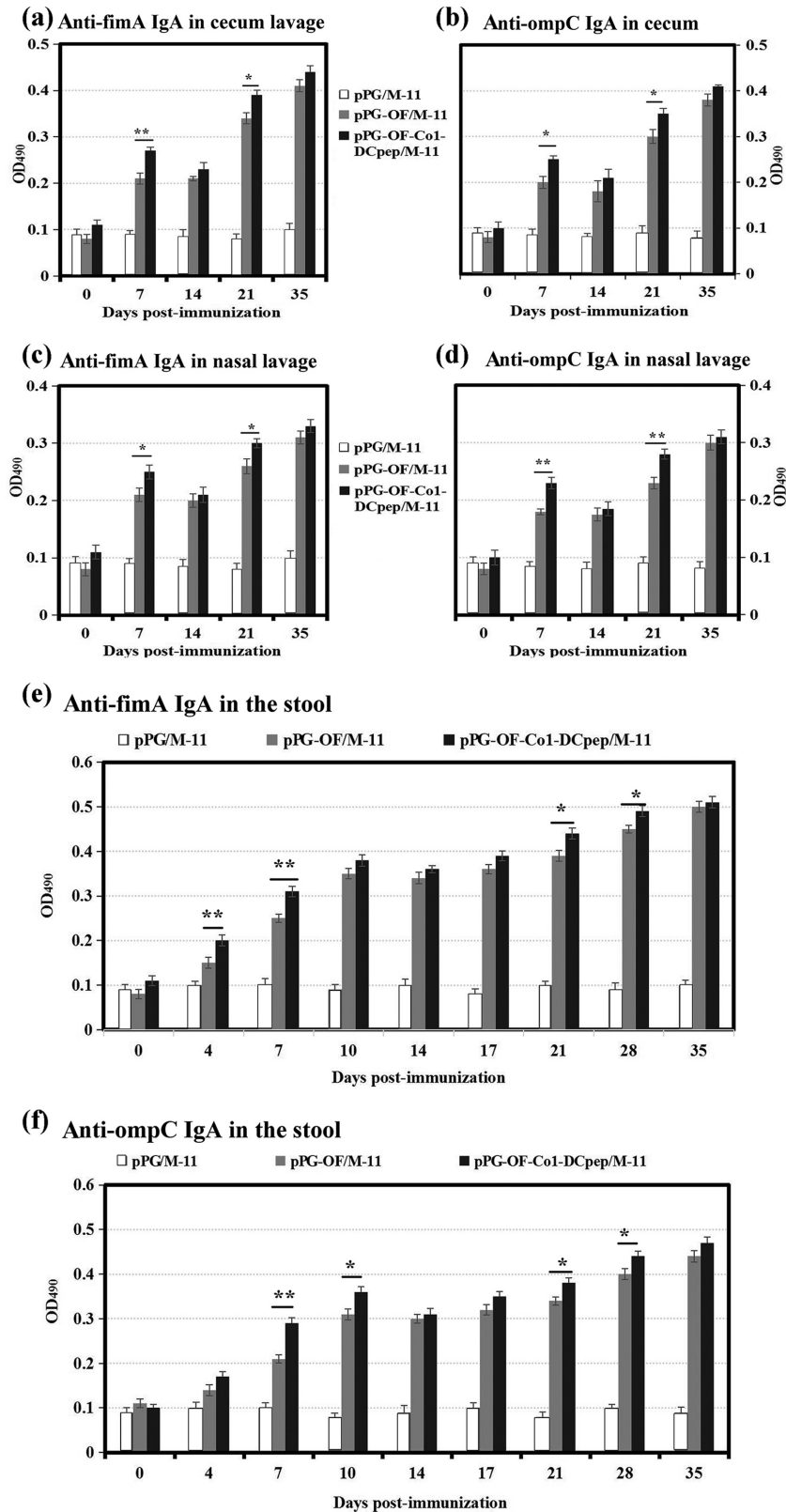


Fig. 7. Anti-fimA-specific sIgA levels in the cecum (a), nasal cavity (c) and faeces (e), and anti-OmpC-specific sIgA levels in the cecum (b), nasal cavity (d) and faeces (f) post-immunization with recombinant *Lactobacillus* strains. Bars represent the mean $\pm$ SE of each group ($n=3$, * $P<0.05$, ** $P<0.01$). The mean was taken from three chickens.

