

Haemagglutinin displayed on the surface of *Lactococcus lactis* confers broad cross-clade protection against different H5N1 viruses in chickens

Han Lei (✉ hlei@swjtu.edu.cn)

College of Medicine, Southwest Jiaotong University <https://orcid.org/0000-0002-5156-4258>

Tong Gao

College of Medicine, Southwest Jiaotong University

Qianhong Cen

Southwest Jiaotong University

Xiaojuan Peng

College of Life Science, Nanchang University

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Abstract

Background The highly pathogenic avian influenza (HPAI) H5N1 virus poses a potential threat to the poultry industry. The currently available avian influenza H5N1 vaccines for poultry are clade-specific. Therefore, an effective vaccine for preventing and controlling H5N1 viruses belonging to different clades needs to be developed.

Results Recombinant *L. lactis*/pNZ8148-Spax-HA was generated, and the influenza virus haemagglutinin (HA) protein of A/chicken/Henan/12/2004 was displayed on the surface of *Lactococcus lactis* (*L. lactis*). Spax was used as an anchor protein. Chickens vaccinated orally with unadjuvanted *L. lactis*/pNZ8148-Spax-HA could produce significant humoral and mucosal responses and neutralizing activities against H5N1 viruses belonging to different clades. Importantly, unadjuvanted *L. lactis*/pNZ8148-Spax-HA conferred cross-clade protection against lethal challenge with different H5N1 viruses in the chicken model.

Conclusion This study provides insights into the cross-clade protection conferred by unadjuvanted *L. lactis*/pNZ8148-Spax-HA, and the results might help the establishment of a promising platform for the development of a safe and effective H5N1 cross-clade vaccine for poultry.

Background

Due to its high mortality and antigen drift rate, the highly pathogenic avian influenza (HPAI) H5N1 virus is associated with severe disease and poses a serious threat to the poultry industry [1]. HPAI H5N1 viruses have undergone significant genetic diversification, and to date, 10 viral clades, denoted clades 0 to 9, have been identified. Among these clades, clade 2 exhibits significant genetic variation and has been classified into numerous subclades [2]. Numerous H5N1 influenza vaccines from candidate clade 1 and 2 viruses have been approved for production [3]. In addition, most licensed inactivated and live-attenuated H5N1 vaccines are produced in embryonated chicken eggs infected with the seed viruses, and the manufacturing process can take up 9 months [4, 5]. Unfortunately, the highly diverse genetic nature and the rapid evolution of H5N1 viruses has resulted in titre reduction in high-quality allantoic fluid [6]. Furthermore, the currently licensed H5N1 influenza vaccines and inactivated egg-derived whole-virus H5N1 vaccines provide inadequate immunogenicity against infection with H5N1 belonging to a different clade [7], and the current vaccine manufacturing capacity would be inadequate during an emerging H5N1 pandemic [8, 9]. Because the rapid generation of a well-matched H5 vaccine would represent a challenging task at the onset of a pandemic, two important issues to resolve include the supply of sufficient H5 vaccine doses in a timely manner and evaluating their cross-clade protection efficacy against newly emerged strains of H5 influenza viruses. Therefore, the development of a cross-clade protective vaccine is an ongoing high-priority effort for preparing the domestic poultry industry against a potential HPAI H5N1 epidemic.

Several vaccine approaches have been developed against different H5N1 clades. A monovalent H5 vaccine with RG-epitope-chimeric H5N1 protects mice from lethal challenge with H5N1 viruses of different clades, including clades 1.0, 2.1, 2.2 and 2.3 [10]. Furthermore, a vesicular stomatitis virus-based influenza vaccine administered via a single immunization confers rapid protection against different H5N1 clades in a mouse model [11].

L. lactis has been used for the expression of heterologous proteins, such as viral antigens, cytokines and enzymes [12, 13]. Importantly, the *L. lactis* expression system is suitable as a promising vaccine platform for the development of animal influenza A viruses. Our previous studies showed that combined with mucosal adjuvant or within an enteric capsule, *L. lactis* expressing H5N1 HA, HA1 or NP is a safe and effective delivery vehicle against homologous H5N1 virus challenge in a mouse or chicken model [14-17]. However, whether unadjuvanted *L. lactis* can provide cross-clade protective immunity against different H5N1 viruses in the chicken model has not yet been investigated.

