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Effects Of Orally Administered Recombinant *Lactobacillus Casei* Expressing HN Protein On Early Growth Performance, Intestinal Health And Protection Against NDV Challenge In Chickens

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Research

Keywords: Lactobacillus casei, Oral immunization, Newcastle disease virus, Hemagglutinin-neuraminidase, Chickens, Intestinal health, Growth performance

Posted Date: July 29th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-48868/v1

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Abstract

Backgroud: Newcastle disease virus (NDV) is considered one of the most important diseases among chickens. In this study, we generated recombinant surface-displayed *Lactobacillus casei* (*L.casei*) expressing the hemagglutinin-neuraminidase (HN) of NDV and a live vector pPG alone (named Lc-pPG-HN and Lc-pPG), and evaluated their effects on early growth development, intestinal health and protection against NDV challenge in chickens. 270 chickens were randomly divided into three groups: Lc-pPG-HN, Lc-pPG and physiological saline (control) group, and chickens from each group were respectively immunized with Lc-pPG-HN, Lc-pPG and physiological saline on 1 and 10 days.

Results: Recombinant *L.casei* expressing the HN protein of NDV (Lc-pPG-HN) was successfully constructed. Orally immunized with Lc-pPG-HN could significantly increase body weight (BW) and immune organs index. Moreover, Lc-pPG-HN improved secretory immunoglobulin A (SIgA) in jejunum, the relative abundance of flora in cecum, histomorphological development of small intestine. In addition, the similar enhancement effects were also observed with hemaggluti-nation inhibition (HI) antibody titer and the expression of cytokines in the serum. The oral administration of Lc-pPG-HN also provided effective protection and alleviated the symptoms of NDV challenge.

Conclusions: Thus, a recombinant *L.casei* vaccine expressing HN may be a potential therapeutic candidate against NDV and improve chickens growth and development.

Introduction

Newcastle disease (ND) is considered one of the most devastating infectious diseases among domestic birds and considerably threatens commercial poultry production [1]. Current live attenuated ND virus (NDV) vaccines are unsatisfactory, and a viral vector vaccine that infects chickens but does not cause disease is needed [2, 3]. The NDV surface envelope is mainly surrounded by the viral hemagglutinin-neuraminidase (HN) glycoprotein, which plays a critical role in viral infectivity and pathogenicity, they produce virus-neutralizing antibody responses and are protective antigens [4]. One study showed that HN also protected chickens during a virulent NDV challenge, although the chickens showed lower neutralizing antibody titers. Thus, live vector vaccines centering on the NDV HN may protect against heterologous pathogens [5]. However, the individual contributions of the HN protein to the growth performance and intestinal health was not clearly known.

To date, many probiotic bacteria have been screened for their potential as animal feed additives to increase the nutritional value and provide additional health benefits. More recently, probiotics have been developed for human use; however, these products must be rigorously tested to ensure their safety and efficacy [6]. Studies have confirmed that probiotics can increase immunity, maintain intestinal health, and reduce pathogen proliferation, thereby enhancing animal and human health [7]. Thus, oral probiotics have significant health and economic benefits. Oral recombinant vaccines using *lactic acid bacteria* (LAB) as carriers are being extensively researched. As normal inhabitants of the intestines, LAB readily colonize the

intestinal mucosa, making LAB vaccines simple to administer while stimulating a good immune response [8]. Previous studies have developed many *Lactobacillus*-associated model vaccines, such as *L. acidophilus* expressing *Helicobacter pylori* adhesin [9], *L.casei* producing porcine epidermal growth factor (pEGF) [10] and expressing muramyl dipeptide and tuftsin [11]. Studies have shown that intranasally or orally administering these vaccines can directly reduce antigen levels [12]. However, while genetic manipulation can enhance the immunogenicity of the proteins used in a vaccine, intestinal digestive enzymes rapidly degrade the vaccine components. Thus, a major focus of oral vaccine research is to optimize the environmental stress tolerance of antigen-presenting vectors and enhance antigen expression in the gut. Interestingly, LAB have immunoadjuvant properties and readily express foreign antigens, making them excellent candidates as vaccine vectors. Recent studies of genetically engineered LAB vaccines show that they have great potential for oral vaccine use [13, 14].

Recently, different type of recombinant *L.casei* have been studied as a novel vaccine in poultry. In particular, some studies showed that NDV HN-expressing *L.casei* via oral administration increased immune responses and did not impair the growth performances [15]. There is still little knowledge on how the dosage and the type of vectored vaccines may affect the growth performances, and intestine health of chickens. Therefore, the aim of this study was to evaluate the effects of recombinant *L.casei* inclusion on growth performance, intestinal health and immune system of chickens. The results suggested that the oral *L.casei* vaccine may provide a promising strategy for controlling viral infections in poultry.

Materials And Methods

Animal ethics statement

270 healthy chickens (Hy-line Variety White) were obtained from a local chickens factory (Runcheng Chickens Breeding Factory, Changchun, China). We followed all animal care protocols regarding the use of live chickens in our experiments, and all chickens had access to food, water, and necessary veterinary care add libitum.

Bacterial strains, plasmids and growth conditions

Lactobacillus casei ATCC 393 was isolated from cheese, a plasmid-free strain grown in De Man, Rogosa and Sharpe grown in De Man, Rogosa and Sharpe (MRS) medium (Oxoid, UK) at 30 °C, without shaking. The *Escherichia coli-Lactobacillus* shuttle vector pPG, a type of cell-surface expression plasmid containing an anchoring matrix-encoding pgsA gene derived from *Bacillus* subtilis behind the target gene. The pPG has the ssUSP secretion signal before the target gene to ensure the target protein secretion. *Escherichia coli* (*E.coli*) competent cells MC1061 was grown in Luria-Bertani (LB) medium at 37 °C with shaking for the cloning of plasmid. Chloramphenicol (Cm) (Sigma, USA) was utilized at final concentration of 10 μg/mL. Newcastle disease virus isolated from the allantoic fluid.

Generation of recombinant*L. casei*expressing HN gene

The HN gene (1617 bp) of NDV strain (GenBank: ABG56153.1) was amplified by PCR using the following primers with *Nco* I or *EcoR* V site underlined (forward primer: 5' CCCATGGATGGACCGCGC GGTTAACAGAGTC 3', Reverse primer: 5' AAGATATCTTAAACTCTATCATCCTTGAGGATC 3'); The PCR product of HN gene was cleaved with *Nco* I/ *EcoR* V restriction endonuclease and inserted into the corresponding sites of pPG to give rise to pPG-HN. Finally, the recombinant pPG-HN and pPG plasmids were transformed into *L.casei* ATCC 393 by electroporation as previously described [16].

Western blot analysis

The expression of recombinant HN gene in *L.casei* was detected by Western blot analysis as described previously [17]. Briefly, Lc-pPG-HN and Lc-pPG were grown in basal MRS (MRS Broth) medium supplemented with 10 μg/mL of chloramphenicol Xylose was added to the culture medium to a final concentration of 10 g/L to induce antigen expression. After induction at 30 °C for 10 h, approximately 1×10^8 cells pellets or supernate (a 10-fold concentration) were examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. The membrane was blocked with 5% skim milk, and then incubated with Rabbit anti-HN serum at dilution of 1:500 with phosphate buffered saline (Bioss, Beijing, China), overnight at 4 °C. Affinity-purified horseradish peroxidase (HRP)-conjugated goat anti-Rabbit immunoglobulin G (IgG) (Bioss, Beijing, China) was used as second antibody. Finally, the blot was visualized by chemiluminescence detection with Western ECL substrate (Termo Scientific) in an Amersham Imager 600 (GE Healthcare, UK).

