Virus Research 263 (2019) 64–72

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Immune responses of mice inoculated with recombinant *Lactobacillus plantarum* NC8 expressing the fusion gene HA2 and 3M2e of the influenza virus and protection against different subtypes of influenza virus

Bo Feng[1], Wen-Tao Yang[1], Seria Masole Shonyela, Yu-Bei Jin, Ke-Yan Huang, Li-Na Shao, Cong Wang, You Zhou, Qiong-Yan Li, Yan-Long Jiang, Hai-Bin Huang, Chun-Wei Shi, Jian-Zhong Wang, Guan Wang, Yuan-Huan Kang, Gui-Lian Yang*, Chun-Feng Wang*

College of Animal Science and Technology, Jilin Provincial Engineering Research Center of Animal Probiotics, Key Laboratory of Animal Production and Product Quality Safety of Ministry of Education, Jilin Agricultural University, Changchun, China

ARTICLE INFO

Keywords:
Influenza virus
Lactobacillus plantarum
Oral immunization
Immune response

ABSTRACT

To evaluate the efficiency of preventing pathogenic avian influenza by vaccination with recombinant *Lactobacillus plantarum* (*L. plantarum*) that expresses conserved antigens, the mucosal and systemic immune responses in animals vaccinated with recombinant *L. plantarum* NC8-409-1 (NC8-pSIP409-pgsA'-HA2) and NC8-409-2 (NC8-pSIP409-pgsA'-3M2e-HA2) were evaluated. Our results showed that recombinant *L. plantarum* NC8-409-1 and NC8-409-2 could substantially stimulate the specific IgA titer in the intestine and the specific IgG antibody titer in the serum. We also found that recombinant *L. plantarum* induced increases in the number of $B220^+$ IgA^+ cells in Peyer's patches (PPs), in lymphocyte proliferation and in the number of IFN-γ^+ producing $CD8^+$ T cells after immunization in mice. Most importantly, the mice that were vaccinated with recombinant *L. plantarum* NC8-409-2 and NC8-409-1 were to some extent protected against infection challenge with the H9N2 and H1N1 viruses. In particular, NC8-409-2 provided up to 80% protection against the H9N2 virus, and NC8-409-1 provided up to 60% protection. Lung tissue pathology was also reduced. Therefore, recombinant *L. plantarum* NC8-409-2 and NC8-409-1 may be candidate oral vaccines against bird flu.

1. Introduction

Avian influenza virus (AIV) is a threat not only to the global poultry industry, where it has caused serious economic losses, but also to public health and safety as a zoonotic agent due to viral contamination in avian-derived food sand the risk of spread (Davis et al., 2015). So far, vaccines are the most effective means of controlling avian flu and have played an important role in decreasing morbidity and mortality in poultry (Yang et al., 2016). With the passage of time, each gene in the virus is likely to mutate, and some of the various influenza virus fragments are altered; therefore, the prevention and control of avian influenza is a very difficult task (Kim et al., 2013). Accordingly, many researchers are examining more effective and inexpensive possibilities for the development of new vaccine candidates, such as live vector vaccines using *L. plantarum* NC8 or baculovirus (Garcia-Crespo et al., 2013). At present, most avian influenza vaccines in use are inactivated vaccines, whose safety is guaranteed but whose immune effect is not ideal. In addition, with the antigen drift and conversion of avian influenza virus, increasing numbers of new subtypes of avian flu present the possibility of outbreaks (Kawase et al., 2010). Therefore, it is important to develop universal vaccines that can induce a wide range of heterologous immune protective effects against different subtypes of AIV (Lee et al., 2013). Avian influenza virus is an important pathogen that causes respiratory diseases, mainly infecting the body through the mucosal pathways, and the mucosal immune system therefore plays a key role in the control of avian influenza infection (Park et al., 2014). Accordingly, developing mucosal vaccine candidates based on these considerations is a reasonable approach (Stoner et al., 2012). At the same time, compared with the traditional vaccination route, mucosal vaccines can not only reduce the host gastrointestinal irritation but also the inflammatory response and can in addition induce the body to produce an effective mucosal immune response (Yang et al., 2017b).

* Corresponding authors at: College of Animal Science and Technology, Jilin Provincial Engineering Research Center of Animal Probiotics, Jilin Agricultural University, 2888 Xincheng Street, Changchun, 130118, China.
E-mail addresses: 4121324@qq.com (G.-L. Yang), wangchunfeng@jlau.edu.cn (C.-F. Wang).
[1] These authors contributed to the study equally.

https://doi.org/10.1016/j.virusres.2019.01.001
Received 22 May 2018; Received in revised form 2 January 2019; Accepted 2 January 2019
Available online 03 January 2019
0168-1702/

Since the surface proteins of the avian influenza virus are susceptible to mutations, vaccines against surface proteins of avian influenza viruses must be constantly updated to match the epidemic strains to provide effective protection (Smee et al., 2012). In contrast, vaccines targeting conserved antigens are able to provide effective protection. At present, HA2, located in the C-terminal stem of hemagglutinin (HA) in avian influenza virus, and the extracellular region M2e of the surface transmembrane protein2 (M2) are highly conserved and can induce not only the cellular immune response but also the cytotoxic lymphocyte (CTL) response (Gao et al., 2013).

Lactobacillus plantarum, a lactic acid bacterium, acts as a probiotic; first, it is normally present in human and animal intestines and does not cause any harm to the body (Schwarzer et al., 2016). Second, lactic acid bacteria can provide the body with a certain amount of nutrients and can inhibit the breeding of harmful bacteria to maintain the balance of the gastrointestinal tract, thereby improving gastrointestinal function (Jie, 2016). Finally, lactic acid bacteria can promote dendritic cell differentiation in the body, which has a certain impact on antigen presentation. In addition, upon the entry of external harmful substances into the intestine, lactic acid bacteria can help to activate macrophages, thereby promoting intestinal macrophage phagocytosis and enhancing the body's natural active immune response (Gao et al., 2017). Thus, lactic acid bacteria can simultaneously enhance the body's specific response and its non-specific reactions, so the use of lactic acid bacteria as a foreign protein delivery carrier is a very wise choice (Varela et al., 2017). *Lactobacillus* have been shown to have strong acceptance in healthy individuals and compatibility with various health problems (Martino et al., 2016). It has been known that certain *L. plantarum* strains have a probiotic effect on animal and human health (Khan and Kang, 2016).

Because the HA2 gene is highly conserved in various avian influenza viruses, it is suitable for the preparation of vaccine targets (Xiang-Jin Meng et al., 1999); meanwhile, although the M2e polypeptide itself has low immunogenicity, it can mediate heterologous antigenic immunity (Park et al., 2016). We previously expressed 3M2e-HA2 using *L. plantarum* fusion, and this fusion protein can be expressed on the surface of *L. plantarum*. Oral immunization of chicks with this recombinant lactic acid bacterium induced a strong immune response that provided good protection for H9N2 AIV-infected chicks (Accepted). To further evaluate the protective effect of recombinant lactic acid bacteria against different subtypes of influenza virus, in this study, we vaccinated mice with recombinant strains of *L. plantarum* NC8 expressing viral 3M2e-HA2. In addition, we then tested the immune indicators of the mice, challenged them with different subtypes of influenza virus, and tested the protective effect on the mice being challenged.