In the present study, we generated and tested the *L. lactis* delivery vector expressing the H5N1 HA of A/Vietnam/1203/2004 (clade 1.0) to demonstrate the feasibility of the *L. lactis* display platform for a well-matched H5N1 vaccine. Chickens vaccinated orally with a prime/boost regimen of unadjuvanted *L. lactis* displayed H5N1 vaccine candidates, which could elicit a significant humoral immune response, a significant mucosal immune response, and a neutralizing antibody response. Most importantly, the vaccinated chickens were protected from lethal challenge with different H5N1 clades.

Methods

Construction of the *L. lactis* vectored vaccine

The Spax (411 bp) gene was used as an anchor domain and amplified by PCR from the *Staphylococcus aureus* (*S. aureus*) genome as described previously [18]. The HA gene fragment (1650 bp) of A/Vietnam/1203/2004 (H5N1) (GenBank accession No. EU122404) without the signal and transmembrane region was amplified by PCR from pcDNA3.1-HA (kindly provided by Institute of Virology, Chinese Academy of Science, Wuhan, China) using the following primers (the GS linker sequence and *Hind* III are underlined): H-F, 5' GGCGGCGGCGGCGCCGATCAGATTTGCATTGGTTAC 3'; and H-R, 5' CCGAAAGCTTTTAAATGCAAATTCAGCATT 3'. The Spax and HA fragments were fused into Spax-HA using the primers S-F (5' CTAGCTAGCAGTCTTCTAACCGAG 3') and H-R via a GS linker. The resulting Spax-HA containing *Nhe* I/*Hind* III was subcloned into an *L. lactis* expression vector, pNZ8148 (Fig. 1A), and then electroporated into competent *L. lactis* NZ9000. The positive clone of *L. lactis*/pNZ8148-Spax-HA was screened and expressed as described previously [16]. *L. lactis*/pNZ8148-Spax was used as a negative control for the subsequent analyses.

Western blot analysis

The HA protein expression level on the surface of recombinant *L. lactis* was determined by Western blot analysis as described previously [15]. Briefly, 10^8 cells of *L. lactis*/pNZ8148-Spax-HA pellets were washed

three times with 500 μ L of sterile phosphate-buffered saline (PBS), resuspended in 50 μ L of 6 \times loading buffer and boiled for 10 min. The treated samples were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). After blocking with 5% non-fat milk at room temperature for 2 h, the membrane was incubated with a monoclonal mouse anti-HA antibody overnight at 4 $^{\circ}$ C and then with affinity-purified horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma-Aldrich Corporation, St. Louis, MO, USA). The membrane was subsequently reacted with the West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA) and imaged using the Molecular Imager ChemiDoc XRS system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Immunofluorescence assay and flow cytometry analysis

The display of HA protein on the surface of *L. lactis* was confirmed by immunofluorescence assay (Olympus IX70, Japan) and flow cytometry (FACS) analysis (BD FACS Calibur, BD Bioscience, San Jose, CA, USA). Briefly, 10^8 cells of *L. lactis*/pNZ8148-Spax-HA were washed three times with sterile PBS containing 0.5% bovine serum albumin (BSA), incubated with monoclonal mouse anti-HA antibody for 1 h at 4 $^{\circ}$ C and then with FITC-conjugated goat anti-mouse IgG for 30 min at 4 $^{\circ}$ C, and resuspended in 500 μ L of sterile PBS. The resulting *L. lactis*/pNZ8148-Spax-HA cells were subjected to immunofluorescence assay and FACS analysis, respectively.

Quantification of HA protein expressed by *L. lactis*/pNZ8148-Spax-HA

The expression of HA protein by *L. lactis*/pNZ8148-Spax-HA was determined by indirect ELISA [19]. Briefly, 10^{12} colony forming units (CFUs) of *L. lactis*/pNZ8148-Spax-HA pellets were resuspended in 100 μ L of a monoclonal mouse anti-HA antibody (0, 10, 25, 50, 75, 100, 125, 150, 175, and 200 μ g/mL) in PBS containing 2% BSA, incubated at room temperature for 2 h, and incubated with goat anti-mouse IgG antibody conjugated to horseradish peroxidase (1 mg/mL) (Sigma-Aldrich Corporation, St. Louis, MO, USA) at room temperature for 1 h. The cells were then washed with sterile PBS and resuspended in 100 μ L of HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma-Aldrich Corporation, St. Louis, MO, USA) in the dark for 25 min, and 100 μ L of 2 mol/L H_2SO_4 was then added to stop the reaction. The OD_{450nm} value of the supernatant was measured using a microplate reader. *L. lactis*/pNZ8148-Spax was used as a negative control.