Immunofluorescence assay

Immunofluorescence was performed to determine the surface-displayed localization of the HN protein from Lc-pPG-HN as previously described [17]. In brief, $1x10^5$ cells of Lc-pPG-HN were resuspended in 50 μL PBS containing 1% bovine serum albumin (BSA) and rabbit anti-HN antiserum (1:1000 dilution) at 37 °C for 1 h Subsequently, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Bioss, Beijing, China) containing 1% Evans blue at 37 °C for 2 h. The cells were visualized using a fluorescence microscopy (Zeiss LSM710). The Lc-pPG was used as negative control.

Vaccine preparation,animal treatment and sample collection

For oral administration, overnight cultures of recombinant *L. casei* grown in De Man, Rogosa and Sharpe medium supplemented with chloramphenicol and xylose were thoroughly mixed with commercial basal diet feed, then oven-dried at 37 °C for 8 h. Recombinant *L.casei* counts were expressed as 1×10^9 CFU/mL and 2×10^9 CFU/mL of sample. Three groups of with 3 replicates tray levels of 30 chickens each orally immunized with Lc-pPG-HN, Lc-pPG or the 0.9% physiological saline feed. Oral vaccinations were conducted on days 1 (1×10^9 CFU/g, 1 mL per chick) and 10 (2×10^9 CFU/g, 1 mL per chick), following the immune protocol administered on 3 consecutive days (Fig. 1). All samples were chosen, and the organs weight and body weight (BW) were measured on 1, 7, 14 and 21 days-old. Hematoxylin and eosin (H&E) staining was used to observe the changes in intestinal morphological structure (duodenum, jejunum and

ileum) and organ pathologies (spleen, bursa of Fabricius, thymus, lung and liver). Serum was stored at −80 °C until further analysis. Pieces of the cecum and jejunum were rapidly excised, frozen in liquid nitrogen, and stored at −80 °C until DNA extraction.

Immune organ measurement

The bursa of Fabricius, thymus and spleen were the immune organs studied. The chickens were weighed, and the immune organs were excised and weighed on 1, 7, 14, and 21 days-old. The data are presented as the relative weight (index) of the given immune organ weight from the total BW. The indices were calculated as immune organ weight/BW × 100%.

Histomorphological investigations

The collected segments of intestine were the loop of duodenum, the tract before Meckel's diverticulum (jejunum) and the tract before the ileocolic junction (ileum). Gut segments were fixed in 10% buffered formalin solution for morphometric analysis. Tissues were routinely embedded in paraffin wax blocks, sectioned at 5 μm thickness, mounted on glass slides and stained with HE. The villus height (VH) was measured from the top of the villus to the crypt mouth; the invagination depth between adjacent crypt mouths was measured and defined as the crypt depth (CD), and the VH/CD ratio was calculated. Morphometric analyses were performed on 5 well-oriented and intact villi and 5 crypts chosen from duodenum, jejunum and ileum [18, 19]. Histopathological lesions were assessed descriptively according to the presence of degeneration, hemorrhaging, congestion, and edema. Organs were graded based on the focal lesion distribution (low severity) and multifocal lesion distribution (moderate severity). Observation was conducted using 200× magnification and 3 view-field repetitions.

Hemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA)

Blood samples from the immunized chickens were clotted at 37 °C and centrifuged at 3000 rpm for 10 min to collect the serum. Two-fold serial dilutions of the serum were prepared in 96-well plates. Next, equal volumes (25 μL) of 4 hemagglutination units (4 HAU) of NDV antigen were added to all rows except the last row. Thirty minutes later, 25 μL of 1% chickens erythrocytes were added and incubated for 30 min. The agglutination was monitored, and the HI titers were determined as the highest serum dilution that completely inhibited NDV agglutination [20]. The jejunum were infused with 0.5 mL phosphate-buffered saline (PBS); pH 7.4, containing 0.1% bovine serum albumin and aprotinin), washed three times, and centrifuged at 2000 rpm at 4 °C for 15 min to collect the supernatant. Commercial ELISA kits for determining secretory immunoglobulin A (SIgA) from jejunal lavage fluid, serum interleukin (IL-2) and interferon (IFN-γ) from chickens (Senbeijia, Nanjing, China) were used to determine the NDV antibody levels and cytokine production [21].

DNA isolation and bacterial quantification with qPCR

The cecum were removed, and 20–30 mg aliquots were weighed. Total cecum DNA was extracted according to the instructions with the DNA ΔSimple Total DNA kit-DP431 (Tiangen, Beijing, China). A 10 μL total reaction volume in a 96-well plate contained 5 μL Maxima SYBR Green qPCR Master Mix (Thermo Scientific/Fermentas, Vilnius, Lithuania), 0.2 μM of each primer specific to the 16S rDNA of *Bifidobacterium spp*. [22], *Lactobacillus spp*. [23], *E.coli* [24] or *universal bacteria* [25], and 2 ng of bacterial DNA template. Table 1 lists the forward and reverse primer sequences. DNA was amplified and detected using the SYBR® Premix Ex Taq^{TM} II real-time RT-PCR (qRT-PCR) kit (Bao Bioengineering Co., Ltd., DaLian, China) with the Applied Biosystems 7500 FAST Real-Time PCR System. During the PCR reaction, samples were subjected to an initial denaturation phase at 95 $^{\circ}$C for 5 min followed by 40 cycles of denaturation at 95 $^{\circ}$C for 15 sec and annealing and extension at 58 $^{\circ}$C for 30 sec. Average Ct values of the four technical replicates obtained from the LightCycler 480 II System software were used for data analysis. PCR efficiency for each primer pair was calculated in the LightCycler 480 II software based on the separate reaction of 5 dilutions (1x, 0.5x, 0.25x, 0.125x and 0.0625x) of pooled bacterial DNA template. The relative abundances of the bacteria in the chyme were calculated as follows: Relative Abundances [%] = (Efficiency universal)$^{\text{Ct universal}}$ / (Efficiency target)$^{\text{Ct target}}$.

Viral challenge

After two oral vaccinations, the chickens in the Lc-pPG-HN and control groups were orally challenged with a 2×10^9 mean chickens infection dose (EID_{50}) of the NDV strain in 0.2 mL viral suspensions per chickens at 21-day-old. The chickens were monitored daily for 10 days after the challenge, and the numbers of dead chickens were recorded.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 software and GraphPad PRISM 7.0. For multiple comparisons one-way ANOVA were preformed, followed by Tukey's test. Data are presented as the mean ± SEM. Differences were considered statistically significant at $P > 0.05$, $P < 0.05$, $P < 0.01$.

Result

Construction of *L.casei* vaccine and expression of HN protein

Extract the total RNA of NDV, and amplify the NDV HN gene by RT-PCR according to primers, the gene fragment is 1617 bp (Fig. 1a). The recombinant plasmids pPG-HN was constructed and transferred into *L.casei* by electroporation. The recombinant plasmids were confirmed by restriction enzyme digestion and sequence analysis (Fig. 1b). To examine whether the properties of the recombinant *L.casei* changed, growth and hereditary stability were analyzed after the recombinant *L.casei* were screened by PCR and DNA sequencing. PCR and sequencing analysis (Fig. 1c) showed that recombinant plasmids were stably inherited over 50 generations.

Expression of HN and immunofluorescence assays

To investigate whether the recombinant *L.casei* is capable of producing HN, cell pellets of Lc-pPG-HN, and Lc-pPG were confirmed by Western blot analyses. The results showed that there was immunoreactive band to 63 kDa were detected in the cell lysates of Lc-pPG-HN (Fig. 2a, lane 2), whereas no band was observed in the Lc-pPG (Fig. 2a, lane 1). These observations suggested that the HN protein is produced and secreted by recombinant *L.casei*. Clear fluorescence signals were observed in Lc-pPG-HN (Fig. 2b, left), making the morphology of *L.casei* faintly visible. There was no signal in cells containing the empty vector (Fig. 2b, right). Bacteria could be seen clearly under oil immersion. This finding suggested that Lc-pPG-HN cells could react with the polyclonal Rabbit anti-HN antibody.