2. Materials and methods

2.1. Construction of recombinant lactic acid bacteria

In order to construct recombinant lactic acid bacteria, the HA2 (GenBank accession number MF620131) of A/Anthropoidesvirgo/Baicheng/219/2013 (H9N2) and 3M2e (GenBank accession number LC120394) of A/Puerto Rico/8/1934 (H1N1) were synthesized by Shanghai Generay Biotech Co., Ltd. (Shanghai, China). Then we digested by Xba I and Hind III and cloned HA2 and HA-3M2e into the pSIP-409-pgsA'-3M2e-HA2 vector, respectively. The positive plasmid was transformed into *Lactobacillus plantarum* strain NC8 (CCUG 61730). Sequencing and firm execution by Shanghai Generay Biotech Co., Ltd. (Shanghai, China).

2.2. Strains of bacteria and viruses

The recombinant *L. plantarum* NC8-pSIP409-pgsA', NC8-409-2, and NC8-409-1, the mouse-adapted H9N2 (A/duck/Xuzhou/07/2003) avian influenza virus and the H1N1 (A/PR/8/34) influenza virus were preserved in the Jilin Provincial Engineering Research Center of Animal Probiotics, Jilin Agricultural University.

2.3. Animals and ethics statement

The animals used in this experiment were purchased from Beijing HFK Biological Science Co., Ltd., and were 6-week-old to 8-week-old, pathogen-free female BALB/c mice. Feed and water were given randomly. The entire animal experiment meets the requirements of the Animal Management and Ethics Committee of Jilin Agricultural University.

2.4. Immunization

To evaluate the immunogenicity of the recombinant *L. plantarum* NC8-pSIP409-pgsA', NC8-409-2, and NC8-409-1, sixty-five BALB/c mice were divided into five groups. The groups of mice were orally immunized with NC8-409-1, NC8-409-2, and NC8-pSIP409-pgsA' suspensions at 2×10^8 colony-forming units (2×10^8 CFU in 200 µl saline) by gavage, and the control groups were administered 0.9% saline (200 µl) by the same method, except for the inactivated H9N2 inactivated vaccine mice, which received intramuscular injections (Fig. 1). The mice were first immunized on days 1, 2, and 3. During the second week, the mice received booster immunizationson days 12, 13, and 14.

2.5. Flow cytometry

Three mice from each group were euthanized 14 days after the final immunization in the vaccination period (Huang et al., 2017). The spleen, mesenteric lymph nodes (MLNs) and Peyer'spatches (PPs) were removed, and single-cell suspensions were prepared. Flow cytometry was performed as described previously (Yang et al., 2016). After preparing the single-cell suspensions from the MLNs and PPs, we transferred 10 µl of $B220^+$ antibodies (BD Biosciences, USA) into a tube containing 1×10^6 PPs, mixed well, and stained the cells at 4 °C for 30 min in the dark. We added 1 mL of phosphate-buffered saline (PBS) to the collected sample. The cell suspension was centrifuged at 2000 rpm and 4 °C for 5 min, and the supernatant was discarded. The above step was repeated. Then, the cells were fixed and permeabilized,

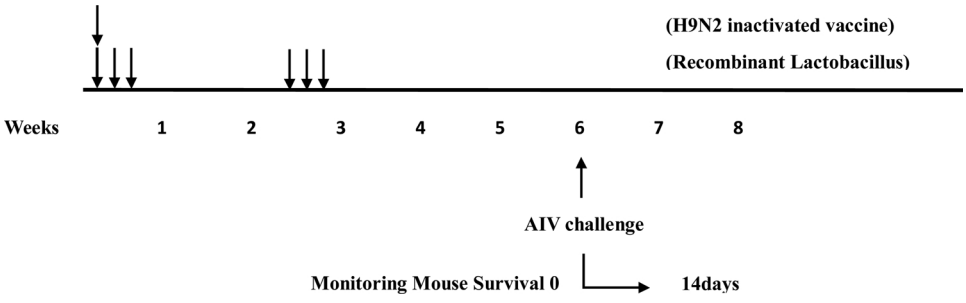

Fig. 1. The groups of mice were immunized with NC8-pSIP409-pgsA', NC8-409-1, NC8-409-2 (2×10^8 CFU in 200 µl saline), 0.9% saline (200 µl) or injected H9N2 inactivated vaccine. Immunization was performed thrice a week (days 1, 2, 3). The groups of mice were boosted thrice during the second week (days 12, 13, 14). The animals were challenged separately with mouse-adapted H9N2 virus and H1N1 virus. Injected H9N2 inactivated vaccine was administered by intramuscular injection on the first day. Survival status was recorded until the 8th week.

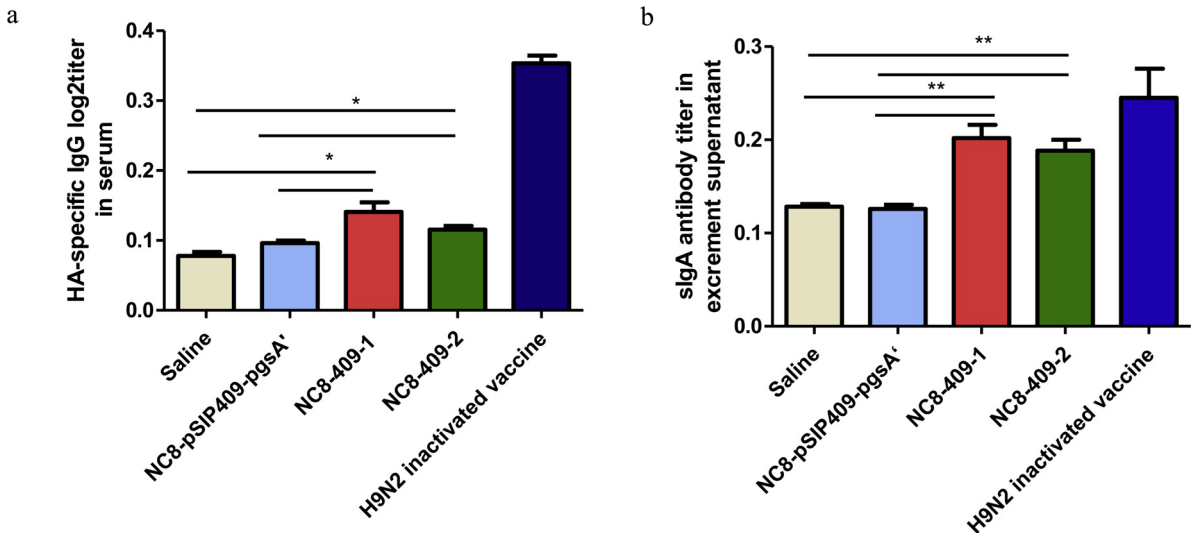

Fig. 2. Recombinant *L. plantarum* stimulates the body to produce specific antibodies. (a) IgG detection by ELISA. (b) sIgA detection by ELISA. The results are presented as the means ± SEM of triplicate tests (n = 3 mice per group) (*P < 0.05, **P < 0.01).

centrifuged twice, and then stained with 10 μL of IgA antibody (BD Biosciences, USA) by the same procedure described above at 4 °C for 30 min in the dark. The MLN single cells were transferred into 96-well cell culture plates, and PMA were added to induce cytokines for 4 h, after which inhibitors were added for 2 h. The cells were centrifuged twice, and 10 μl of $CD3^+$ and $CD4^+$ antibodies were added (BD Biosciences, USA). Then, the cells were fixed, perforated, centrifuged twice and combined with 10 μl of IFN-γ antibody (BD Biosciences, USA).The cells were analyzed using BD fluorescence-activated cell sorting (FACS) in an LSRFortessa™ cell analyzer (BD Biosciences, USA). All data were developed using FlowJo 7.6.2 software.

