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In ovo administration of HA1-expressing *Lactococcus lactis* protects chicks against H9N2 infection via enhancing intestinal development and immune function

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Abstract

In this study, we utilized recombinant *Lactococcus lactis* (*L. lactis*) expressing the H9N2 AIV hemagglutinin (HA) subunit 1 (HA1) protein and evaluated its efficacy through *in ovo* vaccination. At embryonic day 17.5, 300 eggs were randomly assigned to four groups and inoculated with either PBS, an inactivated H9N2 vaccine, an empty *L. lactis* vector, or recombinant *L. lactis*. The results showed that no significant differences in hatchability were observed across all groups ($P > 0.05$). *In ovo* injection of recombinant *L. lactis*-HA1 markedly increased average daily gain (ADG), improved feed conversion ratio (FCR) ($P<0.05$), and elevated indices for the thymus, spleen and bursa of Fabricius, and elevated serum HI titers, HA-specific IgG, IL-2, IL-4, and IFN-γ, as well as increased mucosal SIgA concentrations and strengthened HA1-specific lymphocyte proliferative responses ($P < 0.05$). Furthermore, this treatment increased villus height (VH) and the villus height-to-crypt depth ratio (VH/CD) in both the duodenum and jejunum, and upregulated the transcriptional levels of intestinal barrier-associated genes, including ZO-1, Occludin, Claudin-3, MUC2, and TFF2 ($P < 0.05$). After H9N2 viral challenge, chicks in the recombinant *L. lactis*-HA1 group exhibited milder clinical manifestations and less body weight loss; meanwhile, their oropharyngeal and cloacal viral shedding titers as well as pulmonary viral loads were markedly decreased, with alleviated pulmonary histopathological lesions. Notably, its protective efficacy was statistically comparable to that of the inactivated vaccine. Collectively, *in ovo* administration of HA1-expressing recombinant *L. lactis* accelerates early intestinal maturation and elicits broad-spectrum immune competence in broiler chicks, providing a theoretical basis for developing novel in-ovo lactic-acid-bacterium-vectored vaccines against H9N2 avian influenza virus.

Keywords H9N2 avian influenza virus • Recombinant *L. lactis* • HA1 protein • *In ovo* vaccination • Immune function

1. Introduction

Avian influenza is a zoonotic infectious disease caused by the influenza A virus (IAV), which primarily infects poultry but can also undergo cross-species transmission to infect pigs, mice, and humans (Abdelwhab & Mettenleiter 2023). The H9N2 subtype of avian influenza virus, first isolated in the United States in 1966, has become one of the most prevalent and endemic low-pathogenic avian influenza viruses in global poultry production (Carnaccini & Perez 2020; Gu, Xu, Wang & Liu 2017). H9N2 infection induces immunosuppression and systemic health impairments in poultry, increasing susceptibility to secondary infections with other viruses and *Escherichia coli*, leading to elevated mortality rates and significant economic losses (Abdelaziz, Mohamed, Fayez, Al-Marri, Qasim & Al-Amer 2019). Furthermore, H9N2 viruses frequently contribute internal gene segments to highly pathogenic avian influenza strains, thereby enhancing their capacity to infect humans and posing a substantial public health threat (Cáceres, Rajao & Perez 2021; He, Liu, Hu, Wang, Guo, Wang, Sun, Shi, Ren, Wang, Nie, Shang, Liu, Jiang, Ren, Jin, Li & Zhao 2025). Consequently, the development of effective and safe prevention and control measures against H9N2 is urgently required.

Traditional inactivated vaccines are widely used to control H9N2 infection. However, their cross-protective efficacy against antigenically distinct genotypes or subtypes remains unsatisfactory (Song, Son, Kim, An, Lee, Kwon & Choi 2024). Hemagglutinin (HA), the major envelope glycoprotein of influenza A virus (IAV) and the primary antigen inducing neutralizing antibodies, is encoded by the fourth genomic segment (Wilks, de Graaf, Smith & Burke 2012). After synthesis, HA is proteolytically cleaved into two subunits: the highly genetically diverse HA1 and the relatively conserved HA2 (Shahsavandi, Salmanian, Ghorashi, Masoudi, Fotouhi & Ebrahimi 2011). HA1 harbors the receptor-binding domain and major neutralizing antigenic epitopes (Xiong, Zhao, Yang, Yan & Jiang 2019), rendering it an attractive antigen for eliciting protective immunity against homologous and partially heterologous influenza viruses. Given the advantages of HA1 as a target antigen, investigating safe and efficient delivery systems and immunization routes has become a critical focus for next-generation H9N2 vaccines.

Newly hatched chicks are highly susceptible to pathogens due to their immature immune and digestive systems, which impair immune development and growth performance while increasing breeding costs. During the prehatch period, adequate nutrition can reduce disease incidence by enhancing nutrient supply (Hou & Tako 2018). An *in ovo* injection is an effective nutrient delivery strategy to compensate for energy deficits during hatching. This approach is typically performed at embryonic days 17–18 via the amniotic cavity, as embryos begin swallowing amniotic fluid at this stage, enabling intestinal absorption of injected nutrients to support growth (Siwek, Slawinska, Stadnicka, Bogucka, Dunislawska & Bednarczyk 2018). Since the 1990s, *in ovo* vaccination has been widely adopted in commercial broiler hatcheries to control diseases such as Marek's disease, Newcastle disease, and infectious bronchitis. Accumulating evidence indicates that *in ovo* immunization elicits earlier antibody responses, establishes early immune protection, reduces post-hatch injection stress, and enhances antiviral resistance (Abd El-Ghany 2024). Additionally, *in ovo* administration of probiotics promotes early intestinal colonization and accelerates immune organ maturation, thereby enhancing immune function. Notably, de Oliveira, van der Hoeven-Hangoor, van de Linde, Montijn and van der Vossen (14) reported that this strategy effectively enhances immune protection and disease resistance in post-hatch chicks. *L. lactis*, as a food-grade probiotic, enables efficient delivery of foreign antigens and elicits robust mucosal

immune responses while demonstrating high biosafety, low immunogenicity, and strong colonization capacity in the intestinal tract (Dieye, Hoekman, Clier, Juillard, Boot & Piard 2003). Given the complementary advantages of timing in *in ovo* delivery and antigen presentation in *L. lactis*, combining these two strategies represents a novel and promising approach for developing effective H9N2 avian influenza vaccines.

Few studies have explored in-ovo administration of recombinant *L. lactis* expressing H9N2 AIV HA1 in broilers. Accordingly, this study aimed to evaluate whether in-ovo delivered recombinant *L. lactis*-HA1 could modulate growth performance, intestinal morphological development, and immune-protective efficacy against H9N2 challenge in broiler chicks

2. Materials and methods

2.1 Virus, bacterial and inactivated H9N2 vaccine

The H9N2 influenza A/pigeon/Hebei/02/2017 virus (GenBank accession numbers Mk995886-Mk995893) was isolated from the intestinal tract of a pigeon in Hebei Province and stored in the Key Laboratory of Preventive Veterinary Medicine at Hebei North University. The recombinant *L. lactis* MG1363-pMG36e-HA1 was previously constructed by our research group, with confirmed antigenicity and immunogenicity (Zhang, Xu, Li, Li, Li, Li, Wang, Wang, Wang & Zhang 2023). The virus was propagated by inoculating 10-day-old specific-pathogen-free (SPF) chicken embryos (Ji'nan SAS Poultry Technology Co., Ltd.) for 72 h. The viral titers were measured via plaque assay and stored at -80 °C. To prepare the inactivated H9N2 vaccine, the A/pigeon/Hebei/02/2017 viral strain was propagated in the allantoic cavities of 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs. Allantoic fluid was collected at 72 h post-inoculation, followed by inactivation using 0.2% formalin at ambient temperature over a 24-h period. The inactivated viral supernatant was subsequently emulsified with Montanide ISA70 adjuvant (SEPIC) at a volumetric ratio of 30:70 (v/v).

2.2 Vaccination and challenge

A total of 300 fertilized White Feather Broiler eggs were obtained from a commercial farm (Yizhou Poultry Industry Co., Ltd., Zhangjiakou, China). The eggs were disinfected with 75% ethanol and incubated at 37.5–38.2 °C with 60–70% relative humidity, with automatic rotation every 2 h. Non-viable and infertile eggs were discarded. At embryonic day 17.5, 160 viable embryonated eggs were randomly assigned to four groups (n = 40 per group): PBS group, inactivated H9N2 vaccine (H9N2) group , empty *L. lactis* MG1363-pMG36e vector (L1) group, and recombinant *L. lactis* MG1363-pMG36e-HA1 (L2) group. All eggs received a 0.2 mL *in ovo* injection. The PBS group was administered PBS, the L1 and L2 groups were immunized with the corresponding lactococcal suspensions at a concentration of 10^9CFU/mL, and the vaccine group received the inactivated H9N2 vaccine. All treatments were delivered into the amniotic cavity via *in ovo* injection after surface disinfection, and puncture wounds were sealed with paraffin.

On days 1, 7, 14 and 21 post-hatch, samples including serum, bronchoalveolar lavage fluid (BALF), feces, thymus, spleen, bursa of Fabricius, duodenum and jejunum tissues were harvested to determine growth performance, immune organ indices, HI and HA-specific IgG titers, mucosal SIgA concentrations, serum cytokines, HA1-specific lymphocyte proliferation, intestinal villus morphology, and mRNA expression of intestinal barrier-related genes. Then each group of 10 chicks was intranasally challenged with 10^6 EID_{50} of A/pigeon/Hebei/02/2017 H9N2 virus in 0.1 ml of PBS on day 21 post-hatch. Clinical signs, body weight, viral shedding, pulmonary lesions, and viral lung titers were assessed.

2.3 Measurement of growth performance and hatching rate

Post-hatch hatchability was quantified by tallying hatched chicks. After a 12 h fast on days 1, 7, 14 and 21 post-hatch, five birds per group were weighed for average daily gain (ADG) calculation. Feed conversion ratio (FCR) was determined as body weight gain (BWG)/feed intake (FI).