Effects of oral immunization with recombinant*L.casei*on chickens immune organ index

Oral immunization with Lc-pPG-HN group had significant effect on BW and immune organ index of chickens compared with control and Lc-pPG groups ($P < 0.05$). However, on 14-21 days, the BW in Lc-pPG-HN group was significantly heavier than those in the other two groups ($P < 0.01$, Table 2).

The index of spleen, thymus and the index of fabricius of groups Lc-pPG and Lc-pPG-HN were increased by 5%, 18% ($P < 0.05$), increased by 7.9%, 10% ($P < 0.05$), and 1.3%, 4% ($P < 0.05$) on 21-day-old, respectively (Table 2).

Intestinal histomorphological analyses

The VH, CD and the ratio of VH/CD of duodenum, jejunum and ileum are shown in Table 3. However, the VH and ratio of VH to CD of chickens in the Lc-pPG and Lc-pPG-HN groups during the 14-21 days periods were significantly increased when compared with the control group ($P < 0.05$). Moreover, when compared with the control group, the CD of chickens in the Lc-pPG group during the 14-21 days periods was also significantly decreased ($P < 0.05$).

SIgA expression levels in the jejunal lavage fluid

Jejunal lavage fluid SIgA was measured to evaluate the systemic immune response. SIgA production was dramatically increased in the jejunal lavage fluid of the chickens immunized with Lc-pPG-HN compared with that of the Lc-pPG and control groups on 14 ($P < 0.05$) and 21-days-old ($P < 0.01$) (Fig. 3).

Effects of the intestinal flora

The effects of recombinant *L.casei* on the relative abundance of immune related *Lactobacillus spp.*, *E.coli* and *Bifidobacterium spp.* were examined by qRT-PCR analysis (Fig. 4a, b or c). In Lc-pPG-HN group, significant changes in *Lactobacillus spp.* relative abundance observed after booster immunization ($P < 0.05$) (Fig. 4a). The relative abundance of *E.coli* rapid decreased was detected at 7-day-old compared with the control and Lc-pPG groups, and *Bifidobacterium spp.* reached a fourfold upregulation in caecum at 21-day-old (Fig. 4b or c). Of note, almost no differences in relative abundance were observed between treatments and control groups after prime immunization ($P > 0.05$).

Serum IL-2 and IFN-γ expression levels

The effects of the recombinant *L.casei* on IL-2 levels of serum were shown in Fig. 5a. Chickens that oral administration of Lc-pPG-HN and Lc-pPG resulted in higher IL-2 activity as compared to the controls ($P < 0.01$) after booster immunization at 14-day-old. Likewise, the higher serum IFN-γ level (Fig. 5b) was observed in chickens received Lc-pPG-HN at 21-day-old compared to the controls ($P < 0.05$).

NDV-HN antibody titers by HI

The level of HI anti-HN in the serum of immunized chicken was determined. As the results showing (Fig. 6), significant level of HI was elicited in chickens that received Lc-pPG-HN ($P < 0.05$) as compared to the controls. After booster immunization, the higher antibodies titers in the serum was observed in chickens by oral administration of Lc-pPG-HN compared to the control and Lc-pPG ($P < 0.05$). By contrast, no significant elicitation of antibodies was detected in the control groups. No significant difference ($P > 0.05$) was observed between treatments and controls prior to the prime immunization.

Viral challenge

The NDV strain challenge experiment was performed to evaluate the protective immunity of the recombinant *L.casei.* Of the chickens immunized with Lc-pPG-HN, 80 % survived after being challenged with a low dose of NDV, whereas the control group exhibited the lower survival rate (Table 4).

Pathological organ changes

Clinical signs appeared in infected chickens and were first observed 2 days postchallenge, consisting of green-white soft feces, anorexia, diarrhea, crouch, paralysis, and eyelid edema. Histopathological changes in the visceral organs following the virulent viral challenge were studied separately for NDV.

In the control group, the immune organs showed moderately severe lymphoid depletion in the medulla, and the thinned cortical lymphocytes were loosely arranged, appearing as a few bleeding spots in the medullary lymph (Fig. 7A–F). The control group also presented lymphopenia in the smooth muscle wall of the tertiary bronchi and stenosis in the pulmonary atrium. The pulmonary capillary blood vessels had a few divided red blood cells, a messy cloth-like structure, and a lower cell density with low severity (Fig. 7H). The control group also presented central vein dilation in the liver and liver sinusoid expansion (Fig. 7J). The lung and liver in the Lc-pPG-HN group showed no obvious lesions (Fig. 7G and I). Pathological lesions and histopathological changes were reduced in the vaccinated chickens following the virulent NDV challenge.

Discussion

Newcastle disease (ND) is an economically important disease of poultry industry, and naturally occurring avirulent NDV strains are widely used as live attenuated vaccines all over the world [26]. Several previous studies have suggested that there is a need to develop live-virus-vectored vaccines that do not involve

attenuated NDV strains [27]. In poultry, the small intestine is one of the main mucosal surfaces and immune barriers [28]. As a mucosal vaccine vector, *L.casei* was used very proficiently whereas excellent result was also found when it was used as a carrier in DNA vaccine through oral route [29, 30]. In this study, we engineered and evaluated the recombinant *L. casei* expressing HN protein of NDV to induce an individual development and immunity response in chicken.

In our study, the HN antigen was stably expressed on *L.casei*. HN detection in both the supernatant fluid and cell pellets indicated that the expression system also enabled the *L.casei* to surface display the HN antigen. In analyzing the properties of the recombinant *L.casei*, although overproduction of heterologous proteins often hampers bacterial growth, the HN-expressing strains grew well and were genetically stable. Our results are consistent with those of a previous report that *L. plantarum* expressing the Hirep2 (H2) antigen of *Chlamydia trachomatis* had only limited effects on bacterial fitness [31]. These data enable hypothesizing that HN immunogenicity based on the *L.casei*-expression system can be further investigated via oral administration in a chick model.

Immune organ index and intestinal histomorphology had been widely used for assessing growth performance and intestinal development. For instance, both Ding et al.[32] and Bu [33] found similar increasing growth performance and organs index when feeding chickens with *L.casei* or *Lactobacillus* vaccine. Also, Sumarsih et al [34]. Previous studies also indicated that increased VH and VH/CD are directly correlated with increased epithelial turnover, and longer villi are linked with activation of cell mitosis, with shortening of villi and deeper crypts leading to poor nutrient absorption, increased secretion in the gastrointestinal tract and reduced performance [35, 36]. In our study, chickens oral immunization Lc-pPG-HN consistently increased the VH of jejunum, duodenum and ileum, and the VH/CD significantly increased, which indicated the better digestive and absorption capability [37].

We conducted a oral route of vaccination to evaluate the immunogenicity of our developed vaccine candidate. SIgA is the principal antibody type for host mucosal defense system [38]. The detection of specific IgA from jejunum indicated that recombinant strain Lc-pPG-HN triggered strong mucosal immune response.

Relevant studies have shown that intestinal microbiota of animals plays an important role in the life activities of the host [39]. *L.casei* also determined a modification of the gut microbiota composition in chickens. Furthermore, the results show that Lc-pPG-HN can significantly increase intestinal the number of *Lactobacillus* and *Bifidobacteria* and reduce the number of *Escherichia coli* bacteria. Probiotics, such as *Lactobacillus spp.*, *Bifidobacterium* spp. are living microorganisms that confer health benefits on the host [40]. Accordingly, recombinant *lactic acid bacteria* vaccine promising candidates to study intestinal microbiota/immune system modulation in chickens.