2.6. Antibody assay

Enzyme-linked immune sorbent assay (ELISA) was performed as described previously (Shi et al., 2014; Jin et al., 2017). In brief, 96-well immune plates were incubated with purified influenza antigens (100 μl) overnight at 4 °C, then blocked with 150 μl blocking fluid (PBST containing 10% bovine serum albumin) at 4 °C for 10 h. The plate was washed thrice with PBST. The serum was diluted 64 times, and the fecal supernatant was diluted 32 times. The diluted samples were added to the wells and incubated at 37 °C for 2 h, then washed thrice with PBST. After 3 washes, 100 μl of secondary antibody was added to each well and incubated at 37 °C for 1 h, followed by the addition of 50 μl of diluted streptavidin-HRP. Next, the wells were washed thrice with PBST. The chromogenic substrate was then added and the mixture allowed to stand at 37 °C for 30 min. Finally, the termination solution was added to terminate the reaction. The OD value was measured by a microplate reader. The endpoint titer was evaluated as the highest dilution yielding an absorbance two times higher than the background of the sample.

2.7. Lymphocyte proliferation

For this experiment, 2×10^5 MLN single cells in 20 μl were added to 96-well cell culture plates; each group consisted of three repetitions. Then, the 2 μg/ml specific antigen was added to each well, and the plates were incubated in a cell incubator at 37 °C for 72 h. Next, 10 μl of MTS was added to each well, and the plates were incubated at 37 °C for 4 h. The absorbance (OD492 nm) was measured using a microplate reader. Stimulation (SI) was used to assess the murine lymphocyte proliferation capacity. The formula for calculating the MTT stimulation index is: SI = (Experimental group OD value - blank group OD value) / (negative control group OD value - blank group OD value).

2.8. Viral challenge

At the sixth week, all the mice were injected intraperitoneally with 15 mg/kg mebubarbital (Avertin, Sigma, USA). The mice were then challenged with 10 × LD_{50} of mouse-adapted H9N2 (A/duck/Xuzhou/07/2003) AIV or 5 × LD_{50} of H1N1 (A/PR/8/34) influenza virus by the nasal cavity (Fig. 1).The body weight and survival were evaluated for two weeks.

2.9. Histopathological examination

To assess the pathological damage to the lungs of mice caused by influenza virus, we stained paraffin sections of mouse lung tissue with HE. Fourteen days after the challenge, we euthanized the mice and removed the lungs. The lungs of the mice were then embedded in paraffin and sectioned, and the sections were stained with hematoxylin and eosin and scored. The scoring method was 2 points for inflammatory cell infiltration, 1 point for bleeding points, and 1 for alveolar incompleteness. It was rated by three people blindly

2.10. Statistical analysis

All data in the experiment were from at least three independent experiments, expressed as the mean ± SEM. Differences were tested using Graph-Pad Prism 5.0 software. P values < 0.05 were considered to represent significant differences. Significance was assessed using one-way analysis of variance (ANOVA; Tukey's multiple comparison test).

3. Results

3.1. Recombinant lactobacillus induces specific antibodies in serum

To determine whether the immunogenicity of recombinant *L. plantarum* could stimulate the body to produce specific antibodies, we employed ELISA to test IgG in the serum. Mice orally immunized with NC8-409-2 and NC8-409-1 had clearly higher IgG antibody than the mice that were orally administered saline and NC8-pSIP409-pgsA'. This result proved that recombinant *L. plantarum* can stimulate the body to produce specific antibodies (Fig. 2a).

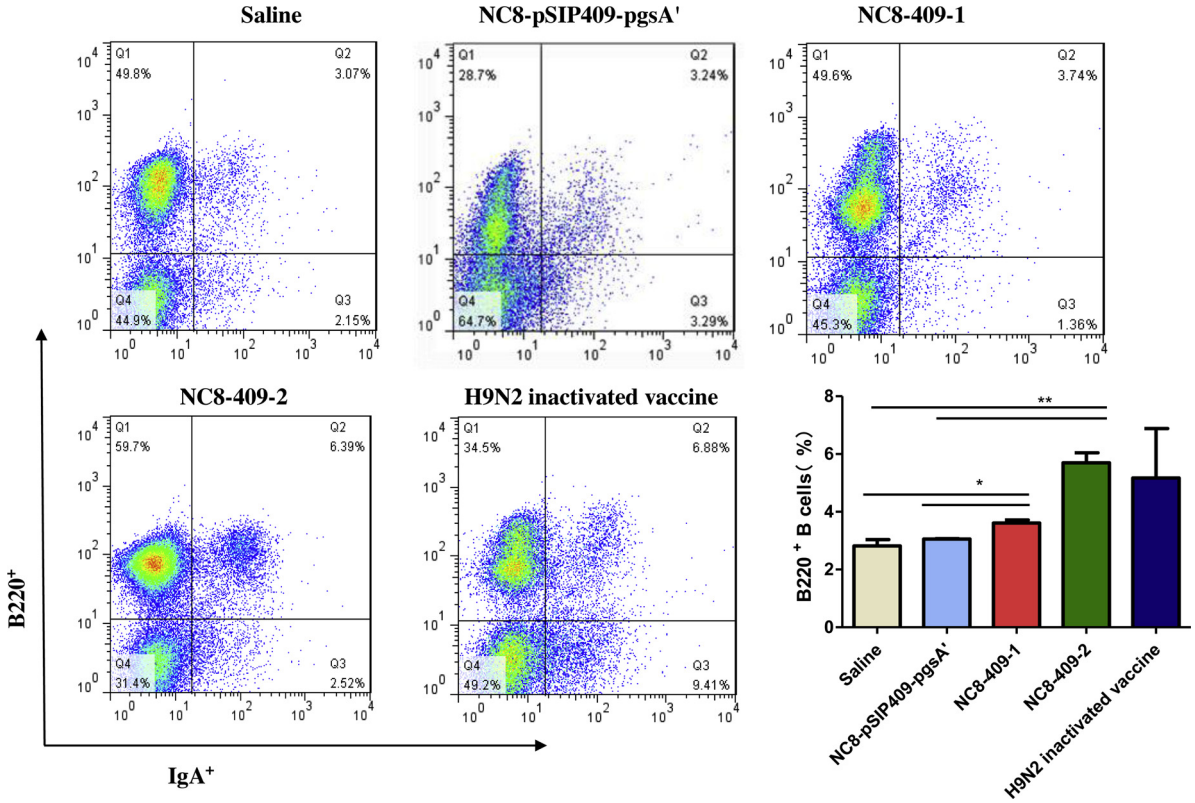

Fig. 3. Four weeks after booster immunization, the number of $B220^+$ IgA^+ cells in PPs was detected by flow cytometry. The results are presented as the means ± SEM of triplicate tests (n = 3 mice per group) (*P < 0.05, **P < 0.01).

3.2. Recombinant lactobacillus induces IgA antibodies in the feces of mice

To investigate whether recombinant *L. plantarum* can induce body mucosal immunity, we used ELISA to detect IgA in the feces of mice. We found which consisted of oral immunization with recombinant *L. plantarum*, the sIgA titers were higher than in the mice that received oral immunization with physiological saline and NC8-pSIP409-pgsA'. This result proved that recombinant *L. plantarum* can stimulate the body to produce a mucosal immune response. However, the process takes some time (Fig. 2b).

3.3. Immunization-induced mouse B cell response

To assess the effect of recombinant *L. plantarum* on mouse B cells in PPs, 2 weeks after the booster immunization, the number of $B220^+$ IgA^+ cells in the mouse small intestine PPs was measured. The results showed that, compared with the group that received saline and NC8-pSIP409-pgsA, the mice immunized with recombinant *L. plantarum* showed a significant increase in the percentage of $B220^+$ IgA^+ cells. However, the group that received the oral administration of recombinant *L. plantarum* NC8-409-2 had the highest percentage (Fig. 3).