Animals, immunization, sample collection and virus challenge

Specific pathogen-free (SPF) white Leghorn chickens (aged 7 days) were purchased from the Veterinary Research Academy of Agricultural Sciences of Jiangxi Province (Jiangxi, China) and housed in ventilated cages (five chickens per cage). The chickens were administered pelleted feed and sterile water and maintained in an SPF environment.

The concentration of *L. lactis*/pNZ8148-Spax-HA was adjusted to 10^{12} CFUs/mL. The chickens (n=11 per group) were vaccinated orally with 2 mL at days 0, 1, and 2 for prime immunization and days 17, 18, and

19 for boost immunization. PBS and *L. lactis*/pNZ8148-Spax cells were used as controls.

At days 15 and 34 after the initial vaccination, blood samples were collected from the wing vein. Sera were separated by centrifugation of the blood at $2,000 \times g$ for 10 min and stored at $-20\text{ }^{\circ}\text{C}$ until use. The intestines were isolated from the vaccinated chickens ($n=3$ per group) and washed with $500\text{ }\mu\text{L}$ of sterile PBS. Faeces were also collected, resuspended in $500\text{ }\mu\text{L}$ of PBS and stored at $-20\text{ }^{\circ}\text{C}$ until use.

Two weeks after the final vaccination, all the vaccinated chickens were transferred into an animal biosafety level-3 (BSL-3) containment facility. Slightly ether narcosis-anaesthetized chickens were intranasally infected with $20\text{ }\mu\text{L}$ of $5 \times 50\%$ lethal dose ($5 \times \text{LD}_{50}$) of HPAI H5N1 virus strains belonging to clade 1.0 (A/Vietnam/1203/2004, VN1203), clade 2.3 (A/Anhui/1/2005, Anhui) or clade 8.0 (A/chicken/Henan/12/2004, Henan). Three chickens in each group were sacrificed at day 3 post challenge to check the virus titres in the lungs, and the other five chickens remaining in each group were used for survival records. The chickens were monitored every alternate day at a fixed time point to record their weight loss and survival. The humane endpoint of the challenge studies was a body weight loss exceeding 25% relative to the weight at the time of challenge inoculation. After final monitoring, all the surviving chickens were euthanized by CO_2 inhalation for 5 min.

All animal immunizations were performed at a BSL-2 facility, and the virus challenge experiments were strictly performed in BSL-3 containment facilities, complied with the Guidelines for the Use and Care of Experimental Animals and were approved by the Institute Animal Care and Use Committee of Nanchang University.

Determination of antibody responses by ELISA

The antibody responses of serum IgG and IgA in the intestinal washes and faeces were determined by ELISA using recombinant HA protein ($2\text{ }\mu\text{g}/\text{mL}$) from A/Vietnam/1203/2004 as a coating antigen as described previously [15]. The optical density (OD) at 405 nm was measured using an ELISA plate reader. The IgG or IgA titre was determined as the lowest dilution with an OD greater than the mean OD of the naïve controls plus two standard deviations.

Neutralization assay

The neutralization activity of serum against different H5N1 viruses was assessed as described previously [15]. Briefly, receptor-destroying enzyme (RDE)-treated sera were serially diluted (2-fold) and incubated with 100 TCID₅₀ of viruses belonging to clade 1.0 (A/Vietnam/1203/2004, VN1203), clade 2.3 (A/Anhui/1/2005, Anhui) or clade 8.0 (A/chicken/Henan/12/2004, Henan) for 1 h at room temperature and plated in duplicate in a 96-well plate with MDCK cells. The neutralizing titre was assessed as the highest antibody dilution for which no cytopathic effect was observed by light microscopy.

Statistical analysis

The statistical significance of the differences was assessed by Student's *t* test and one-way ANOVA with multiple comparisons. Significant differences in the survival curves were determined by log-rank analysis. A *p* value less than 0.05 was considered to indicate statistical significance.

Results

Characterization of HA protein expressed on the surface of *L. lactis*

L. lactis/pNZ8148-Spax-HA was generated by fusing the HA gene of A/Vietnam/1203/2004 (clade 1.0) lacking a signal peptide and a transmembrane domain with Spax via a GS linker (**Fig. 1a**). This Spax was previously shown to serve as effective anchor protein for display [18]. The expression of the HA protein was detected by Western blot analysis. As expected, a specific Spax-HA band was observed at the expected size (approximately 120 kDa) (**Fig. 1b**).

We then performed immunofluorescence assays and a flow cytometry analysis to determine the efficiency of the display of HA protein on the surface of *L. lactis*. *L. lactis*/pNZ8148-Spax-HA was incubated with mouse anti-HA monoclonal antibody for direct labelling. Compared with the *L. lactis*/pNZ8148-Spax control, positive fluorescence signals were observed with *L. lactis*/pNZ8148-Spax-HA (**Fig. 1c and d**). Collectively, these results demonstrated that HA protein was located on the surface of *L. lactis*.