For the detection of cellular response, IL-2 and IFN-γ were selected which are produced by Th1 cells and play a critical role triggering in cell-mediated immune response for the clearance of intracellular pathogens. Oral administration group induced significant ($P < 0.05$) rise of IFN-γ and IL-2 from 14-day-old in comparing control and Lc-pPG groups. Although the level of IFN-γ and IL-2 induction is not so high but

consistent with other observation [41]. From the cytokine study, it is also observed that serum from Lc-pPG group also produced a considerable amount of IL-12 and IFN-γ comparing control PBS group (Fig. 5) which may be due to the ability of *Lactobacillus casei* to induce IL-2 and IFN-γ by itself [42].

The results of the HI assay revealed that the Lc-pPG-HN significantly elicited higher antibody titers than the groups received Lc-pPG or saline at 21 day-old ($P < 0.05$). However, no significant changes were evident for any treatment when compared to the vaccinated Lc-pPG or control group at any time point ($P > 0.05$). Interestingly, The survival study revealed that the Lc-pPG-HN could provide strong protection (80%) for chickens against NDV challenge. However, the lesions observed in NDV-infected chickens were severe and manifested multifocal lesions in the immune organs. The most numerous and consistent gross lesions in these organs involved severe lymphoid depletion and necrosis. H&E staining showed obvious lymphocyte degeneration and necrosis in the immune organs, lung and liver 2 days post challenge. Another study showed that the clinical signs and pathological changes in experimentally infected geese were similar to those of ND induced by viscerotropic velogenic NDV strains in chickens [43]. In addition, anti-HN antibody induced by Lc-pPG-HN demonstrated significant increase and provided protection for vaccinated chicken, immunogenic of Lc-pPG-HN requires to be further identified.

Conclusion

In conclusion, we demonstrated five major points in this paper: (1) We generated a recombinant *L.casei* (surface-displayed) expressing the HN of NDV, and the recombinant plasmid in *L.casei* could be stably inherited over 50 generations; (2) Oral vaccination chickens with the recombinant vaccine candidate induced a higher immune organ index and intestinal development; (3) The recombinant vaccine candidate also stimulated jejunum mucus specific SIgA and serum HI antibody titers, and induced a higher IL-2, IFN-γ levels, eliciting inflammatory response and cell immune response; (4) Relative abundance of *Bifidobacterium* spp, *Lactobacillus* spp, *E. coli* in the oral immunization group with engineered *L.casei* was beneficially modulated in chickens cecum, improving intestine health; (5) Our study clearly demonstrates that the engineered *L.casei* developed in this study provide protection for chickens against infection with NDV. These results suggest that the potential of oral administration as a mucosal vaccine may be an attractive candidate for treating NDV response and improving early growth performance and intestinal health.

Abbreviations

Newcastle disease virus (NDV), *Lactobacillus casei* (*L.casei*), hemagglutinin-neuraminidase (HN), body weight (BW), secretory immunoglobulin A (SIgA), hemaggluti-nation inhibition (HI), *lactic acid bacteria* (LAB), porcine epidermal growth factor (pEGF), De Man, Rogosa and Sharpe (MRS), *Escherichia coli (E.coli*), Luria-Bertani (LB), Chloramphenicol (Cm), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoglobulin G (IgG), fluorescein isothiocyanate (FITC), Hematoxylin and eosin (H&E), villus height (VH), crypt depth (CD), hemagglutination units (HAU), phosphate-buffered saline (PBS), serum interleukin (IL-2) and interferon (IFN-γ)

Declarations

Ethics approval and consent to participate

This study was conducted following the Jilin Agriculture University Institutional Animal Care and Use Committee (JLAU08201409), and the experimental procedures were performed in compliance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023).

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was financially supported by the 13th Five-year Science and Technology Plan Item of Jilin Provincial Education Department (Grant No. JJKH20200355KJ, JJKH20201293JY) and the National Natural Science Foundation of China (Grant No. 31372391).

Authors' contributions

Anqi Ju, Aoyi Duan, Shubao Yang and Yinnan Zhang contributed significantly to analysis and manuscript preparation;

Xin Ma and Weimin Luan performed the data analyses and wrote the manuscript;

Yanjun Qin and Ligang Xue helped perform the analysis with constructive discussions.

Acknowledgements

The authors thank the Animal Development and Immune Regulation Lab of Jilin Agricultural University for technical support.

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Tables

Table 1
The primer sequence of qRT-PCR.

Gene	Primer	sequence
universal bacteria	Forward	TGATATTGCTGCGCTCGTTG
	Reverse	CTTTCTGGCCCATACCAACC
Bifidobacterium spp	Forward	GCGTGCTTAACACATGCAAGTC
	Reverse	CACCCGTTTCCAGGAGCTATT
Lactobacillus spp	Forward	AGCAGTAGGGAATCTTCCA
	Reverse	CACCGCTACACATGGAG
Escherichia coli	Forward	CATGCCGCGTGTATGAAGAA
	Reverse	CGGGTAACGTCAATGAAA

Table 2
Oral immunized chickens body weight and immune organ index.

Items	Day	Groups			*P*-value
		Control	Lc-pPG	Lc-pPG-HN	
Spleen (mg/g)	d 1	0.36 ± 0.51	0.36 ± 0.13	0.39 ± 0.61	0.872
	d 7	0.62 ± 0.24	0.52 ± 0.13	0.68 ± 0.28	0.551
	d 14	0.81 ± 0.19	0.66 ± 0.17	0.72 ± 0.25	0.187
	d 21	0.77 ± 0.87^{a}	0.81 ± 0.12^{b}	0.91 ± 0.01^{b}	0.032
Thymus (mg/g)	d 1	0.98 ± 0.36	1.02 ± 0.15	0.96 ± 0.99	0.677
	d 7	1.15 ± 0.16	0.94 ± 0.12	1.09 ± 0.12	0.113
	d 14	1.03 ± 0.13^{a}	1.46 ± 0.11^{b}	1.51 ± 0.31^{b}	0.005
	d 21	0.21 ± 0.73^{a}	1.70 ± 0.56^{a}	2.00 ± 0.30^{b}	0.019
Bursa of Fabricius (mg/g)	d 1	1.16 ± 0.01^{a}	0.82 ± 0.02^{bc}	0.67 ± 0.02^{b}	0.027
	d 7	1.04 ± 0.01^{a}	1.24 ± 0.03^{b}	1.12 ± 0.07^{a}	0.040
	d 14	1.14 ± 0.01^{a}	1.34 ± 0.03^{b}	1.22 ± 0.07^{a}	0.039
	d 21	1.44 ± 0.09^{a}	1.33 ± 0.10^{a}	1.41 ± 0.16^{b}	0.455
Body Weight (mg/g)	d 1	35.98 ± 2.30^{a}	34.79 ± 2.61^{b}	36.26 ± 2.31^{ab}	0.045
	d 7	32.20 ± 1.92^{a}	38.24 ± 5.73^{a}	45.01 ± 7.58^{b}	0.012
	d 14	44.40 ± 7.70^{a}	53.41 ± 2.18^{ab}	61.01 ± 8.33^{b}	0.007
	d 21	64.52 ± 5.46^{a}	78.27 ± 3.07^{b}	84.40 ± 10.78^{c}	0.009

[a,b,c]Means in the same column followed by different letters differ significantly by the Tukey test, $P < 0.05$, $P < 0.01$. Results are presented as mean ± standard error, SE. n = 5.

Table 3
Effects of intestinal morphology of chickens after oral immunized.