2.4 Measurement of immune organ indices

The thymus, spleen, and bursa of Fabricius from each chicken were collected and weighed on days 1, 7, 14, and 21 post-hatch. The immune organ index was calculated as the organ weight (mg) divided by the body weight (g) to evaluate immune organ development and immune function.

2.5 HI assays

Blood samples were collected at 1, 7, 14, and 21 days post-hatch and centrifuged at 2000 rpm for 15

minutes at 4 ℃ to obtain serum. Serum samples were serially diluted in two-fold increments in bottom 96-well plates. An equal volume of H9N2 antigen containing 4 HA units was added and incubated for 30 min at room temperature, followed by the addition of 1% chicken erythrocytes and a further 30 min incubation. The HI titer was recorded as the reciprocal of the highest serum dilution that completely inhibited hemagglutination.

2.6 Serum IgG antibodies and mucosal SIgA assay

Serum-specific IgG antibody was determined by indirect ELISA as previously described (Dabaghian, Latify, Tebianian, Nili, Ranjbar, Mirjalili, Mohammadi, Banihashemi & Ebrahimi 2014). Briefly, 96-well plates were coated overnight at 4 ℃ with 1 mg/mL H9N2 AIV HA1 peptide (25 amino acids, residues 2-26) in 50 mM sodium bicarbonate buffer. After blocking, serially diluted serum samples were incubated for 1 h at 37 ℃, followed by a 1 h incubation with horseradish peroxidase (HRP)-conjugated goat anti-chicken IgG (Sigma-Aldrich, USA) at 37 ℃. Plates were developed with TMB substrate, and absorbance at 450 nm (OD450) was measured to quantify serum specific IgG. Group mean OD450 values were compared statistically.

The SIgA levels in the feces and BALF were analyzed using the SIgA ELISA kits (Andi Huatai Technology Co., Beijing), according to the manufacturer's instructions.

2.7 Serum cytokines

Commercial ELISA kits (Andi Huatai, Beijing) were used according to the manufacturer's instructions to measure serum levels of IL-2, IL-4, and IFN-γ.

2.8 Lymphocyte proliferation assay

For splenic lymphocyte proliferation assays, lymphocytes were seeded in 96-well plates at 2×10^6 cells/mL (100 μL per well) and cultured for 48 h with 5 μg/mL PHA (positive control), 5 μg/mL synthetic HA1 peptide (specific antigen), or RPMI 1640 medium (negative control). Proliferation was measured using the Cell Proliferation ELISA BrdU Kit (Roche, Germany) according to the manufacturer's instructions, with absorbance read at 450 nm. HA1-specific proliferative capacity was expressed as the stimulation index (SI), calculated as the mean OD ratio of antigen-stimulated to unstimulated cells.

2.9 Intestinal morphological structure

The duodenum and jejunum tissues were fixed in 4% paraformaldehyde and embedded in paraffin blocks for sectioning at 5 μm thickness. Following hematoxylin and eosin (H&E) staining, the intestinal morphological structures were examined microscopically. The height of five villi and the depth of five crypts were measured to calculate the ratio of villus height to crypt depth (VH/CD).

2.10 RNA extraction and quantitative real-time PCR

Total RNA was extracted from jejunal tissues using a Takara RNA extraction kit. Following quality verification, cDNA was synthesized with a Takara reverse transcription kit, and relative mRNA levels of intestinal barrier genes (*MUC2, TFF2, Occludin, ZO-1,* and *Claudin-3*) were quantified by quantitative real-time PCR (qPCR; TransGen Biotech, China). Expression levels were normalized to GAPDH using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used are provided in Table S1.

2.11 Protective immune responses

Challenged chickens were monitored for clinical symptoms, mortality, and body weight changes. To evaluate viral shedding, cloacal and oropharyngeal swabs were collected post-challenge, and viral titers were determined using the $TCID_{50}$ method at 3, 5, and 7 days post-infection (Spackman, Gelb, Preskenis, Ladman, Pope, Pantin-Jackwood & McKinley 2010; Le Gall-Reculé, Cherbonnel, Pelotte, Blanchard, Morin & Jestin 2007). On day 7 post-challenge, five randomly selected chickens from each

group were euthanized for lung tissue collection. Pulmonary viral titers were quantified using the EID_{50} assay, and pathological lung lesions were examined via H&E.

2.12 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 22.0, and data are presented as mean ± SD. Differences among groups were analyzed by a one-way ANOVA followed by Tukey's test, where a p-value < 0.05 was considered statistically significant.

3. Results

3.1 *In ovo* injection of HA1-recombinant *L. lactis* enhances growth performance

As presented in Table 1, no significant difference in hatchability was detected among all experimental groups ($P > 0.05$). ADG remained comparable across all treatments from post-hatch days 1 to 7 ($P > 0.05$), whereas lower FCR values were obtained in the L1 and L2 groups relative to the PBS control group ($P < 0.05$). At the 15-to-21-day stage, the L1 and L2 groups exhibited markedly improved ADG compared with the PBS group ($P < 0.05$), while no statistical difference in FCR existed among the tested groups ($P > 0.05$).

Table 1 Effects of *in ovo* injection with HA1-recombinant *L. lactis* on hatchability and growth performance of chicks.

Item	Groups				*p*-value
	PBS	H9N2	L1	L2	
Hatchability (%)	90.27 ± 2.24	85.48 ± 4.76	87.53 ± 2.80	87.69 ± 2.52	0.262
1-7 days					
ADG (g)	15.23 ± 1.04	14.27 ± 1.62	15.52 ± 0.86	16.21 ± 1.13	0.216
FCR (g/g)	1.89 ± 0.02^{a}	1.84 ± 0.04^{ab}	1.80 ± 0.02^{b}	1.79 ± 0.03^{b}	0.037
8-14 days					
ADG (g)	24.35 ± 2.34	26.79 ± 3.15	25.16 ± 2.17	27.36 ± 3.02	0.054
FCR (g/g)	1.79 ± 0.04	1.78 ± 0.03	1.76 ± 0.02	1.75 ± 0.03	0.648
15-21 days					
ADG (g)	35.46 ± 5.17^{b}	40.53 ± 4.46^{ab}	45.04 ± 3.56^{a}	46.14 ± 5.13^{a}	0.001
FCR (g/g)	1.73 ± 0.03	1.74 ± 0.04	1.72 ± 0.05	1.69 ± 0.03	0.403

In the same row, values with no letters or the same-letter superscripts mean no significant difference ($P > 0.05$), while values with different lowercase letters indicate a significant difference ($P < 0.05$). The results are represented as the mean ± SD (n = 5).

3.2 *In ovo* injection of HA1-recombinant *L. lactis* improves immune organ development

To assess the influences of *in ovo* injection with HA1-recombinant *L. lactis* on chick immune performance, immune organ indices (thymus, spleen, and bursa of Fabricius) were determined. As listed in Table 2, the spleen indices of the H9N2 and L2 groups were markedly elevated compared with the PBS group on post-hatch day 14 ($P < 0.05$). At day 21, higher thymus indices were observed in the H9N2 and L2 groups relative to the PBS group ($P < 0.05$). Meanwhile, spleen and bursa of Fabricius indices from all three experimental treatment groups were significantly increased compared with the PBS control ($P < 0.05$).

Table 2 Effects of *in ovo* injection with HA1-recombinant *L. lactis* on immune organ index.

Item	Groups				*p*-value
	PBS	H9N2	L1	L2	
Thymus index					

1 day	3.52 ± 0.27	3.47 ± 0.18	3.43 ± 0.08	3.55 ± 0.11	0.646
7 days	4.03 ± 0.74	4.27 ± 0.62	4.14 ± 0.43	4.25 ± 0.63	0.532
14 days	4.43 ± 0.82	4.56 ± 0.61	4.48 ± 0.37	4.62 ± 0.86	0.411
21 days	4.70 ± 0.78^{b}	5.24 ± 0.52^{a}	5.02 ± 0.52^{ab}	5.44 ± 0.73^{a}	0.001
Spleen index					
1 day	0.35 ± 0.07	0.38 ± 0.05	0.31 ± 0.02	0.39 ± 0.05	0.627
7 days	0.80 ± 0.04	0.84 ± 0.06	0.80 ± 0.07	0.85 ± 0.04	0.583
14 days	0.96 ± 0.08^{b}	1.20 ± 0.10^{a}	1.16 ± 0.08^{ab}	1.27 ± 0.13^{a}	0.020
21 days	1.04 ± 0.10^{b}	1.38 ± 0.12^{a}	1.34 ± 0.09^{a}	1.46 ± 0.12^{a}	0.013
Bursa of Fabricius index					
1 day	0.86 ± 0.17	0.93 ± 0.22	0.87 ± 0.14	1.03 ± 0.14	0.438
7 days	1.09± 0.07	1.20 ± 0.09	1.13 ± 0.11	1.17 ± 0.14	0.146
14 days	1.27 ± 0.12	1.32 ± 0.16	1.28 ± 0.09	1.29 ± 0.18	0.725
21 days	1.34 ± 0.11^{b}	1.52 ± 0.13^{a}	1.51 ± 0.13^{a}	1.67 ± 0.14^{a}	0.019

In the same row, values with no letters or the same-letter superscripts mean no significant difference ($P > 0.05$), while values with different lowercase letters indicate a significant difference ($P < 0.05$). The results are represented as the mean ± SD (n = 5).

3.3 *In ovo* injection of HA1-recombinant *L. lactis* enhances HI antibody levels

HI antibody levels were measured to verify whether *in ovo* delivery of recombinant *L. lactis* HA1 could trigger specific humoral immune responses in chicks. No significant difference in HI antibody titers was observed across all groups on post-hatch day 1 ($P > 0.05$). As the chicks aged, the L2 group exhibited significantly higher HI antibody titers than the L1 and PBS groups at days 7, 14, and 21 ($P < 0.05$). Although the *in ovo* delivered inactivated H9N2 vaccine induced strong HI antibody responses, no significant difference in antibody titers existed between the H9N2 group and L2 group ($P > 0.05$, Fig.1a).