3.4. Effect of lymphocyte proliferation

To investigate the effect of oral *L. plantarum* lymphocyte proliferation, we detected the SI value of lymphocyte proliferation. The results shown that, compared with the group that received saline and NC8-pSIP409-pgsA', the mice that received oral administration of recombinant lactic acid bacteria exhibited significantly higher lymphocyte proliferation (Fig. 4).

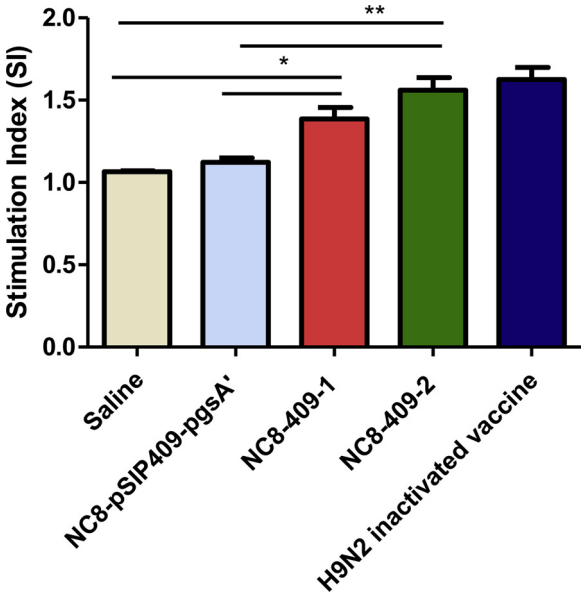

Fig. 4. Recombinant lactate stimulates lymphocyte proliferation. The results are presented as the means ± SEM of triplicate tests (n = 3 mice per group) (*P < 0.05, **P < 0.01).

3.5. Expression of specific cytokines in mice after immunization

To detect the IFN-γ in $CD8^+$ T cells, we used flow cytometry to measure the frequencies of INF-γ-producing $CD8^+$ T cells in mouse MLNs. Compared with the group that received saline and NC8-pSIP409-pgsA', the mice that received recombinant *L. plantarum* exhibited significantly increased frequencies of INF-γ-producing $CD8^+$ T cells. However, the group that received oral administration of recombinant *L.*

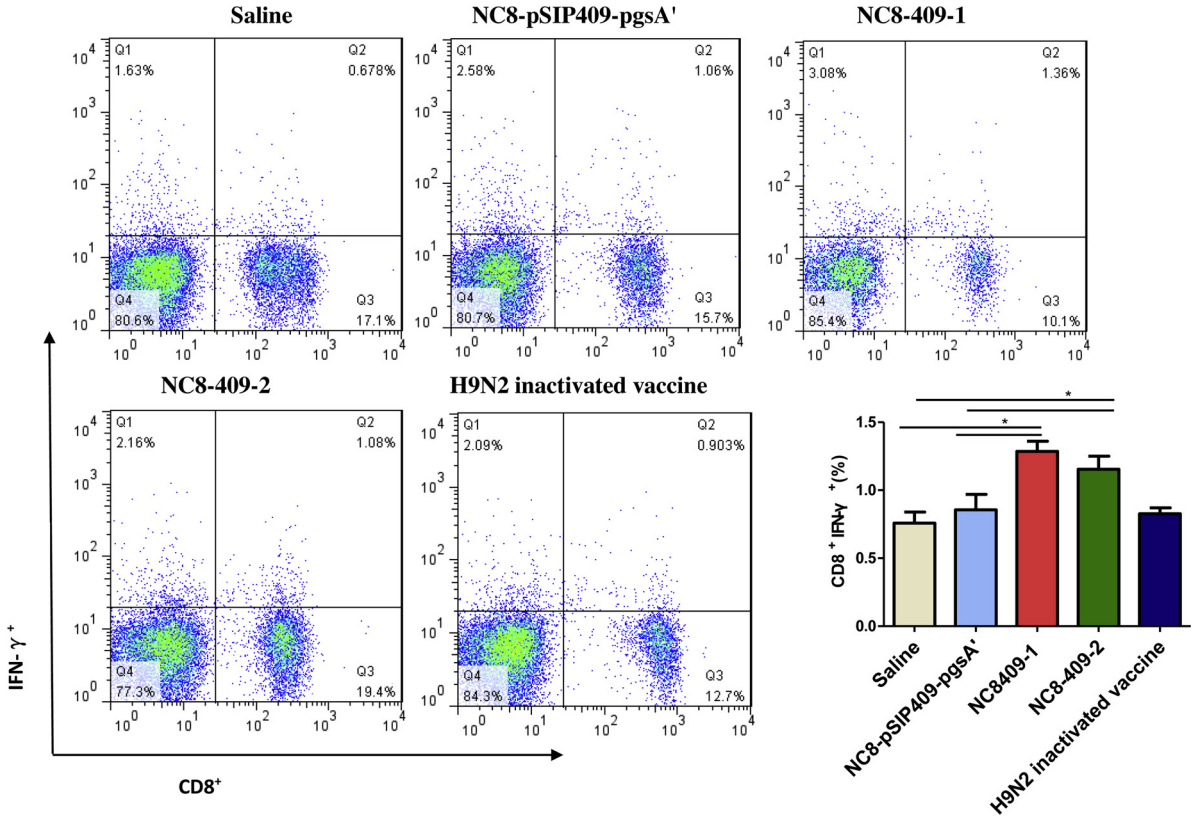

Fig. 5. Four weeks after the end of immunization, the CD4$^+$ IFN-γ$^+$ content in MLNs was tested by flow cytometry. The results are presented as the means ± SEM of triplicate tests (n = 3 mice per group) (*P < 0.05).

plantarum NC8-409-1 had the highest percentage (Fig. 5).

3.6. Protection of the mice challenged with influenza virus

To prove that NC8-409-2 and NC8-409-1 can provide protection against the H9N2 virus, we challenged mouse groups with the virus 3 weeks after final immunization (Fig. 1). Then, we recorded the weight loss for 14 d and the mortality rate. The body weight loss rate of all mice immunized with recombinant *L. plantarum* NC8-409-2 and NC8-409-1 was significantly better than in the groups that received the empty vector and saline. The results also showed the group of NC8-409-2 compared with the group that received the H9N2 inactivated vaccine, their weight loss rate had no significant difference. But it compared with the group of saline, their weight loss rate had significant difference (Fig. 6a). We also recorded the survival of the mice. The mice that received oral recombinant *L. plantarum* had survival rates of approximately 60 % and 80%. In particular, the group of mice that received oral *L. plantarum* NC8-409-2 exhibited approximately 80% protection in terms of survival rate and did not die within 14 days. However, the survival rates of the groups that received empty vector and saline were 40% and 0%, and the group that received the H9N2 inactivated vaccine showed similar results to the groups that received NC8-409-2 and NC8-409-1 (Fig. 6b). In contrast, the mice in the control group that were administered saline all died within 5 days. Meanwhile, 100% survival was observed for the group immunized with inactivated vaccine (Fig. 6b). To test the protective effect of *L. plantarum* NC8-409-2 and NC8-409-1 on pathological changes in the lung in mice, we observed mouse lungs by scoring pathological sections after challenge with the H9N2 virus. The histopathological score of the group that received *L. plantarum* was higher than that of the saline group or the empty vector group and at was similar to the score of the mice injected with the vaccine (Fig. 8a.b).