Quantification of HA protein on the surface of *L. lactis*

Furthermore, as shown in **Fig. 1e**, 10^{12} CFUs of *L. lactis*/pNZ8148-Spax-HA showed optical densities at 125 μ g, which suggested that the expression of HA protein on the *L. lactis* surface reached its saturation limit at 125 μ g/mL.

Antibody responses induced by *L. lactis*/pNZ8148-Spax-HA

The HA-specific antibody responses were determined by ELISA. At day 15, the sera IgG titres from chickens vaccinated orally with *L. lactis*/pNZ8148-Spax-HA showed a slightly significant difference compared with that found in the control chickens vaccinated with PBS and the *L. lactis*/pNZ8148-Spax control. However, a highly significant increase was detected in the *L. lactis*/pNZ8148-Spax-HA group at day 34, whereas after the boost immunization, no significant changes were found in the groups vaccinated with PBS or *L. lactis*/pNZ8148-Spax (**Fig. 2a**). Similarly, a significant level of mucosal IgA antibody was detected in the intestine washes and faeces of chickens vaccinated orally with *L. lactis*/pNZ8148-Spax-HA (**Fig. 2b and c**). These results revealed that a prime-boost regimen of *L. lactis*/pNZ8148-Spax-HA could induce significant humoral and mucosal immune responses in vaccinated chickens.

Neutralization assay

Due to frequent mutation of the H5N1 virus, it is important to determine whether vaccine candidates elicit neutralization antibodies against different H5N1 clades. We thus performed neutralization tests to examine the ability of *L. lactis*/pNZ8148-Spax-HA to generate cross-neutralizing antibody responses against different H5N1 viruses. As shown in **Fig. 2d**, cross-neutralizing antibodies were elicited in the *L. lactis*/pNZ8148-Spax-HA group, and this finding was similar with the HA-specific IgG response. In contrast, no significant cross-neutralizing antibodies were detected in the *L. lactis*/pNZ8148-Spax control group. These data were consistent with the ELISA results: chickens vaccinated orally with *L. lactis*/pNZ8148-Spax-HA using the prime/boost regimen could produce a higher HA-specific IgG antibody and elicit neutralization activities, which might contribute to the prevention of H5N1 clade 1.0 (A/Vietnam/1203/2004, VN1203), clade 2.3 (A/Anhui/1/2005, Anhui) and clade 8.0 (A/chicken/Henan/12/2004, Henan) infection.

Cross-protection against H5N1 virus infection

To further support the cross-protective potential of the *L. lactis*/pNZ8148-Spax-HA vaccine, vaccinated chickens (n=8) were challenged with 20 μ L of $5 \times LD_{50}$ of a H5N1 virus belonging to clade 1.0 (A/Vietnam/1203/2004, VN1203), clade 2.3 (A/Anhui/1/05, Anhui) or clade 8.0 (A/chicken/Henan/12/2004, Henan) and monitored for 14 days. The control groups that received PBS or *L. lactis*/pNZ8148-Spax showed clinical signs of severe disease, a significant body weight loss, and a higher lung virus titre after virus infection and died within 8 days after the lethal challenge (**Fig. 3**). In contrast, the chickens vaccinated orally with *L. lactis*/pNZ8148-Spax-HA survived throughout the study period and had recovered within 14 days after challenge, and no significant weight loss or apparent illness symptoms were noted after virus challenge (**Fig. 3**). A lower virus titre in the lung was observed in the *L. lactis*/pNZ8148-Spax-HA group (**Fig. 3**). Taken together, the results from the virus challenge experiments provided reliable evidence showing that the *L. lactis*/pNZ8148-Spax-HA vaccine candidate conferred cross-clade protection against divergent H5N1 clades in the chicken model.

Discussion

Due to the frequent occurrence of antigenic changes in HPAI H5N1 viruses, there is a risk for an epidemic or potential pandemic of these viruses in poultry. A well-matched H5N1 vaccine is likely an effective measure for fighting a potential H5N1 panzootic. In this study, we generated a well-characterized *L. lactis*/pNZ8148-Spax-HA vaccine candidate based on the *L. lactis* display platform, which was previously found to exhibit several advantages over other vaccine approaches, such as the easy realization of genetic modifications, an efficient and cost-effective manufacturing process, a mucosal delivery route and a proven safety and immunogenicity profile [20].