3.4 *In ovo* injection of HA1-recombinant *L. lactis* enhances the specific IgG Levels

To detect HA-specific IgG levels in chicks after *in ovo* administration of *L. lactis* HA1, serum samples from each group were analyzed using an indirect ELISA. As shown in Fig.1b, the L2 group exhibited significantly higher HA-specific IgG levels at days 7, 14, and 21 post-hatch compared with the L1 and PBS groups ($P < 0.05$). Consistent with the trends observed for HI antibody titers, no significant differences in HA-specific IgG levels were found between the L2 group and the H9N2 group at days 14 and 21 ($P > 0.05$).

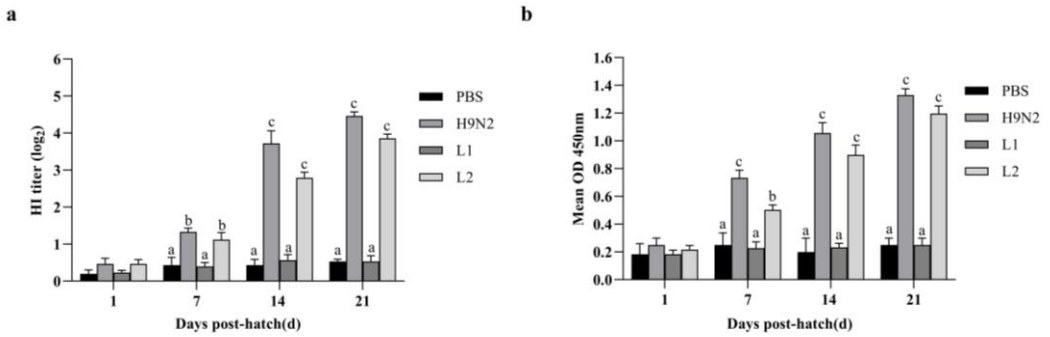

Fig.1 HI antibody levels and the specific IgG levels induced by *in ovo* administration of recombinant *L. lactis* HA1. (a) Antibody titers determined by HI assay; (b) Antigen-specific IgG titers detected by indirect ELISA. The data were shown as means ± SD (n=5). Different letters above the columns indicate significant differences among treatment groups ($P < 0.05$), while identical letters or the absence of letters indicate no significant differences ($P > 0.05$).

3.5 *In ovo* injection of HA1-recombinant *L. lactis* increases serum cytokine levels

IL-2, IFN-γ and IL-4 are key cytokines that mediate cellular and humoral immune responses; their serum concentrations are summarized in Fig.2. Elevated serum IL-2 contents were identified in the L1, L2, and H9N2 groups relative to the PBS group on post-hatch day 14 ($P < 0.05$). On post-hatch day 21, significantly higher IL-2 concentrations were obtained in the H9N2 and L2 groups when compared with the PBS and L1 groups ($P < 0.05$). Meanwhile, IL-4 levels were markedly upregulated in the H9N2 and L2 groups versus the PBS group ($P < 0.05$). Furthermore, the minimum serum IFN-γ content was observed in the PBS group among all experimental groups at day 21 ($P < 0.05$).

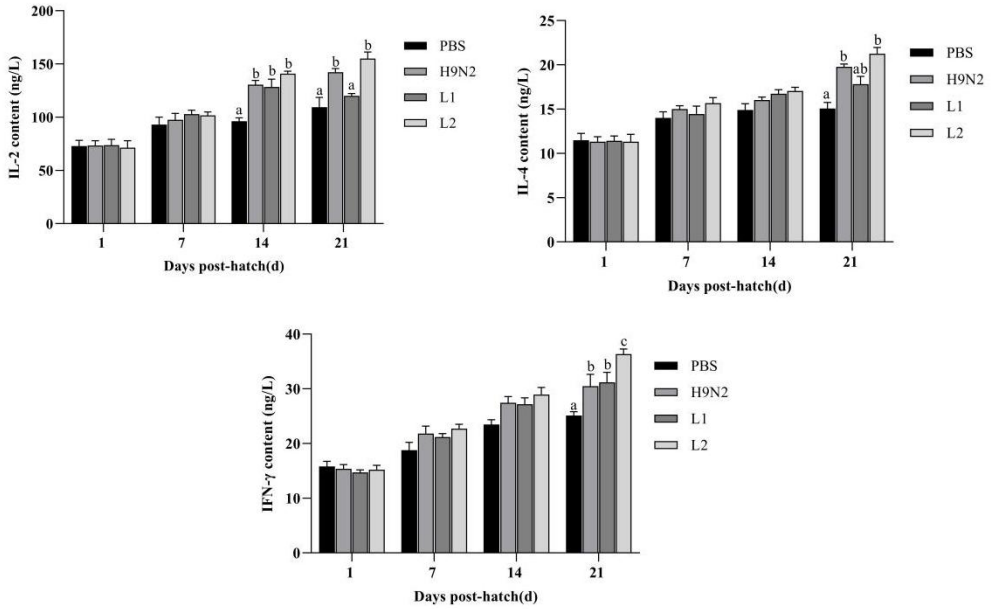

Fig.2 Effects of *in ovo* injection with HA1-recombinant *L. lactis* on serum cytokine levels in chicks. Data are presented as mean ± SD (n = 5). Different letters above the columns indicate significant differences among treatment groups ($P < 0.05$), whereas identical letters or the absence of letters above the columns indicate no significant differences ($P > 0.05$).

3.6 *In ovo* injection of HA1-recombinant *L. lactis* enhanced T-cell proliferation

T-cell proliferation assays were performed to evaluate HA-specific cellular immune responses in chicks after *in ovo* immunization with recombinant *L. lactis*-HA1. As shown in Fig.3, following stimulation with synthetic HA1 peptides, significantly increased T-cell proliferation in response to HA1 was observed in the H9N2 and L2 groups. In contrast, chicks treated with PHA or RPMI exhibited no detectable HA1-specific proliferative responses at days 7, 14, and 21 post-hatch. The stimulation indices for the H9N2 and L2 groups were significantly higher than those for the PBS and L1 groups. Notably, the H9N2 group demonstrated a significantly greater stimulation index compared to the L2 group at day 14 ($P < 0.05$), while no statistically significant intergroup differences were observed at days 7 and 21.

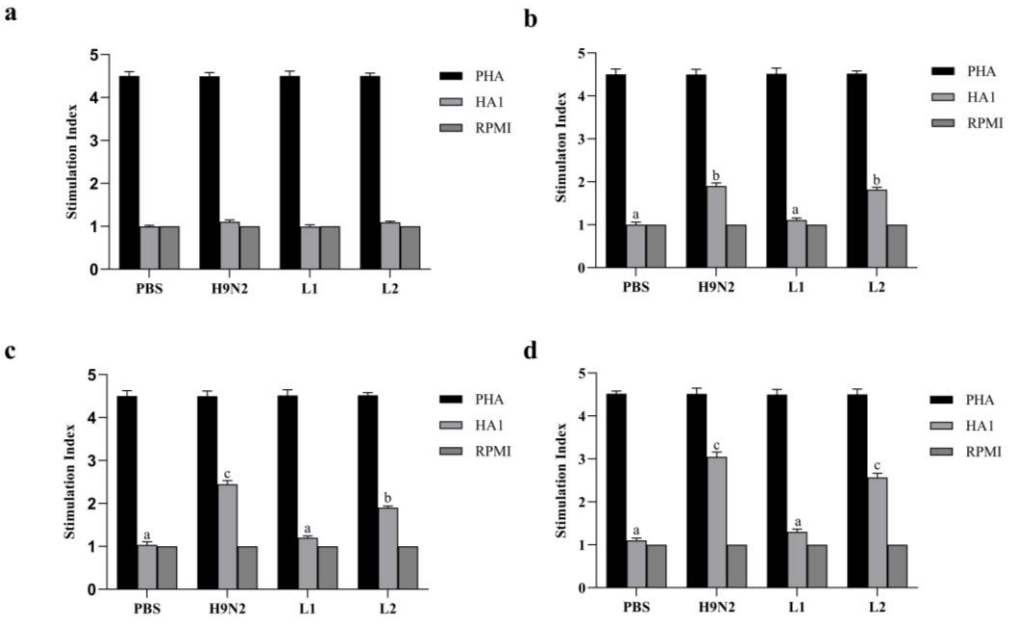

Fig.3 Effects of *in ovo* injection with HA1-recombinant *L. lactis* on T cell proliferation. T cell proliferation was assessed using a Cell Proliferation ELISA BrdU Kit, with results expressed as a stimulation index (SI) for chickens on day 1 (a), day 7 (b), day 14 (c), and day 21 (d). Data are presented as mean ± SD (n = 5). Different letters above the columns indicate significant differences among treatment groups ($P < 0.05$), whereas identical letters or the absence of letters above the columns indicate no significant differences ($P > 0.05$).

3.7 *In ovo* injection of HA1-recombinant *L. lactis* increases mucosal SIgA levels

The changes in the levels of mucosal SIgA are shown in Fig.4. On days 7, 14, and 21 post-hatch, intestinal SIgA concentrations were significantly elevated in the H9N2 and L2 groups compared to the PBS and L1 groups ($P < 0.05$), while no significant difference was observed between the H9N2 and L2 groups ($P > 0.05$) (Fig.4a). BALF SIgA levels showed a similar trend; markedly higher SIgA values were obtained in the H9N2 and L2 groups than in the PBS and L1 groups ($P < 0.05$) (Fig.4b). Taken together, these results indicate that recombinant *L. lactis*-HA1 can effectively enhance mucosal immune responses in chicks.

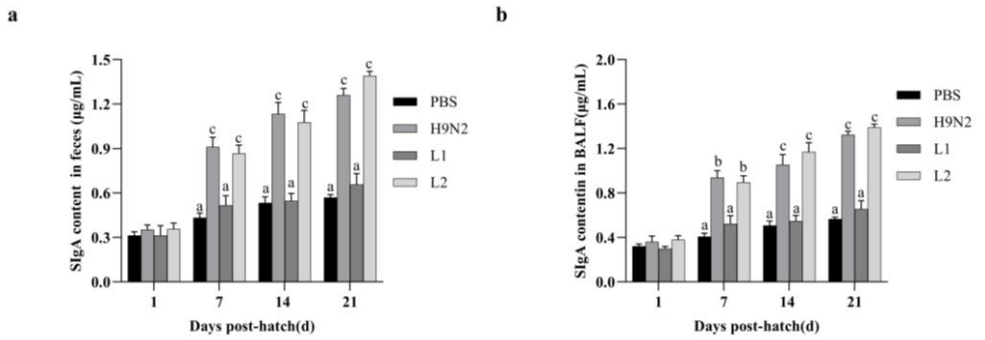

Fig.4 Effects of *in ovo* administration of recombinant *L. lactis* HA1 on SIgA levels in feces and BALF of chicks. SIgA antibodies in (a) the feces and (b) the BALF. Data are presented as mean ± SD (n = 5). Different letters above the columns indicate significant differences among treatment groups ($P < 0.05$), while identical letters or the absence of letters indicate no significant differences ($P > 0.05$).