Items		Day	Groups			*P*-value
			Control	Lc-pPG	Lc-pPG-HN	
Jejunum	VH (μm)	d 14	661.91 ± 1.50^{a}	648.31 ± 17.59^{a}	709.78 ± 17.64^{b}	0.048
		d 21	711.28 ± 1.04^{a}	723.92 ± 7.59^{a}	744.48 ± 28.70^{b}	0.034
	CD (μm)	d 14	103.16 ± 8.23^{a}	100.20 ± 1.87^{a}	104.90 ± 5.61^{b}	0.025
		d 21	103.10 ± 2.43^{a}	102.22 ± 0.89^{a}	96.76 ± 2.18^{b}	0.023
	VH/CD	d 14	7.44 ± 0.73^{a}	6.24 ± 0.28^{b}	6.74 ± 0.82^{b}	0.048
		d 21	6.90 ± 0.34^{a}	7.48 ± 0.08^{a}	7.69 ± 0.19^{b}	0.047
Duodenum	VH (μm)	d 14	855.84 ± 14.34^{a}	846.62 ± 11.21^{a}	855.13 ± 11.21^{b}	0.046
		d 21	833.57 ± 14.06^{a}	830.86 ± 9.59^{b}	939.91 ± 14.61^{b}	0.023
	CD (μm)	d 14	137.27 ± 4.40^{a}	124.59 ± 13.33^{a}	104.61 ± 8.98^{b}	0.047
		d 21	137.68 ± 3.42^{a}	131.27 ± 6.5^{a}	101.13 ± 12.64^{b}	0.049
	VH/CD	d 14	6.19 ± 0.15^{a}	6.67 ± 0.72^{a}	8.17 ± 0.57^{b}	0.042
		d 21	6.82 ± 0.09^{a}	6.86 ± 0.83^{a}	9.33 ± 0.40^{b}	0.048
Ileum	VH (μm)	d 14	511.90 ± 41.45^{a}	498.31 ± 17.22^{a}	559.16 ± 17.68^{b}	0.048
		d 21	565.98 ± 11.23^{a}	587.45 ± 12.00^{ab}	629.64 ± 18.43^{b}	0.006
	CD (μm)	d 14	107.68 ± 2.45^{a}	113.96 ± 1.52^{c}	106.45 ± 1.51^{b}	0.004
		d 21	103.87 ± 2.13^{a}	115.98 ± 0.48^{ab}	105.51 ± 5.83^{ab}	0.024
	VH/CD	d 14	4.74 ± 0.19^{a}	4.37 ± 0.42^{bc}	5.80 ± 0.12^{b}	0.047
		d 21	5.48 ± 0.92^{a}	5.06 ± 0.83^{b}	6.45 ± 0.43^{ab}	0.045

villus height (VH) to crypt depth (CD) ratio (VH/CD). ^{a,b,c}Means in the same column followed by different letters differ significantly by the Tukey test, $P < 0.05$, $P < 0.01$. Results are presented as mean ± standard error, SE.n = 5.

Table 4
Results of virus challenge with the NDV strain.

Vaccinated groups	Sample numbers	2 d	3d	4 d	5 d	6 d	Survived
Lc-pPG-HN	10	0	0	1	1	0	8
Lc-pPG	10	1	2	5	2	0	0
Control	10	1	4	5	0	0	0
Note: Sample numbers are the numbers of chickens after twice oral immunizations.							

Figures

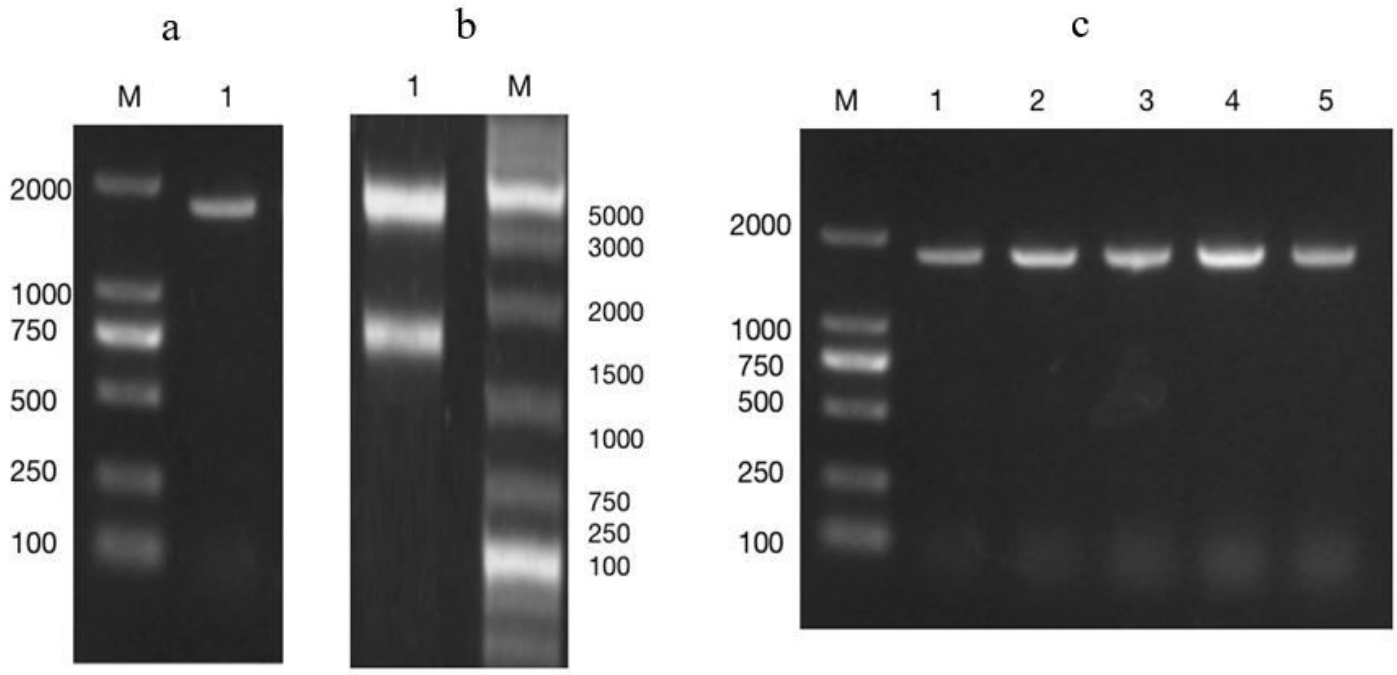


Figure 1

a NDV-HN gene RT-PCR amplification M: DL 2000 DNA Marker 2: The RT-PCR amplification product of NDV-HN. 1b The identification of recombinant L.casei (Lc-pPG-HN) M: DL 5000 DNA Marker1: The production of Lc-pPG-HN digested by restriction enzymes; 1c Characteristics of recombinant L.casei expressing HN antigen. (Lc-pPG-HN) hereditary stability of recombinant L.casei strains were subject to PCR with HN-specific primer pairs confirming that cells were genetically stable after 50 generations. Bands indicated by white (1617 bp) were analyzed by DNA sequencing and were consistent with the putative sequences. L: DNA ladder (bp), Lane 1–5: PCR product of recombinant L. casei HN gene after 50 generations ± SE