To prove that NC8-409-2 and NC8-409-1 can provide protection against the H1N1 virus, we challenged mice with the virus at 3 weeks after final immunization (Fig. 1). Then, we recorded the weight loss for 14 d and the mortality rate. The body weight loss rate of all mice immunized with recombinant *L. plantarum* showed that both the NC8-409-2 and NC8-409-1 groups had significantly better results than the groups that received empty vector and saline. The results showed that compared with that of the mice that received the H9N2 inactivated vaccine, their weight loss rate is basically the same (Fig. 7a). In the same way, we recorded the survival of the mice, and the mice that received oral recombinant *L. plantarum* had a similar survival rate to the mice injected with the vaccine. However, the groups that received the empty vector and saline had a survival rate of only 0% (Fig. 7b). The survival percentage results indicated that the mice immunized with recombinant NC8-409-2 obtained 40% protection and did not die for up to 14 days. In contrast, the mice in the control group administered saline all died within 6 days. Meanwhile, 20% survival was observed for the group immunized with the inactivated vaccine (Fig. 7b). To test the protective effect of *L. plantarum* NC8-409-2 and NC8-409-1 against pathological changes in the lung in mice, we observed the pathological changes in mouse lungs by making pathological sections after challenge with the H1N1 virus and scoring them. The histopathological score of the *L. plantarum* group was higher than that of the saline group and the empty vector group and was similar to the score of the mice injected with the vaccine (Fig. 9a.b).

4. Discussion

In recent years, the livestock and poultry industry has developed rapidly (Feagins et al., 2008). However, AIV is the most important challenge for the progress of the livestock sector (Nagy et al., 2016). Prophylactic vaccination against AIV can help prevent animal diseases, and different vaccination strategies are often applied to combat AIV infection (Bavinck et al., 2009).

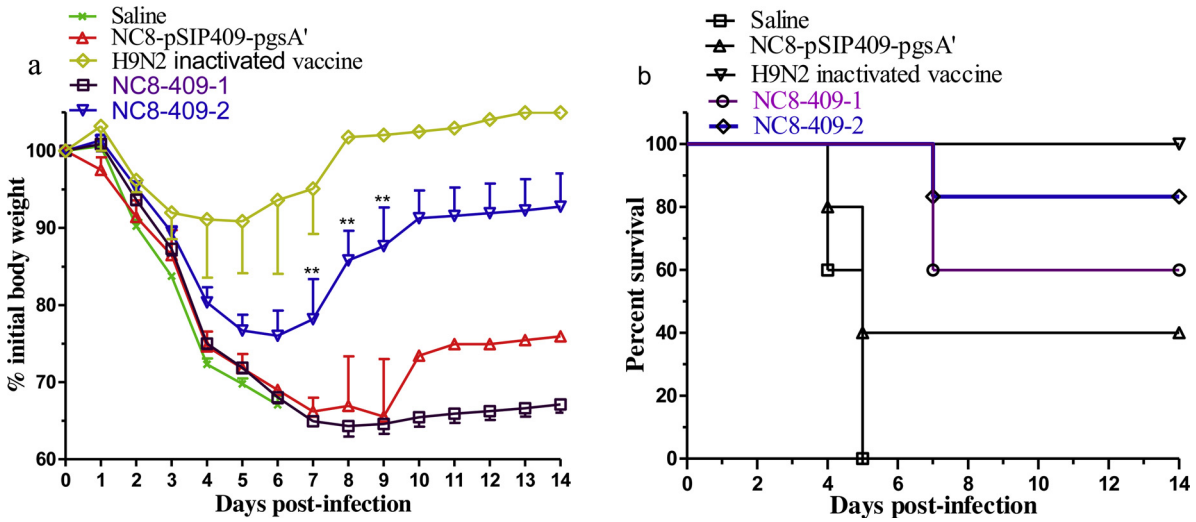

Fig. 6. In the 6th week, BALB/c mice were challenged with H9N2 (A/duck/Xuzhou/07/2003) virus (10 × LD_{50}), and the subsequent body weight (a) and survival (b) were recorded. The results are presented as the means ± SEM of triplicate tests (n = 5 mice per group) (*P < 0.05, **P < 0.01).

The immunization of mice with different recombinant *L. plantarum* was revealed to confer survival benefits following influenza virus infection (Yang et al., 2016). Accordingly, we proposed that immunization with recombinant *L. plantarum* NC8-409-2 would develop protective efficacy.

This study showed that recombinant *L. plantarum* had a comparable antiviral effect against orally administered influenza virus infection, conferring survival protection and preventing considerable weight loss. In a previously reported antiviral protection study, mice treated with the same *L. plantarum* showed varying degrees of protective efficacy after viral challenge; therefore, in our study, we have demonstrated multiple antiviral results of recombinant NC8-409-2 and NC8-409-1, including the prevention of illness as noted by decreased weight losses in animals after challenge with influenza viruses as well as 80% survival protection against H9N2 AIV. However, for the H1N1 virus, the weight loss rate of the recombinant lactic acid bacteria group was better than the group of the H9N2 inactivated vaccine. In terms of protection rate, the protection rate of NC8-409-2 was better than the group of the H9N2 inactivated vaccine. Recombinant NC8-409-2 and NC8-409-1 have been previously shown to be safe and effective vaccines for influenza (Cai et al., 2016). In this study, IgG and mucosal SIgA antibody responses were identified by ELISA and flow cytometry, and the results showed that recombinant NC8-pSIP409-pgsA can induce a significant level of systemic IgG and mucosal SIgA antibody responses, indicating a potential vaccine strategy against AIV infection. Meanwhile, the IgA response increased rapidly after a booster immunization. Secretory IgA antibodies are considered to be the most important component produced in the inherent immune defense of the respiratory mucosa (Jiang et al., 2017).

Recombinant *L. plantarum* with only the HA2 protein have been shown to elicit highly efficient protective immune responses, and recombinant *L. plantarum* containing both the 3M2e and HA2 proteins has, in some studies, showed the same result in immune responses (Shi et al., 2016).

In addition, the study by Youn, Kobayashi and their colleagues and the current study by Shonyela and her colleagues reported that a range of *Lactobacillus* species showed different degrees of protective efficacies and that the dosage and route of administration are important factors in ensuring flu survival (Yang et al., 2017a). We strongly believe that weight changes and survival rates are important in the assessment of infection and of the protective effects of vaccines. The results of this study indicate that immunization with *L. plantarum* NC8 can prevent weight loss and death in mice. As shown by the results of our study, immunization with recombinant *L. plantarum* improved the cytokine IFN-γ results in response to immunization, which was a similar result to that of the study by (Yang et al., 2017c). Our study shows that *L. plantarum* can enhance the activation of cytotoxic $CD8^+$ T cells and enhance the body's adaptive immunity, thereby enhancing the

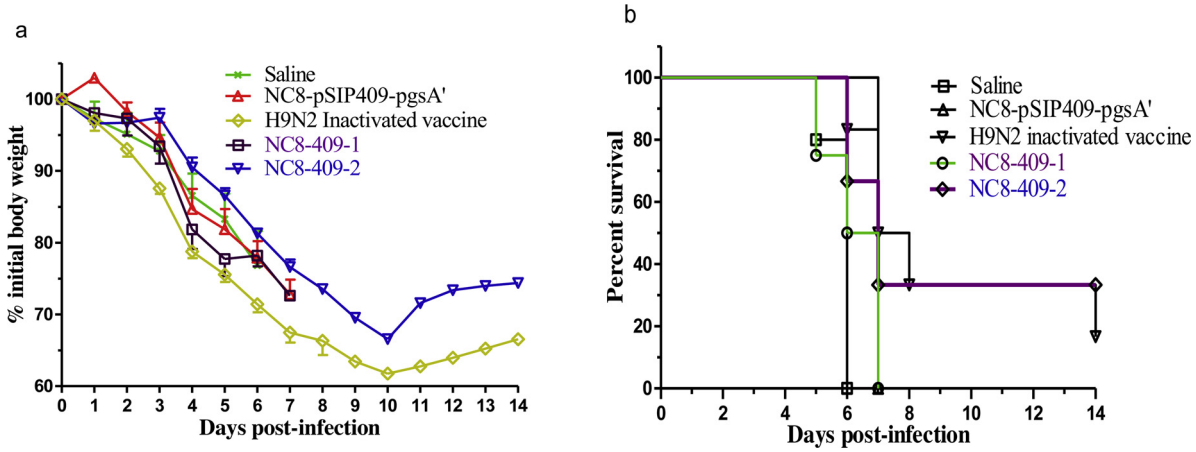

Fig. 7. In the 6th week, BALB/c mice were challenged with H1N1 (A/PR/8/34) influenza virus (5 × LD_{50}), and the subsequent body weight (a) and survival (b) were recorded. The results are presented as the means ± SEM of triplicate tests (n = 5 mice per group).