3.8 *In ovo* injection of HA1-recombinant *L. lactis* improves intestinal development

Intestinal morphology was examined because it is essential for mucosal barrier integrity and immune function, as shown in Table 3. Duodenal VH in the L1 and L2 groups was significantly greater than in the PBS and H9N2 groups on days 7 and 14 ($P < 0.05$). Similarly, compared to the PBS and H9N2 groups, a significant increase in jejunal VH was observed on day 21 in the L2 group ($P < 0.05$). The VH/CD ratio in the duodenum and jejunum was significantly higher in the L1 and L2 groups compared to the PBS and H9N2 groups ($P < 0.05$).

Table 3 Effect of *in ovo* injection with HA1-recombinant *L. lactis* on intestinal morphology.

Item	Groups				*p*-value
	PBS	H9N2	L1	L2	
duodenum					
7 days					
VH	836.58 ± 84.64^{b}	845.89 ± 94.27^{b}	960.17 ± 89.86^{a}	985.21 ± 98.45^{a}	0.032
CD	170.87 ± 31.46	168.92 ± 28.11	162.54 ± 29.15	157.28 ± 24.03	0.654
VH/CD	4.90 ± 0.36^{c}	5.01 ± 0.28^{c}	5.90 ± 0.47^{ab}	6.26 ± 0.39^{a}	0.001
14 days					
VH	968.74 ± 72.34^{b}	940.70 ± 83.16^{b}	1126.54 ± 97.42^{a}	1125.22 ±	0.017
CD	160.35 ± 27.55	163.72 ± 31.48	163.51 ± 19.89	167.08 ± 22.57	0.892
VH/CD	6.04 ± 0.34^{b}	5.75 ± 0.42^{b}	6.88 ± 0.51^{a}	6.73 ± 0.39^{a}	0.032
21 days					
VH	1137.15 ± 113.54	1149.71 ± 94.32	1267.75 ± 133.64	1298.32 ±	0.125
CD	170.71 ± 22.33	186.93 ± 26.31	175.57 ± 32.57	174.66 ± 18.63	0.079
VH/CD	6.66 ± 0.54^{b}	6.13 ± 0.45^{b}	7.22 ± 0.39^{a}	7.43 ± 0.42^{a}	0.022
jejunum					
7 days					

VH	815.87 ± 91.24^{b}	789.56 ± 87.62^{b}	923.54 ± 86.97^{ab}	934.21 ± 102.13^{a}	0.036
CD	160.84 ± 35.21	166.16 ± 27.73	156.71 ± 25.52	147.68 ± 20.83	0.147
VH/CD	5.07 ± 0.51^{bc}	4.75 ± 0.38^{c}	5.89 ± 0.56^{ab}	6.33 ± 0.59^{a}	0.001
14 days					
VH	961.77 ± 124.51^{b}	949.71 ± 113.54^{b}	996.65 ± 124.57^{ab}	1103.67 ±	0.013
CD	165.16 ± 26.75^{ab}	170.12 ± 20.83^{a}	152.53 ± 25.29^{b}	148.87 ± 25.15^{b}	0.025
VH/CD	5.82 ± 0.58^{c}	5.58 ± 0.47^{c}	6.53 ± 0.58^{b}	7.41 ± 0.89^{a}	0.000
21 days					
VH	1070.67 ±	1137.58 ±	1216.15 ± 129.47^{ab}	1287.74 ±	0.011
CD	186.98 ± 20.43	196.67 ± 24.51	178.39 ± 38.83	179.16 ± 19.75	0.078
VH/CD	5.72 ± 0.64^{c}	5.78 ± 0.47^{c}	6.82 ± 0.77^{ab}	7.18 ± 0.86^{a}	0.001

In the same row, values with no letters or the same-letter superscripts mean no significant difference ($P > 0.05$), while values with different lowercase letters indicate a significant difference ($P < 0.05$). The results are represented as the mean ± SD (n = 5).

As illustrated in Fig.5, on day 7, the L1 and L2 groups (Fig.5b, c) exhibited clearer and more intact duodenal morphology, characterized by narrower crypt depth and taller villi compared to the PBS group (Fig.5a). By day 14, the duodenal villi in the L2 group (Fig.5f) were significantly taller and more densely arranged than those in both the PBS and H9N2 groups (Fig.5d, e).

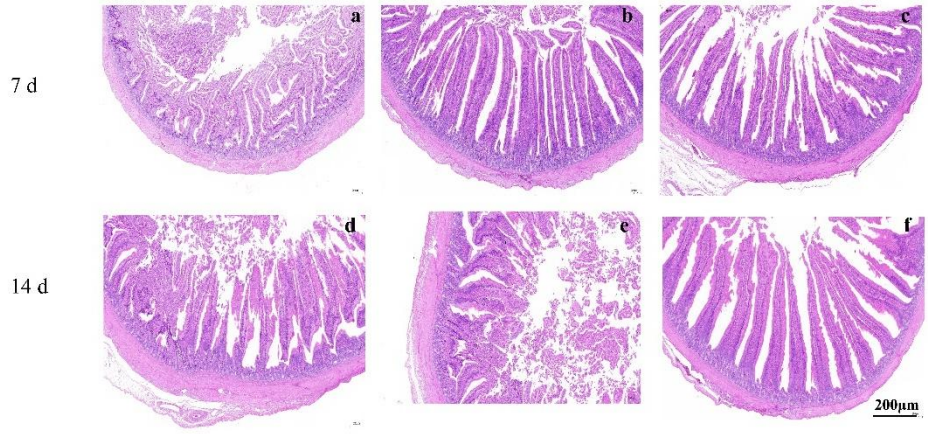

Fig.5 Effects of *in ovo* administration of recombinant *L. lactis* HA1 on the development of duodenal morphological structure. The duodenum in (a–c) the PBS, L1, and L2 groups on day 7 and (d–f) in the PBS, H9N2, and L2 groups on day 14. Tissue sections were stained with H&E. Magnification: ×200. Scale bar: 200 μm.

Regarding the jejunum (Fig.6), on day 7, the villi in the L1 and L2 groups (Fig.6b, c) were notably taller, and the intestinal wall was thicker relative to the H9N2 group (Fig.6a). From day 14 to day 21, the L2 group (Fig.6f, i) maintained regular and intact jejunal morphology, with increased villus height, narrower crypt depth, and a denser arrangement, overall surpassing both the PBS group (Fig.6d, g) and the H9N2 group (Fig.6e, h).

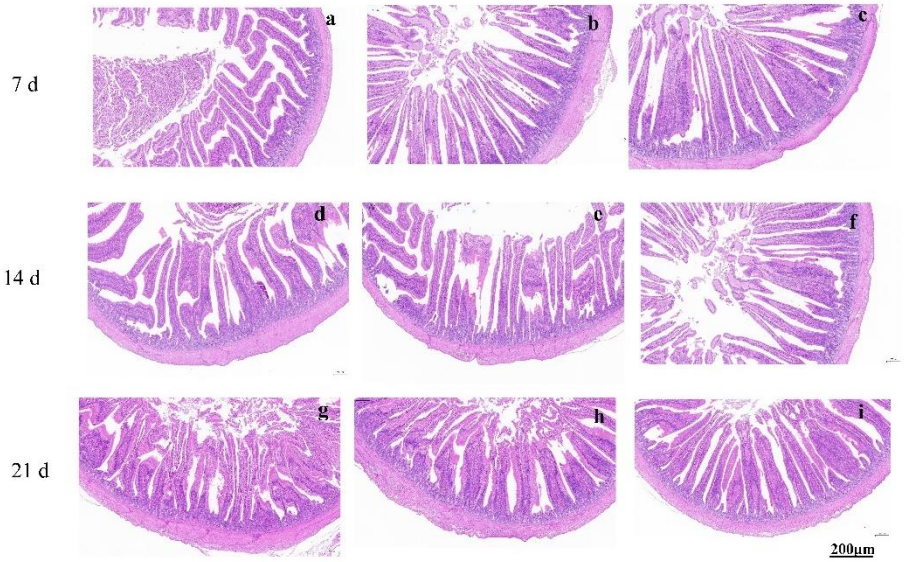

Fig.6 Effects of *in ovo* administration of recombinant *L. lactis* HA1 on the development of jejunal morphological structure. Jejunal sections from (a–c) the H9N2, L1, and L2 groups on day 7; (d–f) the PBS, H9N2, and L2 groups on day 14; and (g–i) the PBS, H9N2, and L2 groups on day 21. Tissue sections were stained with H&E. Magnification: ×200. Scale bar: 200 μm.

3.9 *In ovo* injection of HA1-recombinant *L. lactis* upregulates mRNA expression of intestinal barrier–related genes

To assess the effects of recombinant *L. lactis* HA1 on chick intestinal barrier function, the jejunal segment mRNA abundances of ZO-1, Occludin, Claudin-3, MUC2, and TFF2 were quantified (Fig.7). On post-hatch days 14 and 21, the mRNA levels of tight junction-related proteins (*ZO-1, Occludin, and Claudin-3*) were markedly upregulated in the L2 group compared to the PBS and H9N2 groups ($P < 0.05$). Earlier upregulation of these tight junction genes was observed in the L2 group when compared with the L1 group. Significant elevations in TFF2 mRNA expression were detected in both the L1 and L2 groups on post-hatch days 14 and 21 relative to the PBS and H9N2 groups ($P < 0.05$). The MUC2 transcript abundance of the L2 group increased in a time-dependent manner and was significantly higher than that of the PBS and H9N2 groups on post-hatch day 21 ($P < 0.05$).