To define a more optimized vaccine approach, we investigated the immunogenicity of unadjuvanted *L. lactis*/pNZ8148-Spax-HA administered orally in the chicken model. It is generally accepted that trimeric HA exhibits strong immunogenicity, and previous studies have revealed the promising finding that chickens injected with adjuvanted subunit vaccines consisting of trimeric H5N1 HA exhibit high levels of

cross-neutralizing antibodies against H5N1 clades 1 and 2 [21, 22]. However, we demonstrated the unadjuvanted *L. lactis*/pNZ8148-Spax-HA in which the HA gene lacks the signal and trimerization sequence provided convincing cross-clade protection following prime/boost oral vaccination. Furthermore, the *L. lactis* H5N1 vaccine candidate has advantages compared with the currently used influenza virus vaccines, including easy generation of the vectors and the ability to produce vaccines in cell lines that have already been approved for the manufacturing of human vaccines [23]. In addition, *L. lactis* vaccines are more effective and safer compared with viral vector-based approaches and do not require adjuvants [24]. Thus, the *L. lactis* display platform would have a beneficial effect on the manufacturing of influenza vaccines.

An anchor protein plays an important role in *L. lactis* display-based vaccines. Our previous studies showed that pgsA and Spax could be used as anchor proteins for antigen display [16, 18]. *L. lactis*/pNZ8110-pgsA-HA1 adjuvanted with CTB provides immune protection against homologous H5N1 virus in a mouse model [16]. Additionally, unadjuvanted *L. lactis*/pNZ8110-pgsA-NA and unadjuvanted *L. lactis*/pNZ8008-Spax-HA2 could protect mice from homologous and heterologous virus infection [18, 24]. The comparisons of the display efficiency of *L. lactis*-displayed influenza vaccines obtained with various anchor proteins revealed that Spax was superior to pgsA. Thus, in our present study, Spax was selected for the design of *L. lactis*-displayed H5N1 HA, and unadjuvanted *L. lactis*/pNZ8148-Spax-HA conferred cross-clade protection against different H5N1 viruses in the chicken model. Based on these findings, the *L. lactis* display platform using Spax will contribute to improving the feasibility of developing influenza H5N1 vaccines for poultry that confer protection against different H5N1 clades.

The rapid evolution of new sublineages of influenza A/H5N1 virus poses a great threat to poultry health [25]. A major obstacle in vaccine development against influenza H5N1 virus infection is the rapid evolution of the genetic diversity of these viruses [26]. As a result and given the limitations of the currently approved vaccines for H5N1 viruses in terms of their production timelines and their ability to induce cross-clade protective immune responses, newer vaccine approaches for panzootic preparedness against these viruses are needed [27]. The development of a simple vaccine that provides broad protection against influenza H5N1 is of high priority for preparing the poultry industry against a future influenza panzootic. This study clearly demonstrated that unadjuvanted *L. lactis*/pNZ8148-Spax-HA could serve as an alternative approach that is currently showing promise for the development of a H5N1 cross-clade vaccine for the mass vaccination of poultry. Overall, the *L. lactis* display platform might constitute a new strategy for the development of a universal flu vaccine for poultry and humans.

Conclusions

The findings obtained in this study suggest that the unadjuvanted *L. lactis* display platform can be used to an alternative H5N1 cross-clade vaccine candidate and can overcome the bottleneck of the current manufacturing process because it constitutes a flexible and high-output system for the safe, effective and low-cost production of an H5N1 vaccine for poultry during an influenza H5N1 panzootic.

Declarations

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Authors' contributions

All the authors approved the manuscript for submission. HL, QC, TG and XP contributed to the study design and data interpretation. All the authors contributed to the data analysis and the interpretation of the results. HL wrote the manuscript and produced all the figures.

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Availability of data and materials

The datasets generated and analysed in the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All animal studies complied with the Guidelines for the Use and Care of Experimental Animals and were approved by the Animal Committee of the Institute of Nanchang University.

Competing interests

All authors claim no conflicts of interest.