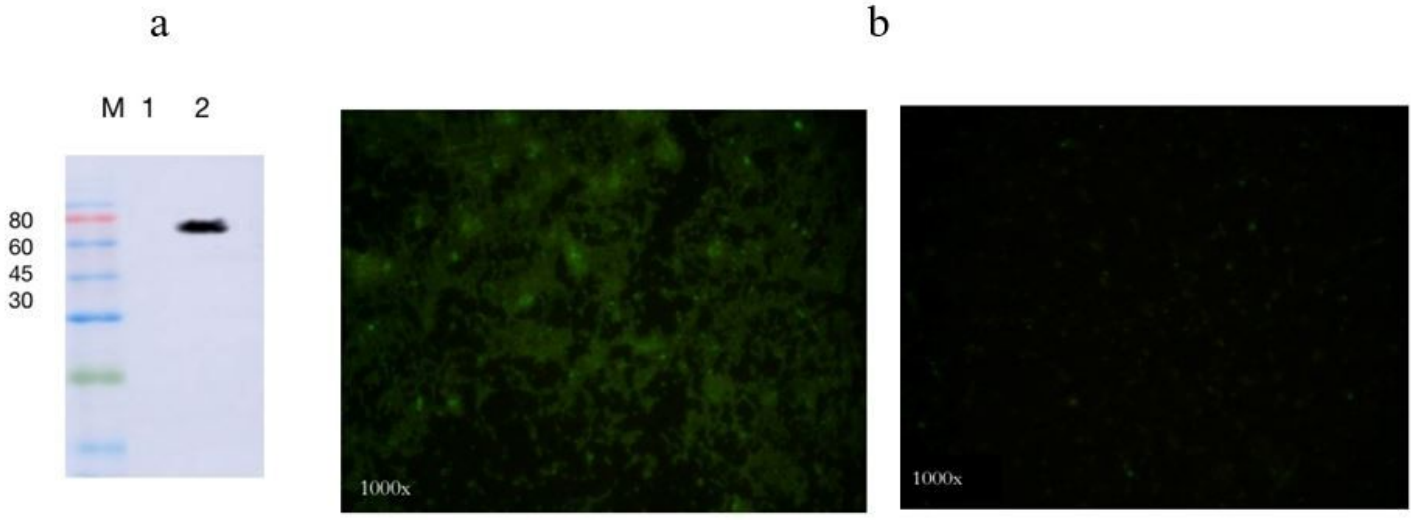


Figure 2

a The identification of recombinant L.casei by Western blotting (left: Lc-pPG-HN M: Blue Plus IV Protein Marker; 1: The expression of induced Lc-pPG; 2: The expression of induced Lc-pPG-HN. 2b The identification of induced Lc-pPG-HN by immunofluorescence and control. (left: Lc-pPG-HN, right: control)

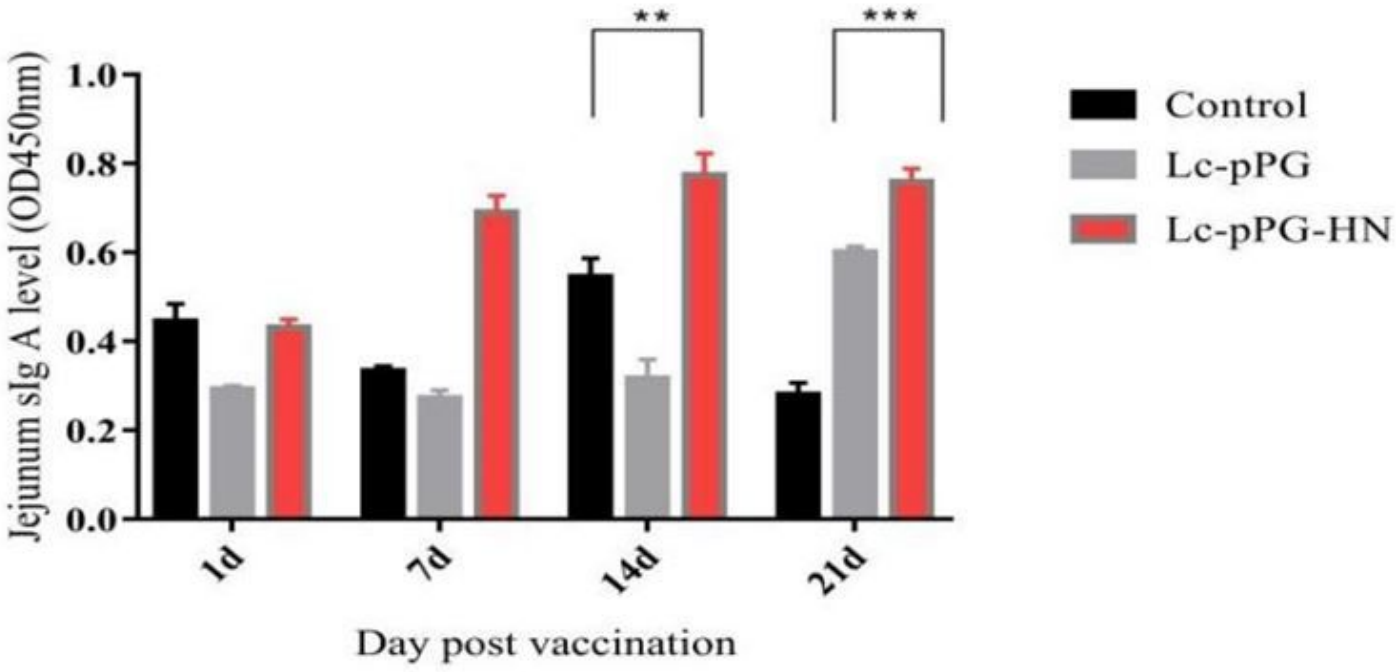


Figure 3

Effect of the immune suppression on level of SIgA in jejunal lavage fluid (OD450 nm). Samples of intestinal content were collected from d 1, 7, 14, 21. Each value represented the mean ± SE of five individuals. **Significant differences ($P < 0.05$), ***Significant differences ($P < 0.01$)