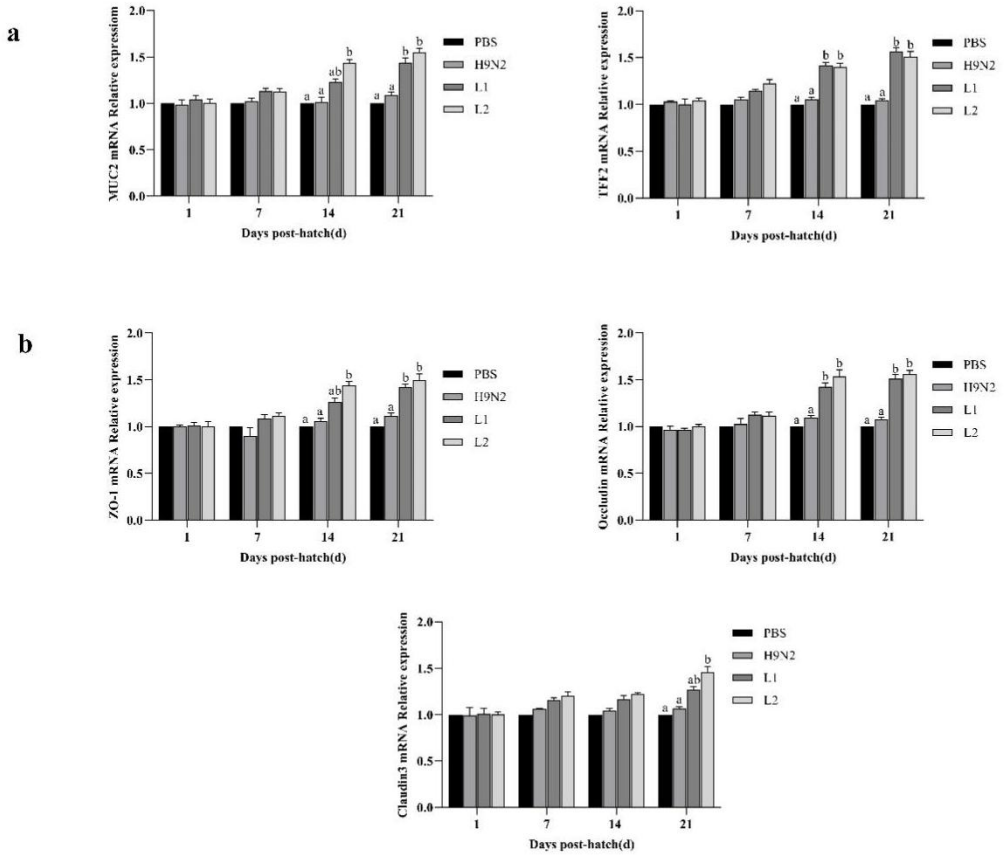

Fig.7 Effects of *in ovo* administration of recombinant *L. lactis* HA1 on mRNA expression levels of intestinal barrier-related proteins. (a) Intestinal tight junction proteins; (b) Intestinal mucosal barrier protein. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method, with GAPDH serving as the endogenous control and the average ΔCt value for the PBS group on each day as the calibrator. Data are presented as mean ± SD (n = 5). Different letters above the columns indicate significant differences among treatment groups ($P < 0.05$), while identical letters or the absence of letters indicate no significant differences ($P > 0.05$).

3.10 *In ovo* injection of recombinant *L. lactis* enhances immune protection against H9N2 challenge

To evaluate the protective efficacy of recombinant *L. lactis* HA1 against H9N2 AIV, clinical signs, body weights, survival rates, viral shedding, viral lung titers, and pulmonary pathologies of each group were statistically analyzed post-challenge. Following H9N2 challenge, no obvious clinical symptoms were observed in any group during the initial period. At 3 days post-challenge, lethargy, reduced feed intake, and respiratory distress were observed in some chicks, with over 50% of affected chicks in the PBS and L1 groups. In contrast, symptoms in the H9N2 and L2 groups were less severe, and fewer chickens exhibited clinical signs. Furthermore, the onset of clinical signs was delayed by 1-2 days, though most clinical signs in the H9N2 and L2 groups were resolved by day 5, with activity and feed intake returning to normal levels. By day 7 post-challenge, symptoms in the PBS and L1 groups had improved significantly, whereas symptoms in the other two groups had returned to normal. No significant differences were observed in clinical symptoms between the H9N2 and L2 groups of chicks.

As shown in Fig.8a, body weights decreased in the PBS and L1 groups three days after H9N2 virus infection, whereas slight weight increases were observed in the H9N2 and L2 groups. From days

3 to 7 post-challenge, the body weights of the L1 and PBS groups exhibited clear downward trends, whereas the H9N2 and L2 groups showed the opposite pattern ($P < 0.05$). From days 7 to 14 after H9N2 infection, all groups exhibited weight gain, with significantly greater weight gains observed in the H9N2 and L2 groups compared to the PBS and L1 groups ($P < 0.05$).

Moreover, there was no significant difference between the H9N2 and L2 groups ($P > 0.05$).

No mortality occurred in the H9N2 and L2 groups following infection with H9N2 AIV, but of the ten chicks in each group, two died in the PBS group and one died in the L1 group (Fig.8b).

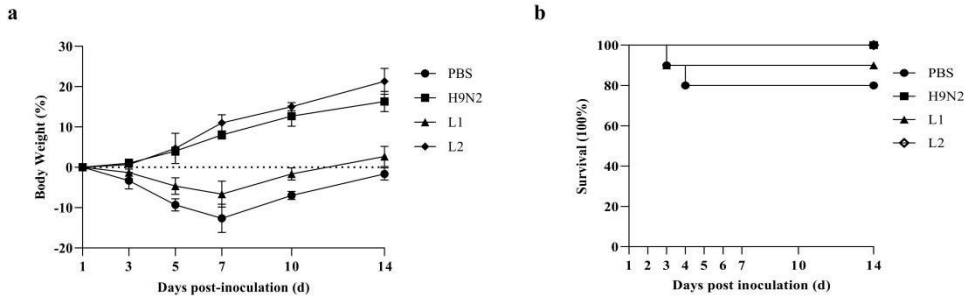

Fig.8 Effects of *in ovo* administration of recombinant *L. lactis* HA1 on weight changes and mortality of chicks following H9N2 challenge. (a) Body weight; (b) Survival probability.

To evaluate viral shedding, oropharyngeal and cloacal swabs were collected on days 3, 5, and 7 post-H9N2 infection for titration in MDCK cells. As shown in Fig.9a, all groups exhibited oropharyngeal viral replication at days 3 and 5 post-challenge. However, the H9N2 group exhibited significantly lower oropharyngeal viral shedding compared to the PBS and L1 groups ($P < 0.05$), with no significant difference observed relative to the L2 group. At 7 days post-challenge, only minimal viral shedding was detected from oropharyngeal swabs in the H9N2 and L2 groups.

Viral shedding from cloacal swabs exhibited a trend similar to that from oropharyngeal swabs, although its viral replication was lower than that of the oropharyngeal swab (Fig.9b).

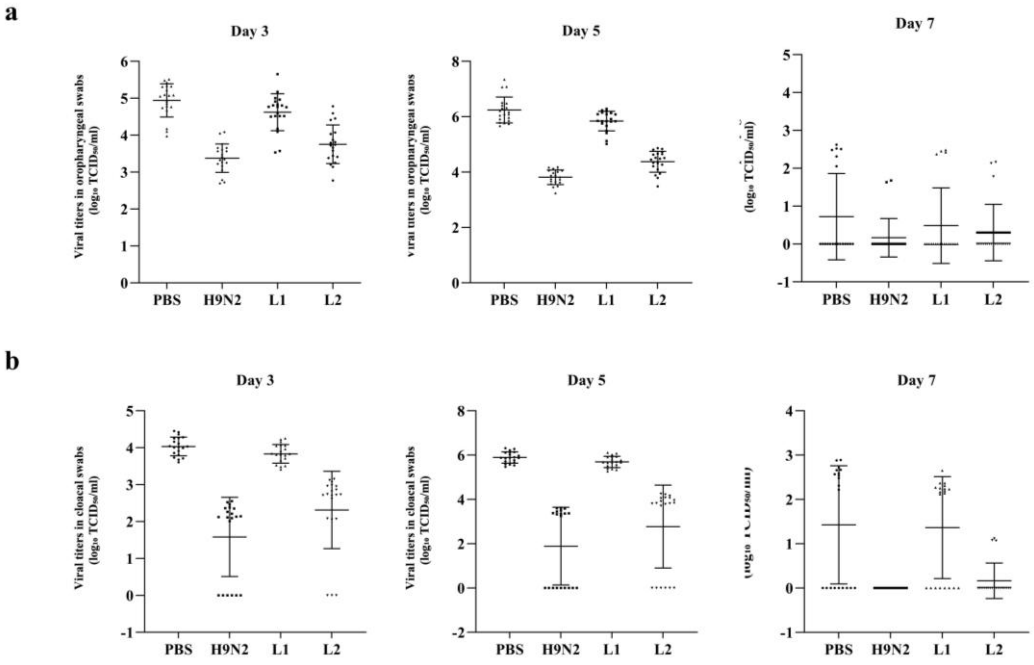

Fig.9 Effects of *in ovo* administration of recombinant *L. lactis* HA1 on viral shedding from the cloacal and oropharyngeal swabs of chicks following H9N2 challenge. Viral titers were detected (a) in

oropharyngeal swabs collected from challenged chickens on days 3, 5, and 7 post-challenge and (b) in cloacal swabs collected from challenged chickens on days 3, 5, and 7 post-challenge.

3.11 *In ovo* injection of HA1-recombinant *L. lactis* reduces viral lung titers after H9N2 challenge

As shown in Fig.10, viral titers in lung tissues were significantly higher in the PBS and L1 groups than in the H9N2 group ($P < 0.05$). Although viral titers in the H9N2 group were numerically lower than those in the L2 group, the difference was not statistically significant ($P > 0.05$). These results indicate that *in ovo* administration of recombinant *L. lactis* HA1 could protect against H9N2 AIV by inhibiting virus replication.

