

1 **Hemagglutinin presented on the surface of *Lactococcus lactis* confers broad**

2 **cross-clade protection against different H5N1 viruses in chickens**

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1 **Abstract**

2 **Background**

3 Highly pathogenic avian influenza (HPAI) H5N1 virus has the potential threat to  
 4 poultry industry. Current avian influenza H5N1 vaccines for poultry are clade-specific,  
 5 Therefore, there is an urgent need to develop an effective vaccine for preventing and  
 6 controlling H5N1 viruses from different clades.

7 **Results**

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

16 **Conclusion**

17 This study provides insight into the cross-clade protection conferred by the  
 18 unadjuvanted *L.lactis*/pNZ8148-Spax-HA that may help establish a promising platform  
 19 for the development of a safe and effective H5N1 cross-clade vaccine in poultry.

20 **Keywords:** *L.lactis*/pNZ8148-Spax-HA, cross protection, H5N1 cross-clade vaccine.

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## 1 Background

2 Since high mortality and antigen drift rate, highly pathogenic avian influenza (HPAI)  
3 H5N1 virus are associated with severe disease and pose a serious threat to poultry  
4 industry [1]. To date, HPAI H5N1 viruses have undergone significant genetic  
5 diversifications resulting in 10 viral clades originating from clade 0 to clade 9. Among  
6 them, clade 2 has shown significant genetic variation into numerous subclades [2].  
7 There are numerous H5N1 influenza vaccines from candidate clade 1 and 2 viruses  
8 have been approved for the production [3], In addition, most of the licensed inactivated  
9 and live-attenuated H5N1 vaccines are produced in embryonated chicken eggs affected  
10 by seed viruses and the manufacturing process can take up 9 months [4, 5].  
11 Unfortunately, the highly diverse genetic nature and the rapid evolution of H5N1  
12 viruses has resulted in titer reduction of quality allantoic fluid [6]. Furthermore, the  
13 protective immunities of currently licensed H5N1 influenza split and inactivated egg-  
14 derived whole virus H5N1 vaccines are inadequate immunogenicity against different  
15 H5N1 clades infection [7] and the current vaccine manufacturing capacity is inadequate  
16 during an emerging H5N1 pandemic [8, 9]. As the rapid generation of a well-matched  
17 H5 vaccine would represent a challenging task at the onset of a pandemic, one important  
18 issue is how to supply enough H5 vaccine doses in a timely manner and evaluate the  
19 efficacy that could provide cross-clade protection against newly emerged strains of H5  
20 influenza viruses. Therefore, the development of cross-clade protective vaccine is  
21 ongoing effort of high priority for domestic poultry to be prepared for a potential  
22 epidemic of HPAI H5N1.

1 Several vaccines approaches have been developed against different H5N1 clades. A  
2 monovalent H5 vaccine with RG-epitope-chimeric H5N1 protected mice from lethal  
3 challenge with H5N1s of different clades, including clade 1.0, 2.1, 2.2 and 2.3 [10]  
4 Furthermore, a vesicular stomatitis virus-based influenza vaccine by a single  
5 immunization conferred rapid protection against different H5N1 clades in the mouse  
6 model [11].

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

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

## 1 **Methods**

### 2 **Construction of *L.lactis* vectored vaccine**

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

### 18 **Western blot analysis**

19 The HA protein expression level on the surface of recombinant *L.lactis* was determined  
 20 by Western blot analysis as described previously [15]. Briefly, 10<sup>8</sup> cells of the  
 21 *L.lactis*/pNZ8148-Spax-HA pellets were washed three times with 500 μL of sterile  
 22 phosphate-buffered saline (PBS), and then re-suspended with 50 μL of 6 × loading