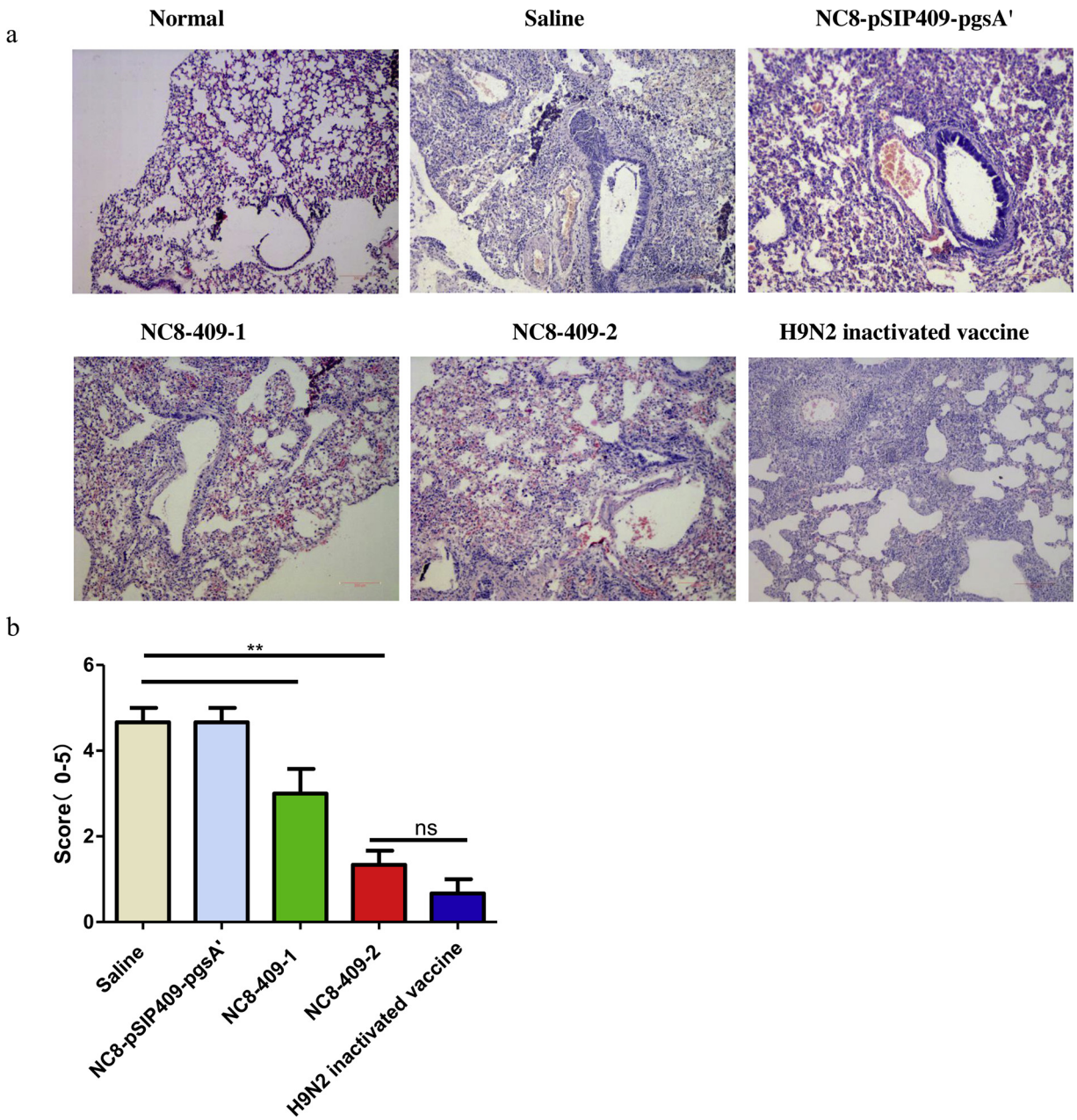

Fig. 8. On the 14th day of the challenge with the H9N2 virus, mice were sacrificed, and the lungs were removed to obtain pathological tissue sections. (a) Pathological tissue section. (b) Scoring of the pathological changes. The results are presented as the means ± SEM of triplicate tests (n = 5 mice per group) (*P < 0.05, **P < 0.01).

regulation of the Th1 immune response. Several studies have also proved that *L. plantarum* can improve the body's immune function (Lu et al., 2016). We observed that oral immunization with NC8-409-2 and NC8-409-1 can significantly elicit IFN-γ production. IFN-γ produced by Th1 lymphocytes plays an important role in the activation of Th1 responses to viral infections. This study proved that the IFN-γ levels in the NC8-409-2 and NC8-409-1 groups were significantly higher than in the control group, which was consistent with previous studies showing that most probiotic bacterial strains can induce IFN-γ (Kang et al., 2011).

In summary, *L. plantarum* have been employed as delivery vehicles against various foreign microorganisms (Liu et al., 2017). The administration of recombinant *L. plantarum* NC8-409-2 offers protection against infection by influenza viruses, perhaps by enhancing immunity. The results of flow cytometry showed that NC8-409-2 and NC8-409-1 could induce intensive favorable immunogenic responses to HA2. The histopathology of the animals used in this study showed that immunization with recombinant *L. plantarum* NC8-409-2 helped to prevent virus-induced lung inflammation. Therefore, immunization with recombinant *L. plantarum* NC8-409-2 prior to infection considerably enhanced protection against AIV.

Additional information

The authors declare that no competing financial interests exist.

Acknowledgments

This work was supported by the National Key Research and Development Program of China (2017YFD0501000, 2017YFD0501200), National Natural Science Foundation of China (31672528, 31602092), Science and Technology Development Program of Jilin Province (20160519011JH, 20170204034NY,