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Figures

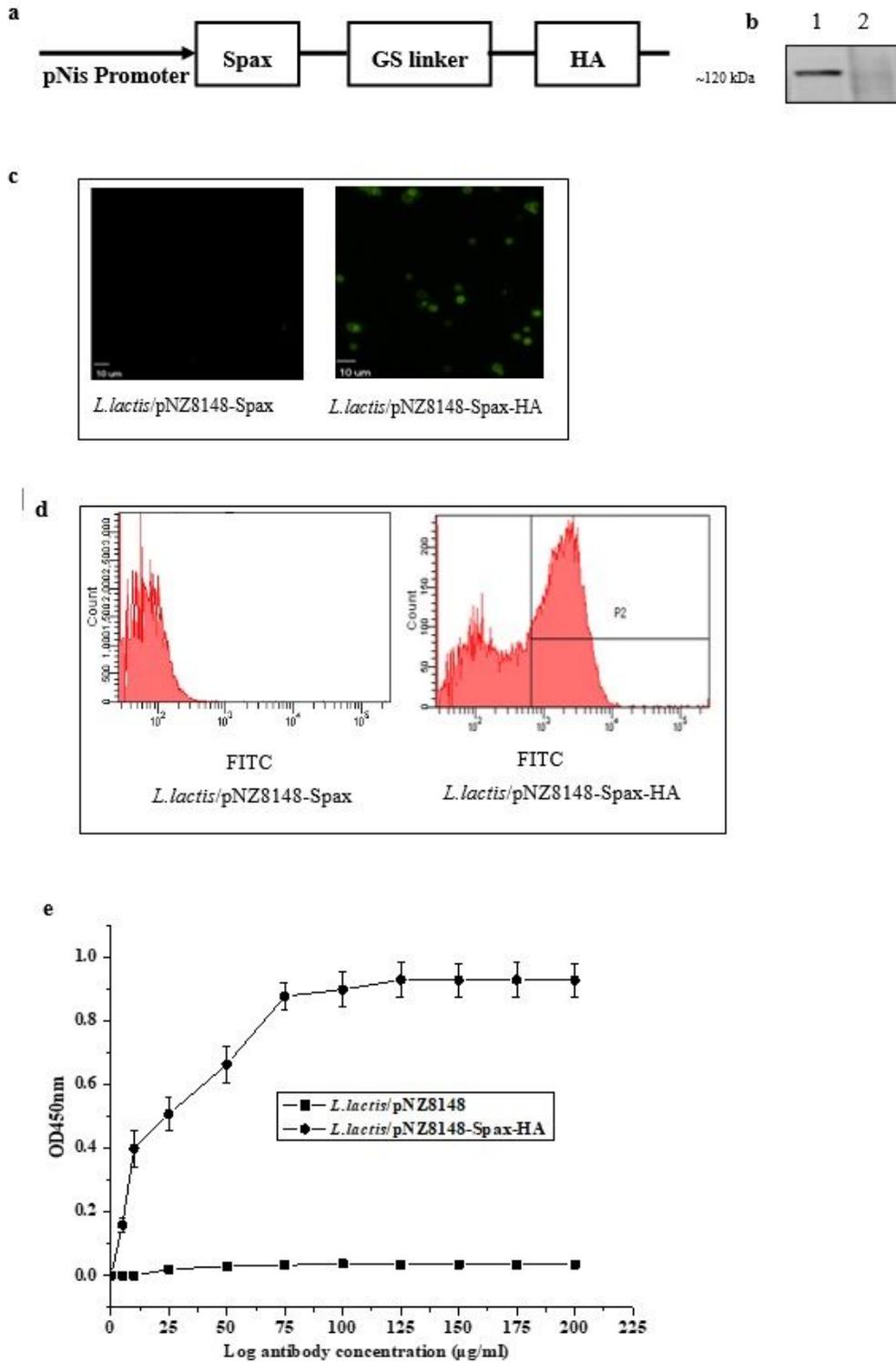


Figure 1

Characterization of the HA protein displayed on the surface of *L. lactis*. (a) Schematic diagram of *L. lactis*/pNZ8148-Spax-HA. A GS linker was inserted between Spax and HA to stabilize the expression of the HA protein. (b) Western blot analysis. Lane 1: *L. lactis*/pNZ8148-Spax-HA; Lane 2: *L. lactis*/pNZ8148-Spax. (c) Immunofluorescence microscopy assay of the HA protein: *L. lactis*/pNZ8148-Spax (left) and *L. lactis*/pNZ8148-Spax-HA (right) (magnification: 1,000×). (d) Flow cytometric analysis of the HA protein

displayed on the surface of *L. lactis*: *L. lactis*/pNZ8148-Spax (left) and *L. lactis*/pNZ8148-Spax-HA (positive rate: 60.5%) (right). A total of 15,000 cells were counted. (e) Quantification of the HA protein expressed by *L. lactis*/pNZ8148-Spax-HA by indirect ELISA. The values were determined from three independent experiments, and the bars indicate the means \pm SDs.