1 buffer and boiled for 10 minutes. Treated samples were run on SDS-polyacrylamide gel  
2 electrophoresis and then transferred to nitrocellulose membrane (Bio-rad, Hercules,  
3 California, USA). After blocking with 5% non-fat milk at room temperature for 2 h, the  
4 membrane was incubated with a monoclonal mouse anti-HA antibody. After incubated  
5 overnight at 4 °C, and then followed by affinity-purified horseradish peroxidase (HRP)-  
6 conjugated anti-mouse IgG (Sigma-Aldrich Corporation, St. Louis, MO, USA). The  
7 membrane was reacted with the West Pico Chemiluminescent Substrate (Thermo Fisher  
8 Scientific Inc., Rockford, IL, USA) and imaged using Molecular Imager ChemiDoc  
9 XRS System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

#### 10 **Immunofluorescence assay and flow cytometry analysis**

11 HA protein displayed on the surface of *L.lactis* was confirmed by immunofluorescence  
12 assay (Olympus IX70, Japan) and flow cytometry (FACS) analysis (BD FACS Calibur,  
13 BD Bioscience, San Jose, CA, USA). Briefly,  $10^8$  cells of the *L.lactis*/pNZ8148-Spax-  
14 HA were washed three times with sterile PBS containing 0.5% bovine serum albumin  
15 (BSA) and then incubated with monoclonal mouse anti-HA antibody at 4 °C for 1h,  
16 followed by a FITC-conjugated goat anti-mouse IgG at 4 °C for 30 min and re-  
17 suspended with 500  $\mu$ L of sterile PBS. Finally, the *L.lactis*/pNZ8148-Spax-HA cells  
18 were used for immunofluorescence assay and FACS analysis, respectively.

#### 19 **Quantification of the *L.lactis*/pNZ8148-Spax-HA expressing HA protein**

20 The *L.lactis*/pNZ8148-Spax-HA expressing HA protein was determined by indirect  
21 ELISA [xxx]. Briefly,  $10^{12}$  colony forming unit (CFU) of the *L.lactis*/pNZ8148-Spax-  
22 HA pellets were re-suspended in 100  $\mu$ L of a monoclonal mouse anti-HA antibody (0,

1 10, 25, 50, 75, 100, 125, 150, 175, 200  $\mu\text{g}/\text{mL}$ ) in PBS containing 2% BSA and  
2 incubated at room temperature for 2 h. Followed by incubation with Goat anti-mouse  
3 IgG antibody conjugated with horseradish peroxidase (1 mg/ml) (Sigma-Aldrich  
4 Corporation, St. Louis, MO, USA) at room temperature for 1 h. After washing with  
5 sterile PBS, the cells were re-suspended in 100  $\mu\text{l}$  of HRP substrate 3,3',5,5'-  
6 tetramethylbenzidine (TMB) (Sigma-Aldrich Corporation, St. Louis, MO, USA) in  
7 the dark for 25 min and then 100  $\mu\text{l}$  of 2 mol/l  $\text{H}_2\text{SO}_4$  was added to stop the reaction.  
8  $\text{OD}_{450\text{nm}}$  value of the supernatant was measured using a microplate reader. The  
9 *L.lactis*/pNZ8148-Spax was used a negative control.

#### 10 **Animals, immunization, sample collection and virus challenge**

11 Specific pathogen-free (SPF) white Leghorn chickens (7 days old) were purchased from  
12 the Veterinary Research Academy of Agricultural Sciences of Jiangxi Province (Jiangxi,  
13 China), and housed in ventilated cages (5 chickens / cage). The chickens were managed  
14 with pelleted feed and sterile water, maintained in a SPF environment.

15 The concentration of the *L.lactis*/pNZ8148-Spax-HA was adjusted to  $10^{12}$  colony  
16 forming unit (CFU) / ml. The chickens (n=11 per group) were vaccinated orally with 2  
17 ml of at days 0, 1, 2 for prime immunization and days 17, 18, 19 for boost immunization.  
18 PBS and the *L.lactis*/ pNZ8148-Spax cells were used as controls.

19 At day 15 and day 34 after the initial vaccination, blood samples were collected from  
20 the wing vein. Sera were separated by centrifugation of blood at  $2,000 \times g$  for 10 min  
21 and stored at  $-20\text{ }^\circ\text{C}$  until use. Intestines were isolated from the vaccinated chickens  
22 (n=3 / group) and washed with 500  $\mu\text{l}$  sterile PBS. Feces were also collected and re-

1 suspended with 500  $\mu$ l PBS, and stored at -20 °C until use.