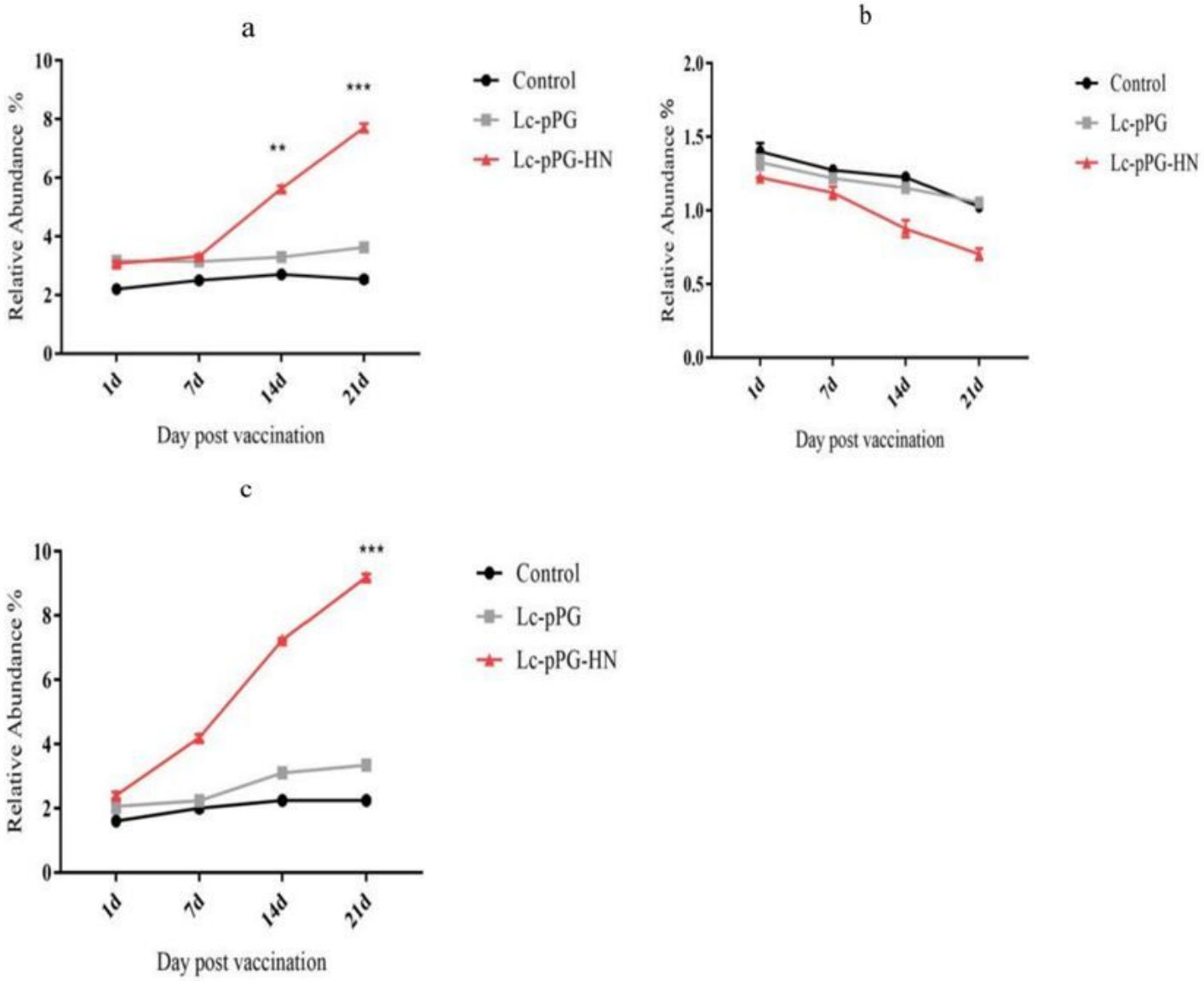


Figure 4

The relative abundance of Lactobacillus spp. (a), Escherichia coli (b) and Bifidobacterium spp.(c) in chyme from caecum in chickens supplemented in oral with recombinant lactobacillus vaccine. Samples of intestinal content were collected from d 1, 7, 14, 21 (n=5). Bacteria quantification was done with qPCR based on bacterial DNA isolated from chyme. Statistical analysis was performed with two-way ANOVA with a Tukey HSD post hoc test. **Significant differences ($P < 0.05$), ***Significant differences ($P < 0.01$).

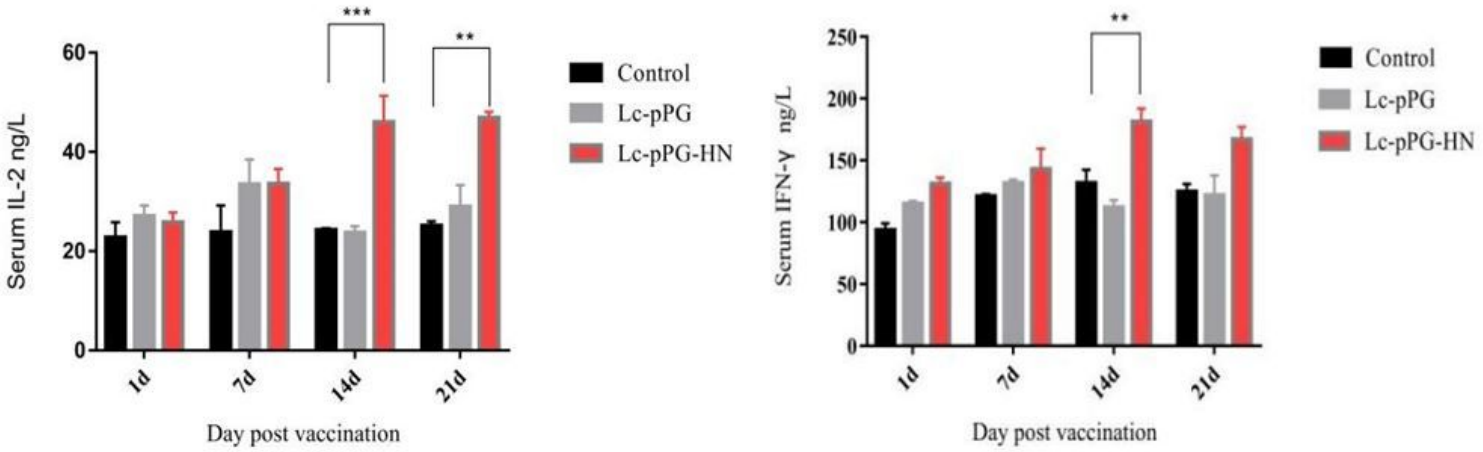


Figure 5

IL-2 and IFN-γ level in serum of immunized chickens (ng/L). a: IL-2; b: IFN-γ. Samples of serum were collected from d 1, 7, 14, 21. Each value represented the mean ± SE of five individuals. **Significant differences ($P < 0.05$).

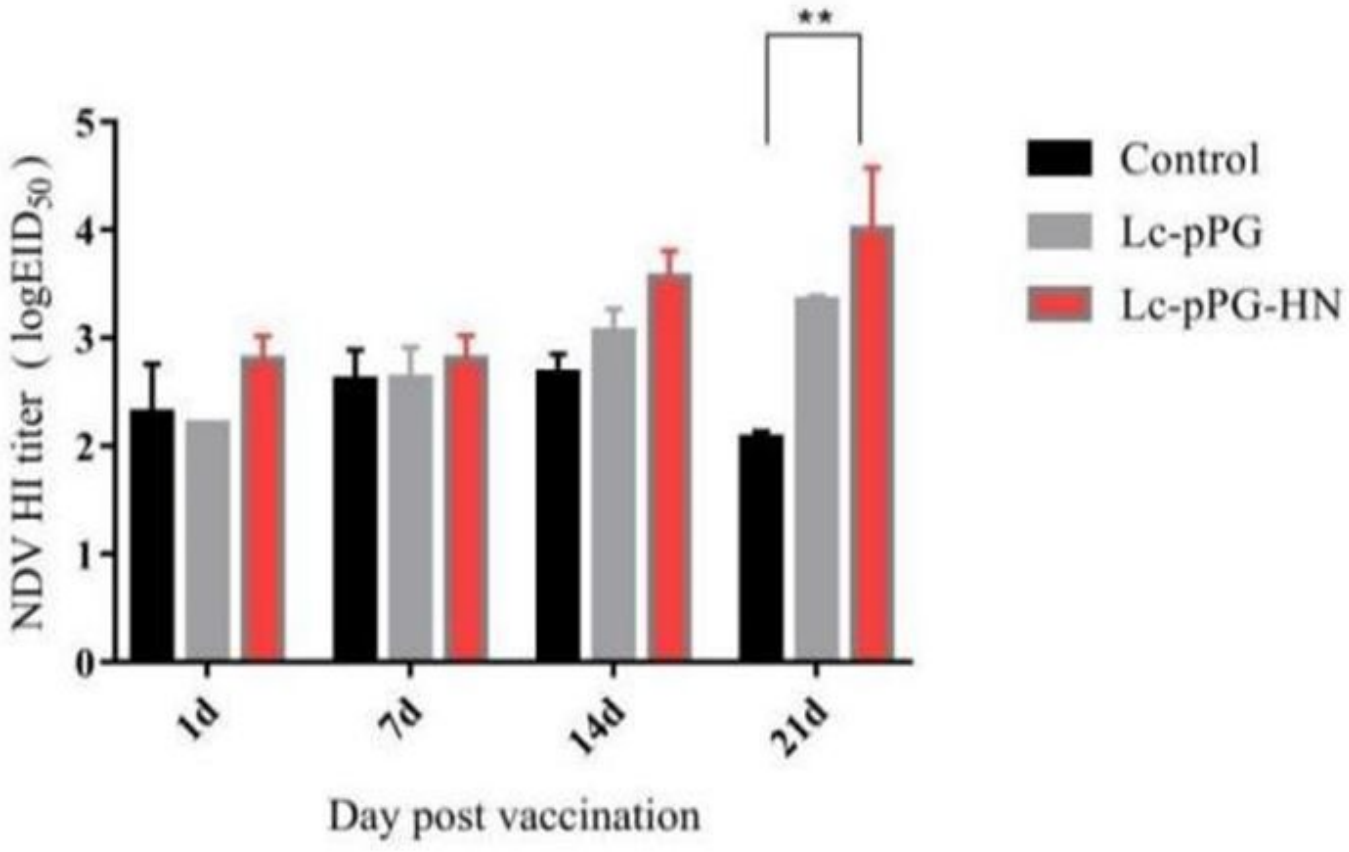


Figure 6

Serum antibody titers to NDV vaccine by hemagglutination inhibition test after the booster vaccination (logEID50). Samples of serum were collected from d 1, 7, 14, 21. Each value represented the mean ± SE of

five individuals. **Significant differences (P < 0.05).