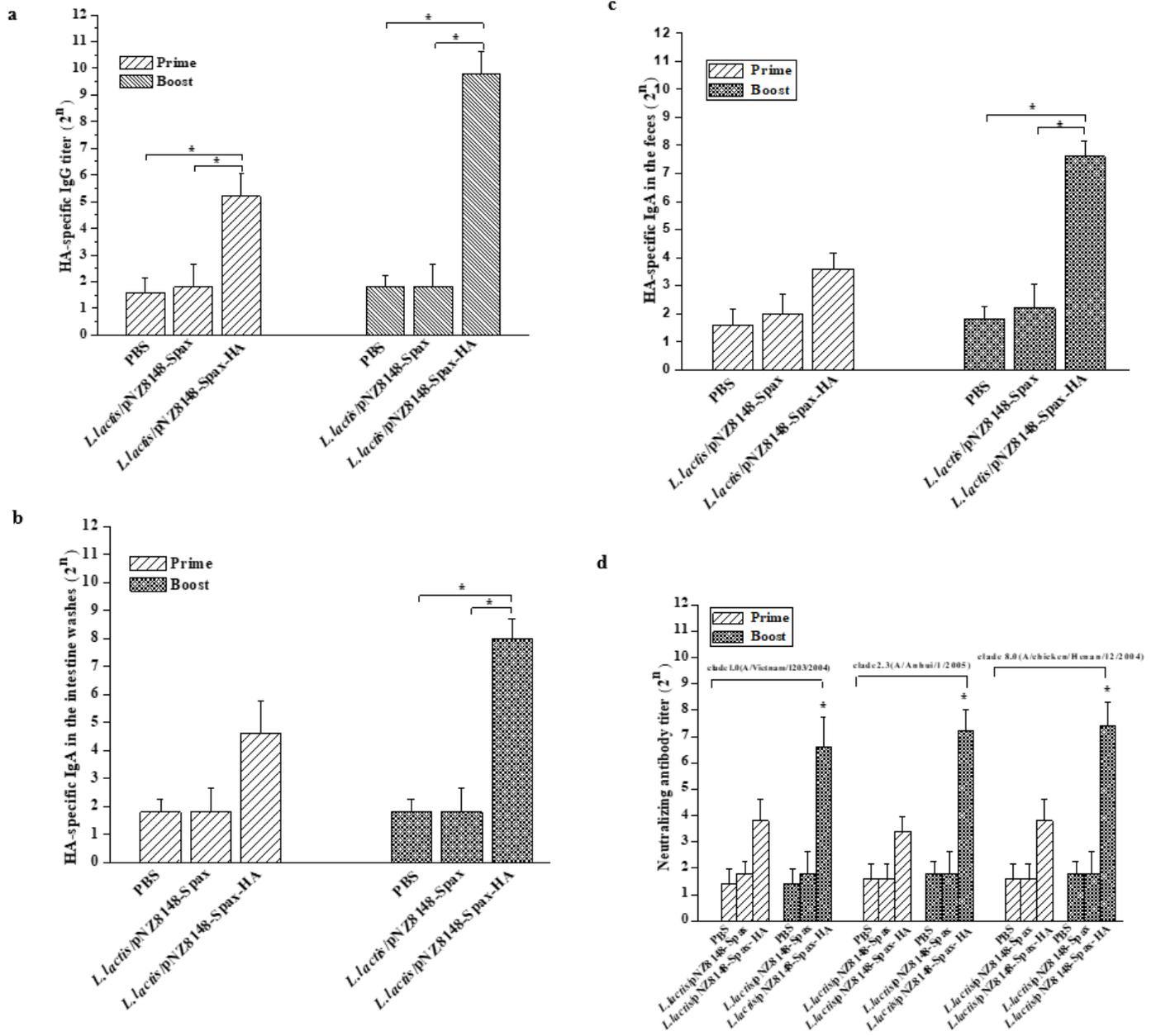


Figure 2

Determination of antibody responses elicited by the *L.lactis*/pNZ8148-Spax-HA in chickens. Sera, feces and intestine washes were collected from chickens vaccinated orally with PBS, *L.lactis*/pNZ8148-Spax or *L.lactis*/pNZ8148-Spax-HA. (a) HA-specific IgG antibody responses in the sera. (b) HA-specific IgA antibody responses in the intestine washes. (c) HA-specific IgA antibody responses in the feces. (D) Neutralizing antibody titer. Data are represented as mean \pm SD. Asterisk indicates significant difference, as compared to PBS and the *L.lactis*/pNZ8148-Spax controls ($p < 0.05$)