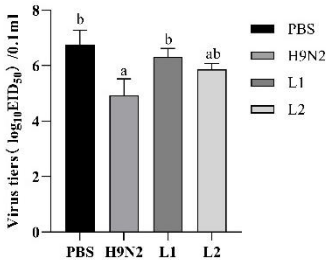

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Fig.10 Effects of H9N2 challenge on viral titers in lung tissue among different groups of chicks inoculated with embryonated eggs. Viral titers in the lungs were measured at 7 days post-challenge, and infectivity was quantified using EID_{50}. Data were shown as mean ± SD (n = 5). Different letters on the column charts indicate significant differences among the treatment groups ($P < 0.05$).

3.12 *In ovo* injection of HA1-recombinant *L. lactis* alleviates pulmonary histopathological damage induced by H9N2 virus

To further evaluate the protective effect of the recombinant *L. lactis* HA1, pathological changes in chickens across all groups were assessed. As illustrated in Fig.11, the PBS group exhibited severe pulmonary structural damage with pronounced erythrocyte infiltration and proteinaceous exudates (Fig.11a), while the L1 group exhibited marked erythrocyte accumulation within the alveoli, though inflammatory infiltration was comparatively less pronounced than in the PBS group (Fig.11c). Lung architecture remained predominantly intact in the H9N2 and L2 groups, with only scattered erythrocytes present (Fig.11b, d), indicating markedly alleviated lung lesions.

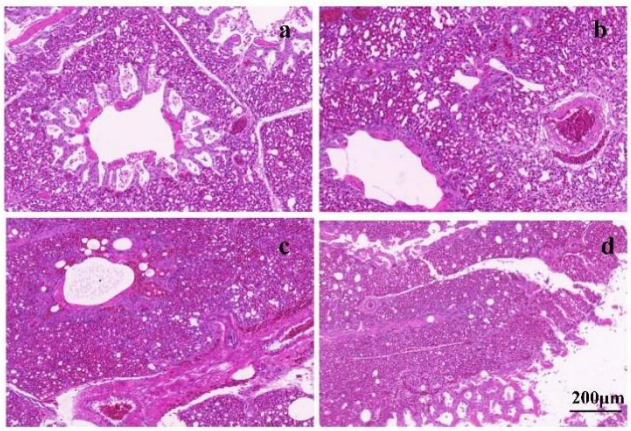

Fig.11 Effects of H9N2 challenge on histopathological changes in lung tissue among different groups of chicks inoculated with embryonated eggs. (a) PBS group; (b) H9N2 group; (c) L1 group; and (d) L2

group. Tissue sections were stained with H&E. Magnification: ×200. Scale bar: 200 μm.

4. Discussion

The H9N2 AIV has been endemic in China for over three decades and remains the predominant influenza virus in poultry populations. Frequent genetic variations in H9N2 strains and delayed vaccine updates have diminished the efficacy of conventional inactivated vaccines, resulting in inadequate immune coverage (Dong, Zhou, Pu & Liu 2022; Song, Son, Kim, Kim, An, Lee, Choi & Kwon 2025). Thus, safe and effective novel vaccines are urgently needed to enhance immune protection against H9N2 AIV. Given that H9N2 AIV primarily infects hosts through the respiratory mucosa, mucosal immunity plays a critical role in blocking early viral invasion. Lactic acid bacteria have been demonstrated to function as safe antigen delivery vectors, capable of efficiently expressing foreign proteins, inducing mucosal immune responses, and establishing immune barriers (Chowdhury, Islam, Yurina & Shimosato 2026; Guo, Yang, Wang, Liu, Wang, Zhang, Li, Wu & Chen 2025). The HA1 subunit of the influenza virus, which contains abundant neutralizing epitopes and exhibits strong immunogenicity, has been widely acknowledged as a promising candidate antigen for subunit vaccines (Li, Peng, Chen, Wang & Wang 2023). Combined with the benefits of *in ovo* injection for early immune stimulation (Abd El-Ghany 2024), this study utilized recombinant *L. lactis* expressing HA1 and assessed its efficacy through *in ovo* vaccine administration, demonstrating enhanced intestinal development, growth performance, and immune function, conferring effective protection against H9N2 AIV challenge.

In ovo interventions have direct effects on embryonic development and post-hatch performance. Therefore, hatchability and growth performance are considered key indicators for evaluating the safety and practical efficacy of *in ovo* vaccination. For this reason, the effects of *in ovo* inoculation on hatchability and growth performance in chicks were evaluated. In the present study, no significant effect on hatchability was observed. Meanwhile, increased ADG was observed from days 15 to 21, whereas a lower FCR was detected at 1 – 7 days post-hatch in chicks administered *in ovo* with *L. lactis* and recombinant *L. lactis* HA1, indicating high biological safety. Similarly, Ghanaatparast-Rashti, Mottaghitalab, Royan and Seighalani (25) found that *in ovo* inoculation of lactobacilli did not affect hatchability but significantly increased FCR, a finding further corroborated by Beck's research (Beck, McDaniel, Wamsley & Kiess 2019). Early intestinal colonization by probiotics following *in ovo* delivery may promote intestinal maturation and nutrient absorption, thereby establishing a favorable physiological foundation for improved growth performance (Duan, Ju, Zhang, Qin, Xue, Ma, Luan & Yang 2021). However, some studies have suggested that *in ovo* probiotic injection may improve hatchability without significantly enhancing growth performance, indicating that the effects may depend on strain, dosage, administration timing, and animal condition, but are not limited to these factors (de Oliveira, van der Hoeven-Hangoor, van de Linde, Montijn & van der Vossen 2014). Thus, broader applications are warranted to further evaluate the practical efficacy of *in ovo* probiotic vaccination.

On this basis, we further evaluated the immune efficacy of recombinant *L. lactis*-HA1 administered via *in ovo* injection. HA antibodies are critical for inhibiting influenza virus attachment to host cells and mediating protective immunity against viral infection (Tutykhina, Sedova, Gribova, Ivanova, Vasilev, Rutovskaya, Lysenko, Shmarov, Logunov, Naroditsky, Tillib & Gintsburg 2013). The HA1 subunit, which harbors numerous neutralizing epitopes and exhibits robust immunogenicity, represents a favorable candidate antigen for influenza subunit vaccines. Accordingly, the present study developed an *in ovo* immunization strategy based on recombinant *L. lactis* expressing HA1 as the target antigen. Experimental results demonstrated that this recombinant strain could elicit robust

antigen-specific antibody responses in chickens. While the inactivated vaccine induced high-titer HI antibodies, the results revealed no significant differences in HI antibody levels between chicken embryos inoculated with the inactivated vaccine and those receiving recombinant *L. lactis*-HA1.

AIV primarily infects hosts by breaching the epithelial barriers of the respiratory and gastrointestinal tracts. SIgA serves as a frontline mucosal defense, blocking viral attachment and colonization on mucosal surfaces to prevent primary infection. Consequently, SIgA abundance is a pivotal biomarker for the systematic assessment of vaccine-mediated mucosal immune protection. Our experimental data indicate that *in ovo* vaccination with recombinant *L. lactis*-HA1 enhanced mucosal immune responses, as validated by increased SIgA concentrations in BALF and intestinal tissues. Consistent with this, the SIgA levels induced by the recombinant *L. lactis*-HA1 strain were statistically comparable to those produced by the inactivated vaccine, demonstrating that embryonic delivery of this recombinant probiotic elicits robust mucosal immunity in chickens.

Beyond mucosal immunity, the intrinsic probiotic properties of *L. lactis* also potentiated cellular immune responses, manifested as elevated secretion of IL-2, IL-4, and IFN-γ, as well as enhanced lymphocyte proliferation capacity. These outcomes align with previous reports indicating that *in ovo* administration of lactic acid bacteria promotes the proliferation and differentiation of chicken T lymphocytes, upregulates Th1-type IFN-γ and Th2-type IL-4, and ultimately improves overall immune competence (Sharma, Kulkarni, Sharif, Hassan, Alizadeh, Pratt & Abdelaziz 2024). Mechanistically, multiple studies have demonstrated that probiotics upregulate IL-2, IL-4, and IFN-γ expression by activating $CD4^+$T cells and modulating the NF-κB and NFAT signaling pathways (Madej & Bednarczyk 2016; Thomas & Versalovic 2010). Notably, recombinant probiotics carrying viral antigens further skewed the immune response toward a Th1-dominant profile through dendritic cell activation within intestinal Peyer's patches (Niu, Xing, Zou, Shi, Huang, Jiang, Wang, Cao, Wang, Zeng, Yang, Yang & Wang 2021). Collectively, the present study confirms that *in ovo* inoculation with recombinant *L. lactis*-HA1 triggers a comprehensive immune activation, reflected by increased specific HA inhibition antibodies, elevated mucosal SIgA, and robust production of IL-2, IL-4, and IFN-γ.

The thymus, spleen, and bursa of Fabricius are primary immune organs in poultry and serve as reliable indicators of immune development and function. Mature immune organs promote T and B lymphocyte proliferation and differentiation, thereby enhancing cellular and humoral immune responses (Chen, Gu, Cao, Tu, Liu & Ju 2024). Previous studies have demonstrated that intestinal-colonizing probiotics can modulate gut microbiota and function as immune stimulants to enhance immune organ development (Balcells, Martínez Monteros, Gómez, Cazorla, Perdigón & Maldonado-Galdeano 2022). Moreover, compared with the empty-vector strain, immunization with recombinant bacteria expressing heterologous proteins elicited significantly higher mucosal and systemic immune responses (Fan, Wu, Yu, Bai & Long 2014). Sławińska, Siwek, Zylińska, Bardowski, Brzezińska, Gulewicz, Nowak, Urbanowski, Płowiec and Bednarczyk (36) demonstrated that *in ovo* administration of synbiotics promotes lymphoid organ development. However, our results revealed that the immune organ indices were significantly elevated in chickens treated with the inactivated H9N2 vaccine, *L. lactis*, and recombinant *L. lactis*-HA1 strain. Among these, the recombinant HA1-expressing group exhibited the most pronounced promoting effect. This beneficial outcome is likely attributed to the ingestion of probiotic bacteria by embryos along with amniotic fluid, which colonize the intestinal tract at an ultra-early embryonic stage and thereby drive the precocious maturation of immune organs.