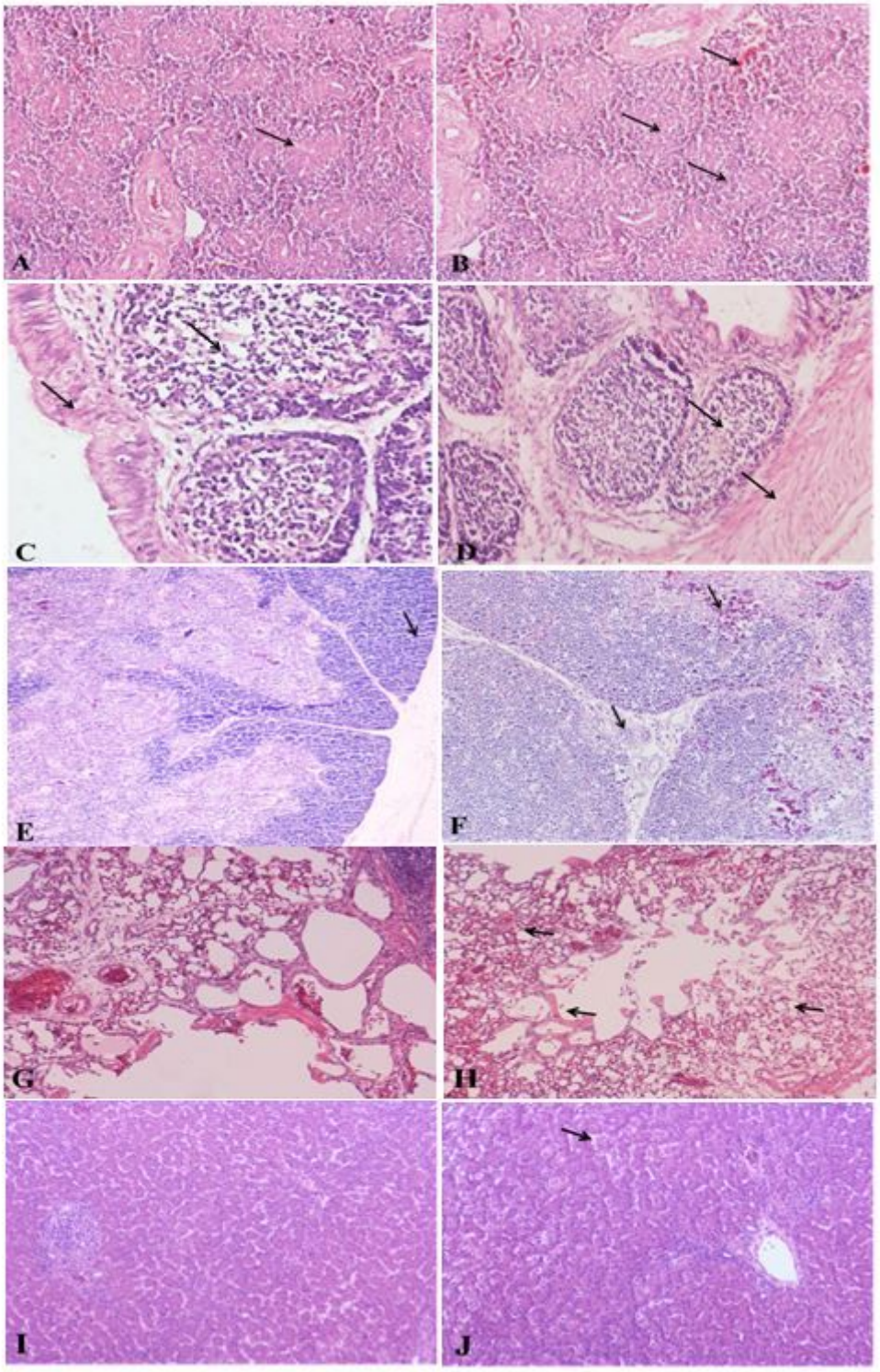


Figure 7

Histopathological lesions in the organs of chickens challenged with NDV. (A, B) spleen with follicular necrosis and lymphoid depletion; (C, D) section of bursa with mild lymphoid depletion and mild peri-follicularedema; (E, F) Thymic cortical thinning lymphocyte arranged loosely, blurred cortical medulla, and infiltration of inflammatory cells (H) Tertiary bronchus smooth muscle wall lymphopenia, congestion; (J) Central vein dilation liver, liver sinus dilation, a small amount of lymphocytes necrosis. No obvious

lesions in the vaccine group of lung (G) and liver (I). Black arrows indicate histopathological changes; HE 200x.

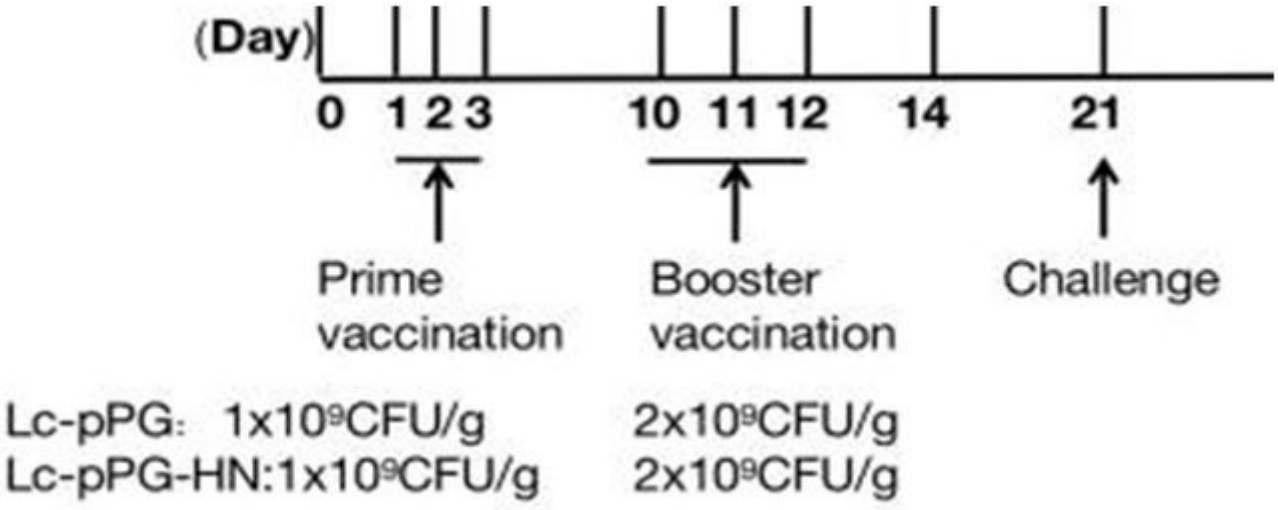


Figure 8

Route of the HN-expressing L.casei in oral immunization