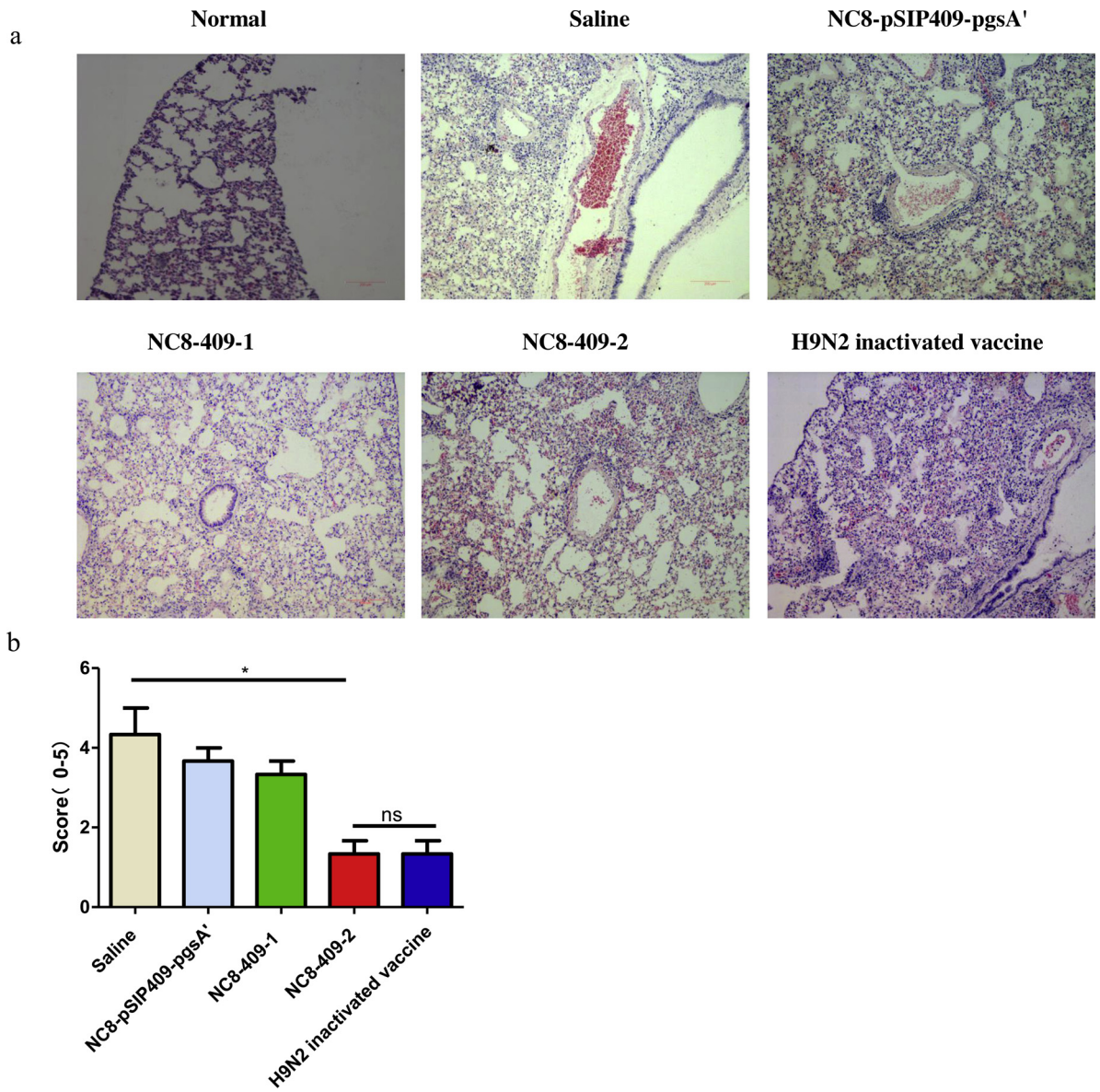

Fig. 9. On the 14th day of the challenge with the H1N1 virus, mice were sacrificed, and the lungs were removed to obtain pathological tissue sections. (a) Pathological tissue section. (b) Scoring of the pathological changes. The results are presented as the means ± SEM of triplicate tests (n = 5 mice per group) (*P < 0.05).

20180520037JH), Special Funds for Industrial Innovation of Jilin Province (2016C063), "Thirteen Five-year Plan" for Sci & Tech Research Program of Jilin Education Department of P.R. China (JJKH20170318KJ).

References

Bavinck, V., Bouma, A., Boven, M.V., Bos, M.E.H., Stassen, E., Stegeman, J.A., 2009. The role of backyard poultry flocks in the epidemic of highly pathogenic avian influenza virus (H7N7) in the Netherlands in 2003. Prev. Vet. Med. 88, 247.

Cai, R., Jiang, Y., Yang, W., Yang, W., Shi, S., Shi, C., Hu, J., Gu, W., Ye, L., Zhou, F., Gong, Q., Han, W., Yang, G., Wang, C., 2016. Surface-displayed IL-10 by recombinant Lactobacillus plantarum reduces Th1 responses of RAW264.7 cells stimulated with poly(I:C) or LPS. J. Microbiol. Biotechnol. 26, 421–431.

Davis, A.S., Taubenberger, J.K., Bray, M., 2015. The use of nonhuman primates in research on seasonal, pandemic and avian influenza, 1893–2014. Antiviral Res. 117, 75–98.

Feagins, A.R., Opriessnig, T., Huang, Y.W., Halbur, P.G., Meng, X.J., 2008. Cross-species infection of specific-pathogen-free pigs by a genotype 4 strain of human hepatitis E virus. J. Med. Virol. 80, 1379–1386.

Gao, R., Cao, B., Hu, Y., Feng, Z., Wang, D., Hu, W., Chen, J., Jie, Z., Qiu, H., Xu, K., Xu, X., Lu, H., Zhu, W., Gao, Z., Xiang, N., Shen, Y., He, Z., Gu, Y., Zhang, Z., Yang, Y., Zhao, X., Zhou, L., Li, X., Zou, S., Zhang, Y., Li, X., Yang, L., Guo, J., Dong, J., Li, Q., Dong, L., Zhu, Y., Bai, T., Wang, S., Hao, P., Yang, W., Zhang, Y., Han, J., Yu, H., Li, D., Gao, G.F., Wu, G., Wang, Y., Yuan, Z., Shu, Y., 2013. Human infection with a novel avian-origin influenza A (H7N9) virus. N. Engl. J. Med. 368, 1888–1897.

Gao, M.L., Hou, H.M., Teng, X.X., Zhu, Y.L., Hao, H.S., Zhang, G.L., 2017. Microbial diversity in raw milk and traditional fermented dairy products (Hurood cheese and Jueke) from Inner Mongolia, China. Genet. Mol. Res.: GMR 16.

Garcia-Crespo, K.E., Chan, C.C., Gabryszewski, S.J., Percopo, C.M., Rigaux, P., Dyer, K.D., Domachowske, J.B., Rosenberg, H.F., 2013. Lactobacillus priming of the respiratory tract: heterologous immunity and protection against lethal pneumovirus infection. Antiviral Res. 97, 270–279.

Huang, K.Y., Yang, G.L., Jin, Y.B., Liu, J., Chen, H.L., Wang, P.B., Jiang, Y.L., Shi, C.W., Huang, H.B., Wang, J.Z., 2017. Construction and immunogenicity analysis of Lactobacillus plantarum expressing a porcine epidemic diarrhea virus S gene fused to a DC-targeting peptide. Virus Res S0168170217307608.

Jiang, Y., Yang, G., Wang, Q., Wang, Z., Yang, W., Gu, W., Shi, C., Wang, J., Huang, H., Wang, C., 2017. Molecular mechanisms underlying protection against H9N2 influenza virus challenge in mice by recombinant Lactobacillus plantarum with surface displayed HA2-LTB. J. Biotechnol. 259, 6–14.

Jin, Y.B., Yang, W.T., Huang, K.Y., Chen, H.L., Shonyela, S.M., Liu, J., Liu, Q., Feng, B., Zhou, Y., Zhi, S.L., 2017. Expression and purification of swine RAG2 in E. coli for production of porcine RAG2 polyclonal antibodies. Biosci. Biotechnol. Biochem. 26, 1.

Jie, L.X., 2016. Optimization of bacteriocin production by Lactobacillus plantarum LF1. International Conference on Medicine and Biopharmaceutical. pp. 1218–1225.