2 Two weeks after the final vaccination, all the vaccinated chickens were transferred into  
3 an animal BSL-3 containment facility. Slight ether narcosis-anesthetized chickens were  
4 intranasally infected with 20  $\mu$ l of  $5 \times 50\%$  lethal dose ( $5 \times LD_{50}$ ) of clade 1.0  
5 (A/Vietnam/1203/2004, VN1203), clade 2.3 (A/Anhui/1/2005, Anhui) or clade 8.0  
6 (A/chicken/Henan/12/2004, Henan) HPAI H5N1 virus strain. Three chickens in each  
7 group were sacrificed at day 3 post challenge to check virus titer in lungs and other five  
8 chickens remained in each group have been used for survival record. Chickens were  
9 monitored every alternate day at fixed time point for recording the weight loss and  
10 survival. The humane endpoint of the challenge studies was body weight loss exceeded  
11 25% relative to the weight at the time of challenge inoculation. After final monitoring,  
12 all survived chickens were euthanized using CO<sub>2</sub> inhalation for 5 minutes.

13 All animal immunizations were carried out at biosafety level 2 (BLS-2) and virus  
14 challenge experiments must be strictly performed under the bio-safety level-3  
15 laboratory (BSL-3) containment facilities complying with the Guidelines for Use and  
16 Care of Experimental Animals and were approved by the Institute Animal Care and Use  
17 Committee of the Nanchang University.

### 18 **Determination of antibody responses by ELISA**

19 Antibody responses of serum IgG and IgA in the intestinal washes and feces were  
20 determined by ELISA using recombinant HA protein (2  $\mu$ g/ml) of  
21 A/Vietnam/1203/2004 as a coating antigen as described previously [15]. Optical  
22 density (OD) was measured at 405 nm using ELISA plate reader. The IgG or IgA titer

1 was determined to be the lowest dilution with an OD greater than the mean OD of naïve  
2 controls plus 2 standard deviations.

### 3 **Neutralization assay**

4 Neutralization activity of serum against different H5N1 viruses was performed as  
5 described previously [15]. Briefly, receptor-destroying enzyme (RDE)-treated sera  
6 were serially diluted (2-fold) and incubated with 100 TCID<sub>50</sub> of clade 1.0  
7 (A/Vietnam/1203/2004, VN1203), clade 2.3 (A/Anhui/1/2005, Anhui) or clade 8.0  
8 (A/chicken/Henan/12/2004, Henan) for 1 h at room temperature and plated in duplicate  
9 onto MDCK cells grown in a 96-well plate. The neutralizing titer was assessed as the  
10 highest antibody dilution in which no cytopathic effect was observed by light  
11 microscopy.

### 12 **Statistical analysis**

13 All analysis for statistically significant differences was performed by the Student *t* test  
14 and one-way ANOVA with multiple comparison. Significant differences in the survival  
15 curves were performed by Log-Rank analysis. A *p* value less than 0.05 was considered  
16 statistically significant.

17

## 18 **Results**

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

20 The *L.lactis*/pNZ8148-Spax-HA was generated by fusing the HA gene of  
21 A/Vietnam/1203/2004 (clade 1.0) lacking signal peptide and transmembrane domain  
22 with Spax via GS linker (**Fig. 1a**). This Spax has been shown previously to be an

1 effective anchor protein for display [18]. Expression of the HA protein was detected by  
2 Western blot analysis. As expected, specific Spax-HA band was shown at expected size  
3 (approximately 120 kDa) (**Fig. 1b**).

4 Next, we performed **immunofluorescence assay and flow cytometry analysis** to  
5 determine the display efficiency of HA protein on the surface of *L.lactis*, the  
6 *L.lactis*/pNZ8148-Spax-HA was incubated with mouse anti-HA monoclonal antibody  
7 for direct labelling. Compared to the *L.lactis*/pNZ8148-Spax control, positive  
8 fluorescence signals were observe and detected in the *L.lactis*/pNZ8148-Spax-HA (**Fig.**  
9 **1c and d**). Collectively, these results demonstrated HA protein was located on the  
10 surface of *L.lactis*.