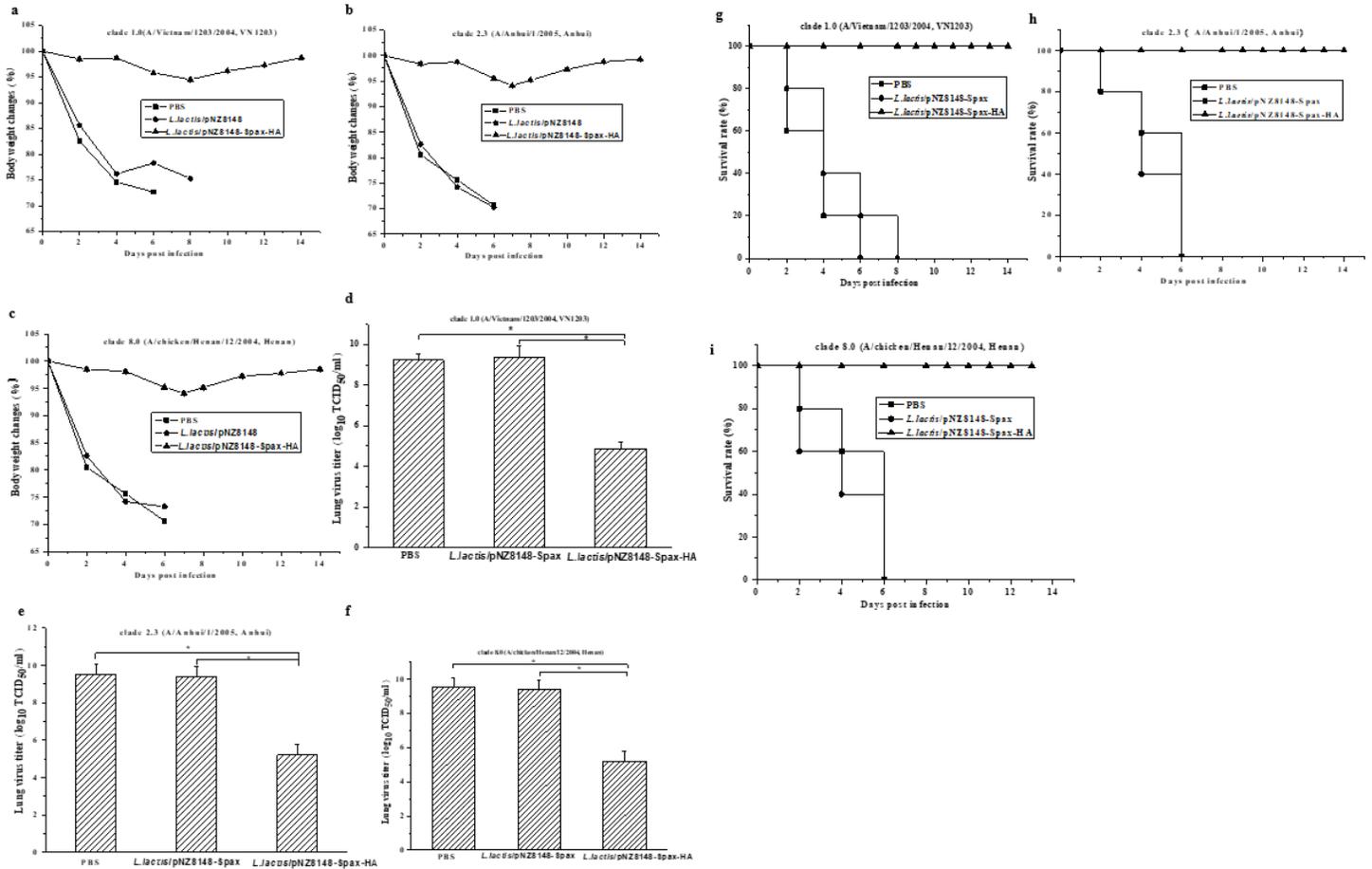


Figure 3

Cross-clade protection of chickens against lethal challenge with different H5N1 viruses. The results are expressed in terms of percent body weight (a, b and c), lung virus titer (d, e and f) and percent survival (g, h and i). Two weeks after the last immunization, chickens were intranasally infected with 20 μ L of 5 \times LD₅₀ of clade 1.0: A/Vietnam/1203/2004 (a, d and g), clade 2.3: A/Anhui/1/2005, Anhui (b, e and f) or clade 8.0: A/chicken/Henan/12/2004 (c, f and i) HPAI H5N1 virus strains. Chickens were monitored throughout a 14-day observation period (n=5 / group). Data for lung virus titers are represented as mean \pm SD. Asterisk indicates significant difference, as compared to PBS and the L.lactis/pNZ8148-Spax controls (p \leq 0.05).