Kang, S.S., Ryu, Y.H., Baik, J.E., Yun, C.H., Lee, K., Chung, D.K., Han, S.H., 2011. Lipoteichoic acid from Lactobacillus plantarum induces nitric oxide production in the presence of interferon-gamma in murine macrophages. Mol. Immunol. 48, 2170–2177.

Kawase, M., He, F., Kubota, A., Harata, G., Hiramatsu, M., 2010. Oral administration of lactobacilli from human intestinal tract protects mice against influenza virus infection. Lett. Appl. Microbiol. 51, 6–10.

Khan, I., Kang, S.C., 2016. Probiotic potential of nutritionally improved Lactobacillus plantarum DGK-17 isolated from Kimchi – a traditional Korean fermented food. Food Control 60, 88–94.

Kim, M.C., Lee, J.S., Kwon, Y.M., O, E, Lee, Y.J., Choi, J.G., Wang, B.Z., Compans, R.W., Kang, S.M., 2013. Multiple heterologous M2 extracellular domains presented on virus-like particles confer broader and stronger M2 immunity than live influenza A virus infection. Antiviral Res. 99, 328–335.

Lee, Y.N., Youn, H.N., Kwon, J.H., Lee, D.H., Park, J.K., Yuk, S.S., Erdene-Ochir, T.O., Kim, K.T., Lee, J.B., Park, S.Y., Choi, I.S., Song, C.S., 2013. Sublingual administration of Lactobacillus rhamnosus affects respiratory immune responses and facilitates protection against influenza virus infection in mice. Antiviral Res. 98, 284–290.

Liu, Y.Y., Yang, W.T., Shi, S.H., Li, Y.J., Zhao, L., Shi, C.W., Zhou, F.Y., Jiang, Y.L., Hu, J.T., Gu, W., Yang, G.L., Wang, C.F., 2017. Immunogenicity of recombinant Lactobacillus plantarum NC8 expressing goose parvovirus VP2 gene in BALB/c mice. J. Vet. Sci. 18, 159–167.

Lu, W., Chen, S., Lai, C., Lai, M., Fang, H., Dao, H., Kang, J., Fan, J., Guo, W., Fu, L., Andrieu, J.M., 2016. Suppression of HIV replication by CD8(+) regulatory T-cells in elite controllers. Front. Immunol. 7, 134.

Martino, M.E., Bayjanov, J.R., Caffrey, B.E., Wels, M., Joncour, P., Hughes, S., Gillet, B., Kleerebezem, M., van Hijum, S.A.F.T., Leulier, F., 2016.

Nagy, A., Lee, J., Mena, I., Henningson, J., Li, Y., Ma, J., Duff, M., Li, Y., Lang, Y., Yang, J., Abdallah, F., Richt, J., Ali, A., Garcia-Sastre, A., Ma, W., 2016. Recombinant Newcastle disease virus expressing H9 HA protects chickens against heterologous avian influenza H9N2 virus challenge. Vaccine 34, 2537–2545.

Park, J.K., Lee, D.H., Cho, C.H., Yuk, S.S., To, E.O., Kwon, J.H., Noh, J.Y., Kim, B.Y., Choi, S.W., Shim, B.S., Song, M.K., Lee, J.B., Park, S.Y., Choi, I.S., Song, C.S., 2014. Supplementation of oil-based inactivated H9N2 vaccine with M2e antigen enhances resistance against heterologous H9N2 avian influenza virus infection. Vet. Microbiol. 169, 211–217.

Park, S., Lee, I., Kim, J.I., Bae, J.Y., Yoo, K., Kim, J., Nam, M., Park, M., Yun, S.H., Cho, W.I., Kim, Y.S., Ko, Y.Y., Park, M.S., 2016. Effects of HA and NA glycosylation pattern changes on the transmission of avian influenza A(H7N9) virus in guinea pigs. Biochem. Biophys. Res. Commun. 479, 192–197.

Schwarzer, M., Makki, K., Storelli, G., Machucagayet, I., Srutkova, D., Hermanova, P., Martino, M.E., Balmand, S., Hudcovic, T., Heddi, A., 2016. Lactobacillus plantarum strain maintains growth of infant mice during chronic undernutrition. Science 351, 854–857.

Shi, S.H., Yang, W.T., Yang, G.L., Cong, Y.L., Huang, H.B., Wang, Q., Cai, R.P., Ye, L.P., Hu, J.T., Zhou, J.Y., Wang, C.F., Li, Y., 2014. Immunoprotection against influenza virus H9N2 by the oral administration of recombinant Lactobacillus plantarumNC8 expressing hemagglutinin in BALB/c mice. Virology 464–465, 166–176.

Shi, S.-H., Yang, W.-T., Yang, G.-L., Zhang, X.-K., Liu, Y.-Y., Zhang, L.-J., Ye, L.-P., Hu, J.-T., Xin, X., Qi, C., Li, Y., Wang, C.-F., 2016. Lactobacillus plantarum vaccine vector expressing hemagglutinin provides protection against H9N2 challenge infection. Virus Res. 211, 46–57.

Smee, D.F., Julander, J.G., Tarbet, E.B., Gross, M., Nguyen, J., 2012. Treatment of oseltamivir-resistant influenza A (H1N1) virus infections in mice with antiviral agents. Antiviral Res. 96, 13–20.

Stoner, T.D., Krauss, S., Turner, J.C., Seiler, P., Negovetich, N.J., Stallknecht, D.E., Frase, S., Govorkova, E.A., Webster, R.G., 2012. Susceptibility of avian influenza viruses of the N6 subtype to the neuraminidase inhibitor oseltamivir. Antiviral Res. 93, 322–329.

Varela, M., Figueras, A., Novoa, B., 2017. Modelling viral infections using zebrafish: innate immune response and antiviral research. Antiviral Res. 139, 59–68.

Xiang-Jin Meng, S.D., Engle, Ronald E., Friendship, Robert, Lyoo, Young S., Theerapol Sirinarumitr, K.U., Wang, Dong, Wong, Doris, Yoo, Dongwan, Yanjin Zhang, R.H.P., Emerson, Suzanne U., 1999. Prevalence of antibodies to the hepatitis E virus in pigs from countries where hepatitis E is common or is rare in the human population. J. Med.

Yang, W.T., Shi, S.H., Yang, G.L., Jiang, Y.L., Zhao, L., Li, Y., Wang, C.F., 2016. Cross-protective efficacy of dendritic cells targeting conserved influenza virus antigen expressed by Lactobacillus plantarum. Sci. Rep. 6, 39665.

Yang, G., Yao, J., Yang, W., Jiang, Y., Du, J., Huang, H., Gu, W., Hu, J., Ye, L., Shi, C., Shan, B., Wang, C., 2017a. Construction and immunological evaluation of recombinant Lactobacillus plantarum expressing SO7 of Eimeria tenella fusion DC-targeting peptide. Vet. Parasitol. 236, 7–13.

Yang, W.T., Yang, G.L., Wang, Q., Huang, H.B., Jiang, Y.L., Shi, C.W., Wang, J.Z., Huang, K.Y., Jin, Y.B., Wang, C.F., 2017b. Protective efficacy of Fc targeting conserved influenza virus M2e antigen expressed by Lactobacillus plantarum. Antiviral Res. 138, 9–21.

Yang, W.T., Yang, G.L., Yang, X., Shonyela, S.M., Zhao, L., Jiang, Y.L., Huang, H.B., Shi, C.W., Wang, J.Z., Wang, G., Zhao, J.H., Wang, C.F., 2017c. Recombinant Lactobacillus plantarum expressing HA2 antigen elicits protective immunity against H9N2 avian influenza virus in chickens. Appl. Microbiol. Biotechnol. 101, 8475–8484.