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

12 Further, As shown in **Fig. 1e**,  $10^{12}$  colony forming unit (CFU) of the *L.lactis*/pNZ8148-  
13 Spax-HA showed the optical densities at 125  $\mu\text{g}$  reach a stable point suggesting that  
14 expression of HA proteins on the *L.lactis* surface was at its saturation limit at 125  $\mu\text{g}/\text{ml}$ .

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

16 HA-specific antibody responses were measured by ELISA. Sera IgG titers from  
17 chickens vaccinated orally with the *L.lactis*/pNZ8148-Spax-HA showed a slight  
18 significant difference compared to PBS and the *L.lactis*/pNZ8148-Spax controls at day  
19 15. However, a highly significant increase was detected in the *L.lactis*/pNZ8148-Spax-  
20 HA group at day 34, whereas there were still no significant changes in the PBS or  
21 *L.lactis*/pNZ8148-Spax controls after the boost immunization (**Fig. 2a**). Similarly,  
22 significantly mucosal IgA antibody was detected in the intestine washes and feces of

1 chickens vaccinated orally with the *L.lactis*/pNZ8148-Spax-HA (**Fig. 2b and c**). These  
2 results revealed the *L.lactis*/pNZ8148-Spax-HA could induce significant humoral and  
3 mucosal immune responses in vaccinated chickens based on prime-boost regimen.

#### 4 **Neutralization assay**

5 Due to frequent mutation of H5N1 virus, it is important to determine if vaccine  
6 candidate elicits neutralization antibody against different H5N1 clades. Therefore, we  
7 performed neutralization tests to examine the ability of the *L.lactis*/pNZ8148-Spax-HA  
8 to generate cross-neutralizing antibody responses against different H5N1 viruses. As  
9 shown in **Fig. 2d**, cross-neutralizing antibodies were elicited in the detected in the  
10 *L.lactis*/pNZ8148-Spax-HA group that was similar to the HA-specific IgG response. In  
11 contrast, no significant cross-neutralizing antibodies were detected in the  
12 *L.lactis*/pNZ8148-Spax control group. These data were consistent with ELISA assay,  
13 chickens vaccinated orally with the *L.lactis*/pNZ8148-Spax-HA after prime/boost  
14 regimen could produce a higher HA-specific IgG antibody and also elicit neutralization  
15 activities which might contribute to preventing H5N1 clade 1.0 (A/Vietnam/1203/2004,  
16 VN1203), clade 2.3 (A/Anhui/1/2005, Anhui) and clade 8.0 (A/chicken/Henan/12/2004,  
17 Henan) infection.

#### 18 **Cross-protection against H5N1 virus infection**

19 Finally, in order to support the cross-protective potential of the *L.lactis*/pNZ8148-Spax-  
20 HA vaccine, the vaccinated chickens (n=8) were challenged with 20  $\mu$ L of  $5 \times LD_{50}$  of  
21 clade 1.0 (A/Vietnam/1203/2004, VN1203), clade 2.3 (A/Anhui/1/05, Anhui) or clade  
22 8.0 (A/chicken/Henan/12/2004, Henan) H5N1 virus, and monitored for 14 days. The

1 control groups that received PBS or the *L.lactis*/pNZ8148-Spax showed clinical signs  
2 of severe disease, significant body weight loss, a higher lung virus titer at post virus  
3 infection, and died within day 8 after the lethal challenge (**Fig. 3**). In contrast, chickens  
4 vaccinated orally with the *L.lactis*/pNZ8148-Spax-HA survived completely and  
5 recovered by 14 days and no significant weight loss or apparent illness symptoms were  
6 noted at post virus challenge (**Fig. 3**). A lower level of lung virus titer was observed  
7 in the *L.lactis*/pNZ8148-Spax-HA group (**Fig. 3**). Taken together, virus challenge  
8 experiment provided the reliable evidence that the *L.lactis*/pNZ8148-Spax-HA vaccine  
9 candidate conferred cross-clade protection against divergent H5N1 clades in the  
10 chicken model.