Intestinal morphological integrity is essential for nutrient absorption, mucosal barrier function, and immune homeostasis. A well-developed intestinal structure not only promotes efficient nutrient utilization but also, more importantly, strengthens host defense against pathogenic infections. Accordingly, this study explored the effects of *in ovo* administration of recombinant *L. lactis* on intestinal development. Intestinal morphological changes in the duodenum and jejunum were assessed via H&E staining, with VH, CD, and the VH/CD ratio further determined. The results showed that chicks treated *in ovo* with *L. lactis* and recombinant *L. lactis*-HA1 had significantly higher VH and VH/CD ratio than those in the PBS and inactivated H9N2 vaccine groups, indicating enhanced intestinal maturation and digestive capacity (Alagbe, Sung, Pasternak & Adeola 2026). Multiple previous reports have demonstrated that in-ovo delivery of probiotics or synbiotics promotes intestinal villus growth and mucosal architecture maturation (Madej, Woźniak-Biel, Gaweł & Bobrek 2025; Shehata, Seddek, Khamis, Elnesr, Nouri, Albasri & Paswan 2024). Similarly, our findings support that early intervention with *L. lactis* via *in ovo* injection effectively promotes intestinal morphological development

Beyond structural morphology, the intestinal epithelial tight junction complex and mucus barrier constitute core components that maintain intestinal barrier integrity. Impaired intestinal barrier function increases mucosal permeability, permitting harmful substances and pathogens to enter systemic circulation, thereby triggering intestinal inflammation, immune dysregulation, and multi-organ dysfunction recombinant probiotics carrying viral (Gilani, Chrystal & Barekatain 2021). Therefore, to further clarify the regulatory effects of recombinant *L. lactis* on intestinal barrier function, we measured the mRNA expression levels of key tight junction proteins (*ZO-1, occludin, claudin-3*) and mucosal repair factors (*MUC2, TFF2*). *ZO-1* functions as a scaffold protein that links *occludin* and claudins to enhance intercellular adhesion (Bauer, Zweimueller-Mayer, Steinbacher, Lametschwandtner & Bauer 2010). *Occludin* contributes to tight junction stability (Al-Sadi, Khatib, Guo, Ye, Youssef & Ma 2011), while claudin-3 regulates paracellular permeability (Suzuki 2020). *MUC2* constitutes the protective mucus layer, whereas *TFF2* facilitates mucosal repair and defense (Song, Chai, Chen, Zhang, Zhang & Zhou 2023; Runkle & Mu 2013). Together, these molecules maintain the stability of the intestinal mucosal barrier. Studies have reported that numerous specific probiotics have been shown to enhance barrier integrity. These probiotics exert their effects through diverse mechanisms, including the upregulation of *occludin*, *claudins*, and *ZO-1* (Ferris, Subitoni Antonio & Al-Sadi 2025). Similarly, *in ovo* probiotic administration can enhance intestinal barrier function by upregulating tight junction and mucin-related genes (Madej, Woźniak-Biel, Gaweł & Bobrek 2025; Mangan, Połtowicz, Metges & Siwek 2025). In agreement with these reports, our results demonstrated that recombinant *L. lactis*-HA1 significantly up-regulated the mRNA expression of ZO-1, Occludin, Claudin-3, MUC2 and TFF2 in comparison with the PBS and inactivated H9N2 vaccine groups. These results indicate that *in ovo* administration of recombinant *L. lactis* HA1 effectively enhances intestinal barrier integrity at the molecular level, which provides a foundation for improved growth and immune function.

Lactic acid bacteria possess several advantages, including safety, non-toxicity, the ability to adhere to and colonize mucosal epithelial layers (e.g., respiratory and digestive tracts), the capacity to express and secrete heterologous proteins, and the induction of strong mucosal immune responses (Chowdhury, Islam, Yurina & Shimosato 2026). Consequently, *in ovo* vaccination using recombinant lactic acid bacteria has emerged as a significant research direction in poultry immunization. This study further evaluated the protective efficacy of the *in ovo* administration of recombinant *L. lactis* HA1

against H9N2 AIV infection. The results showed that chicks receiving *in ovo* administration of recombinant *L. lactis*-HA1 exhibited significantly alleviated clinical symptoms, reduced weight loss and mortality, decreased viral shedding in oropharyngeal and cloacal swabs, lowered pulmonary viral load, and mitigated histopathological lung lesions. Consistent with this finding, Rauw, Gardin, Palya, Anbari, Lemaire, Boschmans, van den Berg and Lambrecht (48) reported that *in ovo* recombinant F protein vaccines reduce viral shedding and provide better protection than oral attenuated vaccines. Similarly, *in ovo* immunization with recombinant vaccines against infectious laryngotracheitis viruses can significantly reduce clinical infection rates and mitigate tissue damage in poultry (Jiang, Feng, Zeng, Wang, Zhang, Shang, Wang, Wang, Yao, Li, Suo, Luo & Wen 2024). These findings collectively validate the feasibility and potential application of the *in ovo* vaccination strategy.

5. Conclusion

In conclusion, this study showed that *in ovo* administration of recombinant *L. lactis*-HA1 exerted no adverse effects on hatchability while improving growth performance. This treatment enhanced immune organ development, and improved intestinal morphological integrity and barrier-related gene expression. It induced robust humoral immunity characterized by elevated HI titers and HA-specific IgG, enhanced HA1-specific lymphocyte proliferation, and boosted mucosal SIgA production. Following H9N2 AIV challenge, vaccinated chicks exhibited milder clinical signs, decreased viral shedding and pulmonary viral burden, and attenuated pulmonary histopathological lesions. Collectively, this recombinant strain represents a promising candidate for novel in-ovo lactic-acid-bacterium-vectored vaccines against H9N2 avian influenza.

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CRediT authorship contribution statement

Jiaxin Yuan: Conceptualization, Methodology, Formal analysis, Data curation. **Tingting Liu:** Data curation, Writing-review and editing. **Dayi Liu**: Writing-original draft, Editing. **Tianci Liu:** Methodology, Data curation. **Tong Xu:** Writing-review and editing. **Longfei Li:** Supervision, Funding acquisition.

Conflict of interest

The authors declare no conflict of interest.

Ethics statement

All researchers have received training in animal ethics and welfare, and all experiments were approved by the Animal Care and Use Committee of Hebei North University (HBNU2025022007).

Data availability statement

Data that support the findings of this study are available on reasonable request.

References

Abdelwhab EM, Mettenleiter TC. 2023. Zoonotic Animal Influenza Virus and Potential Mixing Vessel Hosts. Viruses 15https://doi.org/10.3390/v15040980

Carnaccini S, Perez DR. 2020. H9 Influenza Viruses: An Emerging Challenge. Cold Spring Harb Perspect Med 10:a038588. https://doi.org/10.1101/cshperspect.a038588

Gu M, Xu L, Wang X, Liu X. 2017. Current situation of H9N2 subtype avian influenza in China. Vet Res 48:49. https://doi.org/10.1186/s13567-017-0453-2

Abdelaziz AM, Mohamed MHA, Fayez MM, Al-Marri T, Qasim I, Al-Amer AA. 2019. Molecular survey and interaction of common respiratory pathogens in chicken flocks (field perspective). Vet World 12:1975–86. https://doi.org/10.14202/vetworld.2019.1975-1986

Cáceres CJ, Rajao DS, Perez DR. 2021. Airborne Transmission of Avian Origin H9N2 Influenza A Viruses in Mammals. Viruses 13https://doi.org/10.3390/v13101919

He M, Liu L, Hu J, Wang Z, Guo Z, et al. 2025. The H5N6 Virus Containing Internal Genes From H9N2 Exhibits Enhanced Pathogenicity and Transmissibility. Transbound Emerg Dis 2025:6252849. https://doi.org/10.1155/tbed/6252849

Song JH, Son SE, Kim HW, An SH, Lee CY, et al. 2024. A Model H5N2 Vaccine Strain for Dual Protection Against H5N1 and H9N2 Avian Influenza Viruses. Vaccines (Basel) 13https://doi.org/10.3390/vaccines13010022

Wilks S, de Graaf M, Smith DJ, Burke DF. 2012. A review of influenza haemagglutinin receptor binding as it relates to pandemic properties. Vaccine 30:4369–76. https://doi.org/10.1016/j.vaccine.2012.02.076

Shahsavandi S, Salmanian AH, Ghorashi SA, Masoudi S, Fotouhi F, Ebrahimi MM. 2011. Development of rHA1-ELISA for specific and sensitive detection of H9 subtype influenza virus. J Virol Methods 171:260–3. https://doi.org/10.1016/j.jviromet.2010.11.012

Xiong J, Zhao P, Yang P, Yan Q, Jiang L. 2019. Evolutionary dynamics of the H7N9 avian influenza virus based on large-scale sequence analysis. PloS one 14:e0220249. https://doi.org/10.1371/journal.pone.0220249

Hou T, Tako E. 2018. The In Ovo Feeding Administration (Gallus Gallus)-An Emerging In Vivo Approach to Assess Bioactive Compounds with Potential Nutritional Benefits. Nutrients 10https://doi.org/10.3390/nu10040418

Siwek M, Slawinska A, Stadnicka K, Bogucka J, Dunislawska A, Bednarczyk M. 2018. Prebiotics and synbiotics - in ovo delivery for improved lifespan condition in chicken. BMC Vet Res 14:402. https://doi.org/10.1186/s12917-018-1738-z

Abd El-Ghany WA. 2024. In Ovo Vaccination Technology: An Alternative Approach to Post-Hatch Vaccination in Modern Poultry Operations. Microbiology Research 16https://doi.org/10.3390/microbiolres16010007

de Oliveira JE, van der Hoeven-Hangoor E, van de Linde IB, Montijn RC, van der Vossen JM. 2014. In ovo inoculation of chicken embryos with probiotic bacteria and its effect on posthatch Salmonella susceptibility. Poultry science 93:818–29. https://doi.org/10.3382/ps.2013-03409