Growth properties of chickens with orally administered recombinant *Lactobacillus* strains

The body weight of the experimental chickens that received pPG-OF-Co1-DCpep/M-11, pPG-OF/M-11 and pPG/M-11 was significantly higher ($P < 0.05$) than that of control chickens that received a standard diet supplemented with PBS; however, there were no significant differences ($P > 0.05$) in growth among the experimental groups (Table S3).

Immune responses induced in chickens by oral administration of the recombinant *Lactobacillus* strains

The immunogenicity of the recombinant *Lactobacillus* strains pPG-OF/M-11 and pPG-OF-Co1-DCpep/M-11 in chickens after oral immunization was evaluated by detecting the presence of anti-OmpC/FimA IgG and IgA by ELISA in the mucosal and systemic immune responses, respectively. There were significantly higher levels ($P < 0.01$) of antigen-specific systemic IgG (Fig. 6) and antigen-specific mucosal secretory IgA (sIgA) (Fig. 7) in chickens that had received the recombinant strains orally compared to the control group. At 7 and 21 days post-immunization, the IgG and sIgA were significantly higher in the pPG-OF-Co1-DCpep/M-11 group than in the pPG-OF/M-11 group, but no differences were observed at 14 and 35 days post-immunization. After booster immunization, the sIgA levels increased rapidly and the values at 28 and 35 days post-immunization were significantly higher ($P < 0.01$) than the peak value of the first immunization. Further, the recombinant pPG-OF-Co1-DCpep/M-11 strain induced higher antigen-specific immune responses at both the mucosal and systemic levels ($P < 0.05$) compared to pPG-OF/M-11.

Protection efficiency of the recombinant *Lactobacillus* strains

A challenge experiment was performed using an APEC strain (*E. coli* CVCC1553) to evaluate the protection efficiency of the genetically engineered *Lactobacillus* strains pPG-OF/M-11 and pPG-OF-Co1-DCpep/M-11. The results showed an 80 % protection rate in the pPG-OF-Co1-DCpep/M-11 group and necropsy of surviving chickens revealed that three chickens had slight congestion of the intestinal mucosa. In the pPG-OF/M-11 group, 70 % of chickens survived, but four chickens among them had slight congestion of the intestinal mucosa (with the absolute value corresponding to 100 %). In contrast, chickens in the PBS and pPG/M-11 groups developed severe clinical signs of infection (congestion and ulceration in the intestinal mucosa and loose stools) and died at 5 days post-challenge (Fig. 8).

DISCUSSION

Avian colibacillosis is a major threat to the poultry industry, and is responsible for significant economic losses. It has been reported that 10–15 % of avian alimentary tract colibacillosis cases are caused by APEC serotypes [31], and both virulent and avirulent *E. coli* can persist in the intestinal

tract [32, 33]. Although inactivated vaccines and fimbriae-based subunit vaccines are commonly used to protect poultry from APEC [34], new vaccines are urgently needed due to the presence of diverse serotypes and complex pathogenic types. Using probiotics with good adhesion and colonization ability as carriers for antigen delivery can not only compete with the pathogenic bacteria for binding epitopes, but also trigger antigen-specific immune responses, suggesting that they may be promising candidates in the development of vaccines against APEC.

Some symbiotic gut microbes that are stably associated with specific vertebrate species are predicted to evolve host-specific adaptations [35]. For example, *Lactobacillus reuteri* from rodent lineages show specific adaptations to mice. Therefore, an appropriate delivery vector may be the linchpin of mucosal vaccines. In the present study, eight chicken-borne *Lactobacillus* strains were isolated from the intestinal tracts of chickens, and of these, *L. saerimneri* M-11 showed the best adherence ability as evaluated by an *in vitro* basement-membrane matrix assay [36, 37]. Moreover, *L. saerimneri* M-11 did not harbour any wild plasmids, showed sensitivity to chloramphenicol (which was used as a selection marker for constructing the recombinant strains) and promoted the maturation of chBM-DCs effectively – suggesting good natural adjuvant activity. Therefore, chicken-borne *L. saerimneri* M-11 was selected to deliver APEC antigens in an oral vaccine against avian colibacillosis.