11

## 12 **Discussion**

13 Due to frequently occurring antigenic changes with HPAI H5N1 viruses, there exists  
14 an epidemic or potential pandemic risk in poultry. A well-matched H5N1 vaccine is  
15 likely the effective measure to fight potential H5N1 panzootic. In this study, we  
16 generated the well-characterized *L.lactis*/pNZ8148-Spax-HA vaccine candidate based  
17 on the *L.lactis* display platform as it shows advantages over other vaccine approaches  
18 such as ease of genetic modification, efficient and cost-effective manufacturing,  
19 mucosal delivery route and proven safety and immunogenicity profile [19].

20 To define a more optimized vaccine approach, we investigated the immunogenicity of  
21 the unadjuvanted *L.lactis*/pNZ8148-Spax-HA via oral administration in the chicken  
22 model. It is accepted generally that the trimeric HA has strong immunogenicity.

1 Promising results from previous studies in chickens via injection route showing that  
2 adjuvanted subunit vaccines consisting of the trimeric H5N1 HA induced high levels  
3 of cross-neutralizing antibodies against H5N1 clade 1 and 2 [20, 21]. However, we  
4 demonstrated the unadjuvanted *L.lactis*/pNZ8148-Spax-HA in which HA gene without  
5 the signal and trimerization sequence performed convincing cross-clade protection  
6 following prime/boost oral vaccination. Further, the *L.lactis* displayed H5N1 vaccine  
7 candidate has advantage compared to the currently used influenza virus vaccines  
8 including the ease of generation of the vectors as well as the vaccine production in cell  
9 lines which are already approved for manufacturing of human vaccines [22]. In addition,  
10 the *L.lactis* displayed vaccines are efficacious without safety issues compared to the  
11 viral vectored approaches and do not require adjuvants [23]. Thus, the *L.lactis* displayed  
12 platform would have a beneficial effect on influenza vaccine manufacturing.

13 Anchor protein plays an important role in the *L.lactis* displayed vaccines. Our previous  
14 studies have shown that pgsA and Spax could be used as anchor proteins for antigen  
15 display [16, 18]. The *L.lactis*/pNZ8110-pgsA-HA1 adjuvanted with CTB provided  
16 immune protection against homologous H5N1 virus in a mouse model [16]. Also, the  
17 unadjuvanted *L.lactis*/pNZ8110-pgsA-NA and the unadjuvanted *L.lactis*/pNZ8008-  
18 Spax-HA2 could protect mice from homologous and heterologous virus infection [18,  
19 23]. Compare to the display efficiency via the anchor protein, we found that the Spax  
20 is superior over the pgsA in the *L.lactis* displayed influenza vaccines. Thus, in our  
21 present study, the Spax is chosen to design the *L.lactis* displayed H5N1 HA and  
22 unadjuvanted *L.lactis*/pNZ8148-Spax-HA conferred cross-clade protection against

1 different H5N1 viruses in the chicken model. Based on these findings, the *L.lactis*  
2 displayed platform via the Spax will contribute to providing feasibility in influenza  
3 H5N1 vaccine development for poultry against different H5N1 clades.

4 The rapid evolution of new sublineages of influenza A/H5N1 virus poses a great threat  
5 to poultry health [24]. A major obstacle in vaccine development against influenza H5N1  
6 virus infection is the rapid evolution of genetic diversity [25], as a result, newer vaccine  
7 approaches for panzootic preparedness against these viruses are needed, given the  
8 limitations of vaccines currently approved for H5N1 viruses in terms of their production  
9 timelines and the ability to induce cross-clade protective immune responses [26]. The  
10 availability of a simple and broadly protective vaccine for influenza H5N1 is a high  
11 priority in preparedness for a future influenza panzootic in poultry industry. The study  
12 demonstrated clearly that the unadjuvanted *L.lactis*/pNZ8148-Spax-HA could serve as  
13 an alternative approach for the development of H5N1 cross-clade vaccine in poultry for  
14 a mass vaccination, which is currently showing particular promise. Overall, the *L.lactis*  
15 displayed platform may contribute a new strategy for the development of the universal  
16 flu vaccine in poultry and human.

