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The recombinant vaccine of *Lactobacillus plantarum* elicits immune protection against H1N1 and H9N2 influenza virus infection

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Abstract

Influenza A virus (IAV) causes annual epidemics and occasional pandemics, resulting in significant economic losses and numerous fatalities. Current vaccines, typically administered through injection, provide limited protection due to the frequent antigenic shift and drift of IAV strains. Therefore, the development of alternative broad-spectrum vaccine strategies is imperative. Lactic acid bacteria (LAB) represent promising candidates for vaccine engineering due to their low cost, high safety profile, and suitability for oral administration. In this study, we identified a strain of *Lactobacillus plantarum* (Lp) that is resistant to acid and bile salts and capable of colonizing the intestines of mice. Subsequently, we employed the RecE/T gene editing system to integrate headless hemagglutinins (*mini-HA*) into the genome of Lp, generating Lp-mini-HA-SP. Remarkably, immunization with Lp-mini-HA-SP elicited serum IgG antibody responses and conferred immune protection against H9N2 and H1N1 influenza virus challenges. Collectively, our findings offer a novel approach for the development of orally administered IAV vaccines and hold significant potential for future drug development endeavors.

Keywords: Influenza virus; *Lactobacillus plantarum*; mini-HA

1. Introduction

Influenza A virus (IAV) is indeed a significant respiratory pathogen that poses a threat to both humans and animals, leading to seasonal epidemics and potential pandemics [1-2]. Vaccination remains a crucial strategy in preventing infections caused by IAV, but due to the constant antigenic changes in the virus through shifts and drifts, the effectiveness of current vaccines is limited [3]. This limitation has highlighted the urgent need for the development of a universal vaccine that can target various subtypes of IAV.

The hemagglutinin (HA) which locates in the surface of IAV virions is the primary antigen recognized by the host and induces the immune responses [4]. The HA is composed of head domain and HA stalk domain (mini-HA). Among which, the mini-HA is highly conserved between different subtypes of IAV, and many antigen sites are retained within mini-HA. Meanwhile, the mini-HA expressed from *E. coli* or insect cells has been shown to provide immune protection against infection of multiple strains of IAV [5-6]. Therefore, mini-HA has the potential to be the targeting antigen for developing the broad-spectrum vaccines against IAV.

The administration of vaccine usually depends on the injection [3, 7], which requires a complex process and extra cost. The development of oral influenza virus vaccine would simplify the way of immunization and improve the efficiency of animal vaccination, suggesting that alternative vaccine expression platforms need to be developed. Recent studies reveal that gut microbiota, in which *Lactobacillus plantarum* (Lp)reside as a major population, are safe and benefit for the host [8]. Furthermore, dozens of bacteria

have been utilized for gene editing by the RecE/T homologous assisted recombination technique [9-13], providing the possibilities for integrating exogenous gene into the Lp. Therefore, developing the orally administrated vaccines against IAV infection based on the Lp holds great promise.

In this study, we identified a kind of Lp that is resistant to acid and bile salts, and that can colonize the intestines of mice. Whereafter, we established a RecE/T gene editing system to knock in antigen fragments and constructed a mini-HA-secreted Lp gene-edited strain (Lp-mini-HA-SP). It was revealed that Lp-mini-HA-SP exhibited immunological protection effects against both H9N2 and H1N1 IAV challenge in mice. Taken together, the recombinant Lp vaccine we developed offers immune protection effects against different IAV subtypes infection, providing a novel strategy for the development of orally administrated vaccines.

2. Materials and Methods

2.1 Ethics statement

All animal experiments were approved by the Committee on the Ethics of Animal Experiments of Huazhong Agricultural University (HZAUMO-2024-0126).

2.2 Bacterial strains, cells, viruses, and mice

Lactobacillus plantarum (Lp) were isolated from kimchi and cultured at 37℃ with deMan rogosa sharpe (MRS) medium. The antibiotics were supplemented with 50 μg/mL kanamycin for Luris-Bertani broth (LB) and 10 μg/mL erythromycin and

chloramphenicol for MRS. *E. coli* DH10B was used as clone construction, and grown at 30℃ on LB.

Caco2 cells were purchased from ATCC and cultured in Minimum Essential Medium (MEM) (11090081, Invitrogen) supplemented with 20% fetal bovine serum (S711-001S, LONSERA), 2mM L-Glutamine (G0200, Solarbio), 1% nonessential amino acids (N1250, Solarbio) and 1% Pyruvic acid sodium salt (P8380, Solarbio). Caco2 cells were incubated at 37℃ in a 5% CO_2 humidified atmosphere.

Influenza A/Puerto Rico/8/1934 (H1N1) was stored in our laboratory, Influenza A/Chicken/hubei/XY/2019 (H9N2) was isolated and stored in our laboratory. All viruses were amplified in 10-day-old SPF embryonic chicken eggs (Sparfas) and stored at -80℃.