We constructed two recombinant *Lactobacillus* strains, pPG-OF/M-11, expressing OmpC and FimA proteins of APEC, and pPG-OF-Co1-DCpep/M-11, expressing OmpC and FimA fused with DCpep and Co1. The two recombinant *Lactobacillus* strains showed tolerance to low pH and 0.1 % bile salt, similar to wild-type *L. saerimneri* M-11. The presence of pPG-OF and pPG-OF-Co1-DCpep did not affect growth or the ability to colonize the intestinal tract. Our data showed that recombinant *Lactobacillus* strains successfully colonized the chickens' ileum, cecum and colon

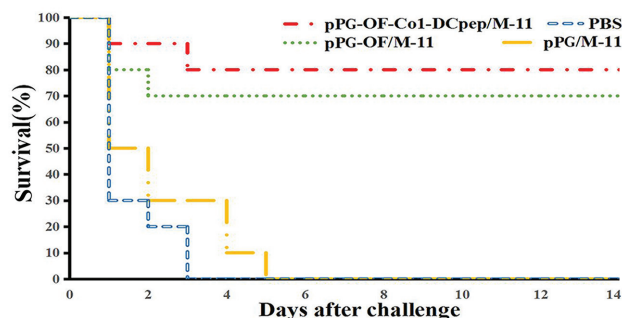


Fig. 8. Protection efficiency in chickens orally immunized with recombinant pPG-OF-Co1-DCpep/M-11 and pPG-OF/M-11 against avian *E. coli* CVCC 1553 challenge. Twenty chickens in the pPG-OF-Co1-DCpep/M-11 and pPG-OF/M-11 groups were orally challenged with 078 *E. coli* CVCC1553 at 35 days post-immunization.

for 21 days, especially the cecum, which contains a large cluster of aggregated lymphoid tissues [38]. Therefore, we effectively promoted intestinal mucosal immunity against APEC by supplying chickens with the recombinant *Lactobacilli* probiotics [39].

To evaluate the immunogenicity of recombinant *Lactobacilli*, chickens were orally immunized with the aforementioned strains, and the antibody (IgG and sIgA) levels were then determined. Our data showed that both pPG-OF/M-11 and pPG-OF-Co1-DCpep/M-11 can effectively induce antigen-specific mucosal and systemic immune responses. Nevertheless, the activity of mammalian DCpep and Co1 was not significant in chickens. DCs and M cells are involved in avian and mammalian immune activity, but chickens have a different repertoire of molecules and immune gene families compared to mammals, such as chemokine/chemokine receptors and Toll-like receptors (TLRs) [40]. Correlative studies on avian DCs and M cells are still required. Despite the fact that pPG-OF-Co1-DCpep/M-11 was not as efficient as expected in protecting against an APEC challenge (two more chickens survived than in the pPG-OF/M-11 group), this strategy may provide a theoretical basis for the development of avian mucosa-targeted vaccines.

Chicken-borne *L. saerimneri* M-11 strain, showing good colonization as an antigen carrier, delivered FimA and OmpC as an oral vaccine against APEC infection. Our results showed that a recombinant *L. saerimneri* M-11 strain expressing the FimA and OmpC antigens (pPG-OF/M-11), effectively induced antigen-specific immune responses at both the mucosal and systemic levels, and provided effective protection in chickens against an APEC challenge. This suggests that the *Lactobacillus* delivery system may be a promising strategy for the development of vaccines against APEC infections.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

Animal experiments were carried out in accordance with the recommendations in the institutional and national guidelines for animal care and use. The protocol was approved by the Committee on the Ethics of Animal Experiments of Northeast Agricultural University, Harbin, People's Republic of China (2016NEFU-315, 13 April 2017). All procedures were performed under ether anaesthesia, and made to minimize suffering.

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