17

## 18 **Conclusions**

19 These findings suggest that the unadjuvanted *L.lactis* display platform can be  
20 considered as an alternative H5N1 cross-clade vaccine candidate to overcome the  
21 bottleneck of current manufacturing process, and provide a safe, effective and low cost  
22 production of H5N1 vaccine for poultry in a flexible and high output system during an

1 influenza H5N1 panzootic.

2

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5

### 6 **Authors' contributions**

7 All authors approved the manuscript. HL, QC, TG and XP contributed to study design

8 and data interpretation. All contributed to data analysis and results interpretation. HL

9 wrote the manuscript and produced all figures.

10

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15

### 16 **Availability of data and materials**

17 The datasets generated and analyzed during the current study are available from the

18 corresponding author on reasonable request.

19

### 20 **Ethics approval and consent to participate**

21 All animal studies complied with the Guidelines for Use and Care of Experimental

22 Animals and were approved by the Animal Committee of the Institute of Nanchang

1 University consent to participate.

2

### 3 **Competing interests**

4 All authors claim no conflict of interests.

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1 **Fig. 1. Characterization of the HA displayed on the surface of *L.lactis*.** (a) Schematic diagram  
2 of the *L.lactis*/pNZ8148-Spax-HA. A GS linker inserted between Spax and HA to stabilize the HA  
3 protein expression. (b) Western blot analysis. Lane 1: *L.lactis*/pNZ8148-Spax-HA; Lane 2: *L.lactis*-  
4 pNZ8148-Spax. (c) Immunofluorescence microscopy assay of the HA protein. *L.lactis*/pNZ8148-  
5 Spax (left) and *L.lactis*/pNZ8148-Spax-HA (right) (magnification: 1, 000×). (d) Flow cytometric  
6 analysis of the HA display on the surface of *L.lactis*. *L.lactis*/pNZ8148-Spax (left) and  
7 *L.lactis*/pNZ8148-Spax-HA (positive rate: 60.5%) (right). 15,000 cells were counted. (e)  
8 Quantification of the *L.lactis*/pNZ8148-Spax-HA expressing HA protein by indirect ELISA. The  
9 values were determined from three independent experiments. Bar indicates mean  $\pm$  SD.

10

11 **Fig.2. Determination of antibody responses elicited by the *L.lactis*/pNZ8148-Spax-HA in**  
12 **chickens.** Sera, feces and intestine washes were collected from chickens vaccinated orally with PBS,  
13 *L.lactis*/pNZ8148-Spax or *L.lactis*/pNZ8148-Spax-HA. (a) HA-specific IgG antibody responses in  
14 the sera. (b) HA-specific IgA antibody responses in the intestine washes. (c) HA-specific IgA  
15 antibody responses in the feces. (D) Neutralizing antibody titer. Data are represented as mean  $\pm$  SD.  
16 Asterisk indicates significant difference, as compared to PBS and the *L.lactis*/pNZ8148-Spax  
17 controls ( $p < 0.05$ )

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19 **Fig. 3. Cross-clade protection of chickens against lethal challenge with different H5N1 viruses.**  
20 The results are expressed in terms of percent body weight (a, b and c), lung virus titer (d, e and f)  
21 and percent survival (g, h and i). Two weeks after the last immunization, chickens were intranasally  
22 infected with 20  $\mu$ L of  $5 \times LD_{50}$  of clade 1.0: A/Vietnam/1203/2004 (a, d and g), clade 2.3:

1 A/Anhui/1/2005, Anhui (b, e and f) or clade 8.0: A/chicken/Henan/12/2004 (c, f and i) HPAI H5N1  
2 virus strains. Chickens were monitored throughout a 14-day observation period (n=5 / group). Data  
3 for lung virus titers are represented as mean  $\pm$  SD. Asterisk indicates significant difference, as  
4 compared to PBS and the *L.lactis*/pNZ8148-Spax controls ( $p < 0.05$ ).