Dieye Y, Hoekman AJ, Clier F, Juillard V, Boot HJ, Piard JC. 2003. Ability of Lactococcus lactis to export viral capsid antigens: a crucial step for development of live vaccines. Appl Environ Microbiol 69:7281–8. https://doi.org/10.1128/aem.69.12.7281-7288.2003

Zhang R, Xu T, Li Z, Li L, Li C, et al. 2023. Vaccination with recombinant Lactococcus lactis expressing HA1-IgY Fc fusion protein provides protective mucosal immunity against H9N2

avian influenza virus in chickens. Virol J 20:76. https://doi.org/10.1186/s12985-023-02044-9
Dabaghian M, Latify AM, Tebianian M, Nili H, Ranjbar AR, et al. 2014. Vaccination with recombinant 4 × M2e.HSP70c fusion protein as a universal vaccine candidate enhances both humoral and cell-mediated immune responses and decreases viral shedding against experimental challenge of H9N2 influenza in chickens. Veterinary microbiology 174:116–26. https://doi.org/10.1016/j.vetmic.2014.09.009
Spackman E, Gelb J, Jr., Preskenis LA, Ladman BS, Pope CR, et al. 2010. The pathogenesis of low pathogenicity H7 avian influenza viruses in chickens, ducks and turkeys. Virol J 7:331. https://doi.org/10.1186/1743-422x-7-331
Le Gall-Reculé G, Cherbonnel M, Pelotte N, Blanchard P, Morin Y, Jestin V. 2007. Importance of a prime-boost DNA/protein vaccination to protect chickens against low-pathogenic H7 avian influenza infection. Avian Dis 51:490–4. https://doi.org/10.1637/7592-040206r.1
Dong J, Zhou Y, Pu J, Liu L. 2022. Status and Challenges for Vaccination against Avian H9N2 Influenza Virus in China. Life (Basel) 12https://doi.org/10.3390/life12091326
Song JH, Son SE, Kim H, Kim SJ, An SH, et al. 2025. Development of an H9N2 vaccine strain with enhanced neuraminidase immunogenicity via dual hemagglutinin and neuraminidase modification. J Vet Sci 26:e81. https://doi.org/10.4142/jvs.25069
Chowdhury MR, Islam A, Yurina V, Shimosato T. 2026. Genetically Modified Lactic Acid Bacteria: a Promising Mucosal Delivery Vector for Vaccines. Probiotics Antimicrob Proteins 18:3094–112. https://doi.org/10.1007/s12602-025-10667-3
Guo L, Yang L, Wang K, Liu W, Wang S, et al. 2025. Synbiotic microencapsulation of Lactiplantibacillus plantarum-lentinan for enhanced growth in broilers. AMB Express 15:128. https://doi.org/10.1186/s13568-025-01929-y
Li Z, Peng C, Chen L, Wang P, Wang F. 2023. Construction and Immunogenicity Evaluation of Recombinant Bacillus subtilis Expressing HA1 Protein of H9N2 Avian Influenza Virus. Curr Microbiol 81:25. https://doi.org/10.1007/s00284-023-03548-x
Ghanaatparast-Rashti M, Mottaghitalab M, Royan M, Seighalani R. 2025. Effect of in ovo injection of Lactobacillus isolates from the gastrointestinal tract of Iranian native poultry on hatchability, immune response, and growth performance in broilers. Animal Production Research 14https://doi.org/10.22124/ar.2025.31136.1909
Beck CN, McDaniel CD, Wamsley KGS, Kiess AS. 2019. The potential for inoculating Lactobacillus animalis and Enterococcus faecium alone or in combination using commercial in ovo technology without negatively impacting hatch and post-hatch performance. Poultry science 98:7050–62. https://doi.org/10.3382/ps/pez441
Duan AY, Ju AQ, Zhang YN, Qin YJ, Xue LG, et al. 2021. The Effects of In Ovo Injection of Synbiotics on the Early Growth Performance and Intestinal Health of Chicks. Front Vet Sci 8:658301. https://doi.org/10.3389/fvets.2021.658301
Tutykhina IL, Sedova ES, Gribova IY, Ivanova TI, Vasilev LA, et al. 2013. Passive immunization with a recombinant adenovirus expressing an HA (H5)-specific single-domain antibody protects mice from lethal influenza infection. Antiviral research 97:318–28. https://doi.org/10.1016/j.antiviral.2012.12.021
Sharma S, Kulkarni RR, Sharif S, Hassan H, Alizadeh M, et al. 2024. In ovo feeding of probiotic lactobacilli differentially alters expression of genes involved in the development and immunological maturation of bursa of Fabricius in pre-hatched chicks. Poultry science

103:103237. https://doi.org/10.1016/j.psj.2023.103237
Madej JP, Bednarczyk M. 2016. Effect of in ovo-delivered prebiotics and synbiotics on the morphology and specific immune cell composition in the gut-associated lymphoid tissue. Poultry science 95:19–29. https://doi.org/10.3382/ps/pev291
Thomas CM, Versalovic J. 2010. Probiotics-host communication: Modulation of signaling pathways in the intestine. Gut Microbes 1:148–63. https://doi.org/10.4161/gmic.1.3.11712
Niu H, Xing JH, Zou BS, Shi CW, Huang HB, et al. 2021. Immune Evaluation of Recombinant Lactobacillus plantarum With Surface Display of HA1-DCpep in Mice. Front Immunol 12:800965. https://doi.org/10.3389/fimmu.2021.800965
Chen Y, Gu X, Cao K, Tu M, Liu W, Ju J. 2024. The role of innate lymphoid cells in systemic lupus erythematosus. Cytokine 179:156623. https://doi.org/10.1016/j.cyto.2024.156623
Balcells F, Martínez Monteros MJ, Gómez AL, Cazorla SI, Perdigón G, Maldonado-Galdeano C. 2022. Probiotic Consumption Boosts Thymus in Obesity and Senescence Mouse Models. Nutrients 14https://doi.org/10.3390/nu14030616
Fan H, Wu X, Yu F, Bai Y, Long B. 2014. Oral immunization with recombinant Lactobacillus acidophilus expressing the adhesin Hp0410 of Helicobacter pylori induces mucosal and systemic immune responses. Clin Vaccine Immunol 21:126–32. https://doi.org/10.1128/cvi.00434-13
Sławińska A, Siwek M, Zylińska J, Bardowski J, Brzezińska J, et al. 2014. Influence of synbiotics delivered in ovo on immune organs development and structure. Folia Biol (Krakow) 62:277–85. https://doi.org/10.3409/fb62_3.277
Alagbe EO, Sung JY, Pasternak JA, Adeola O. 2026. Postnatal small intestinal morphological development in Cobb 500 broiler chickens. Poultry science 105:106412. https://doi.org/10.1016/j.psj.2026.106412
Madej JP, Woźniak-Biel A, Gaweł A, Bobrek K. 2025. Boosting Broiler Health and Productivity: The Impact of in ovo Probiotics and Early Posthatch Feeding with Bacillus subtilis, Lactobacillus fermentum, and Enterococcus faecium. Microorganisms 13https://doi.org/10.3390/microorganisms13061219
Shehata AM, Seddek NH, Khamis T, Elnesr SS, Nouri HR, et al. 2024. In-ovo injection of Bacillus subtilis, raffinose, and their combinations enhances hatchability, gut health, nutrient transport- and intestinal function-related genes, and early development of broiler chicks. Poultry science 103:104134. https://doi.org/10.1016/j.psj.2024.104134
Gilani S, Chrystal PV, Barekatain R. 2021. Current experimental models, assessment and dietary modulations of intestinal permeability in broiler chickens. Anim Nutr 7:801–11. https://doi.org/10.1016/j.aninu.2021.03.001
Bauer H, Zweimueller-Mayer J, Steinbacher P, Lametschwandtner A, Bauer HC. 2010. The dual role of zonula occludens (ZO) proteins. J Biomed Biotechnol 2010:402593. https://doi.org/10.1155/2010/402593
Al-Sadi R, Khatib K, Guo S, Ye D, Youssef M, Ma T. 2011. Occludin regulates macromolecule flux across the intestinal epithelial tight junction barrier. American journal of physiology. Gastrointestinal and liver physiology 300:G1054–64. https://doi.org/10.1152/ajpgi.00055.2011
Suzuki T. 2020. Regulation of the intestinal barrier by nutrients: The role of tight junctions. Anim Sci J 91:e13357. https://doi.org/10.1111/asj.13357
Song C, Chai Z, Chen S, Zhang H, Zhang X, Zhou Y. 2023. Intestinal mucus components and secretion

mechanisms: what we do and do not know. Exp Mol Med 55:681–91. https://doi.org/10.1038/s12276-023-00960-y

Runkle EA, Mu D. 2013. Tight junction proteins: from barrier to tumorigenesis. Cancer Lett 337:41–8. https://doi.org/10.1016/j.canlet.2013.05.038

Ferris MM, Subitoni Antonio L, Al-Sadi R. 2025. Probiotics and the intestinal tight junction barrier function. Frontiers in cell and developmental biology 13:1671152. https://doi.org/10.3389/fcell.2025.1671152

Mangan M, Połtowicz K, Metges CC, Siwek M. 2025. Modulatory effects of in ovo delivery of galactooligosaccharide and Lactiplantibacillus plantarum on antioxidant capacity, gene expression, and selected plasma metabolite parameters of broiler chickens. J Appl Genet 66:421–34. https://doi.org/10.1007/s13353-024-00931-7

Rauw F, Gardin Y, Palya V, Anbari S, Lemaire S, et al. 2010. Improved vaccination against Newcastle disease by an in ovo recombinant HVT-ND combined with an adjuvanted live vaccine at day-old. Vaccine 28:823–33. https://doi.org/10.1016/j.vaccine.2009.10.049

Jiang L, Feng H, Zeng Z, Wang Z, Zhang G, et al. 2024. Research Notes: Evaluation of recombinant Newcastle disease virus expressing gB protein of infectious laryngotracheitis virus as bivalent in ovo vaccine. Poultry science 103:104397. https://doi.org/10.1016/j.psj.2024.104397