The 6-8-weeks-old female C57BL/6J and BALB/c mice were purchased from the laboratory animal central, Huazhong agricultural university. The study considered that female mice are more susceptible to the influenza virus, so female mice were used in the study, and the sex of mice did not affect the experimental results.

2.3 Plasmids and cloning constructions

pLH01 and pLCNICK were both shuttle plasmids that could be transcribed and replicated in *E. coli* and Lp. Among them, pLCNICK provided homologous segments containing *mCherry/mini-HA-SP*. As a helper plasmid, pLH01 was mainly capable of expressing homologous recombinant proteins RecE and RecT to assist the integration of *mCherry/mini-HA-SP* into the genome of Lp. The mini-HA was acquired from the A/Chicken/hubei/XY/2019 (H9N2) by removing of head domain of HA, and the HA stalk

domains were connected by GS linker (**Supplementary material 1**). The mini-HA was then synthesized and a secretory signaling peptide was located in the 5' end of the mini-HA, fused with the Flag and His tag. We connected the upstream and downstream 1.0 kb fragments of knock out gene, P5 promoter, USP45 signal peptide and *mCherry/mini-HA* gene sequence by homologous recombination, and the recombinant fragments were attached to the pLCNICK by T4 DNA ligase.

2.4 Antibodies and compounds

The antibodies used in this study as follows: anti-Flag mouse monoclonal antibody (mAb) (F1804, Sigma), anti-His mouse mAb (CW0286, CWBIO). The secondary antibody used in the western blot and enzyme linked immunosorbent assay (ELISA) assay was horse radish peroxidase-conjugated anti-mouse (BF03001, Beijing Biodragon Inmmunotechnologies).

5(6)-carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) (MCE, HY-D0938) was a fluorescent dye that could penetrate cell membrane and it would be detected under the excitation light of 488 nm.

2.5 Transformation by electroporation

The helper plasmid pLH01 was delivered into Lp with the electrical transform condition at 2.0 kV, 200 Ω and 25 μF, and the transformants were screened on 10 μg/mL chloramphenicol plate. Then, Lp containing pLH01 would grow in 1% glycine MRS medium until the $OD_{600\ nm}$ value reached 0.3, and the inducing peptide (amino acid sequence is MAGNSSNFIHKIKQIFTHR) was added to the medium at a final

concentration of 100 ng/mL to induce the expression of RecE/T. Once the culture's OD_{600nm} value reached 0.6, the cells containing RecE/T would be collected and subjected to prepare the competent cells.

The recombinant plasmid of pLCNICK was electrically transferred into 100 µL of Lp competent cell containing RecE/T under the same electrotransformation conditions as described above. Then 1 mL of the recovery medium (MRS with 500 mM sucrose, 20 mM $MgCl_2$, and 2 mM $CaCl_2$) would be added and the mixture would be recovered with 3 h. The sample was centrifuged at 4500 rpm for 5 min, and 100 µL medium was retained to resuspend the cell precipitates, then the samples were plated on the MRS supplemented with 10 µg/mL erythromycin and chloramphenicol at 37℃ for 3-5 days. A single colony grew on the MRS plate and was identified by PCR screen.

2.6 Gram staining of Lactobacillus plantarum

The slide was evenly coated with 100 µL of *L. plantarum* to dry and fix its conformation. Then, the slide was dyed with 50 µL of crystal violet solution (0.1 g crystal violet, 95% ethanol solution for 2 mL, 1% ammonium oxalate for 8 mL) drops for 1 min and rinsed with water. Followed by staining with 50 µL of iodized solution (0.033 g iodine, 0.067 g potassium iodide, water for 10 mL) for 1 min and rinsing with water. Decolorizing the slide with 100 µL of decolorizing solution (95% ethanol solution) for 20-60 sec and rinsed with water, then the excess water was removed. Next, the slide was dyed with 100 µL of saffron solution (0.025 g safranin, 95% ethanol solution for 1 mL, water for 9 mL) for 1 min and rinsed with water, and excess water was removed. Finally,

the slide was subjected to microscopy analysis for observation.

2.7 Acid and bile salt tolerance of Lactobacillus plantarum

Lp was inoculated into the 50 mL of MRS medium with a pH range from 1 to 6 or with bile salt concentration at 0-0.5% for 12 h, respectively. 100 μL of medium were collected and coated on three individual MRS solid medium plates without antibiotics, and the plates were incubated at 37℃ for 24 h. The number of colonies was counted and statistical analyzed.

2.8 Fluorescent labeling of Lactobacillus plantarum

Lp was inoculated into 50 mL of MRS medium and cultured at 37℃ for 18 h. The sample was centrifuged at 4500 rpm for 5 min, strain precipitates were collected and washed three times with sterile phosphate buffer saline (PBS), the precipitates were resuspended with PBS, and a fluorescent dye cFDA-SE (MCE, HY-D0938) was added to the suspension of Lp at a final concentration of 20 μM. The samples were kept at 37℃ for 20 min in the dark. Then, the sample was centrifuged with 5000 rpm for 10 min at 4℃, and washed three times with sterile PBS to remove excess cFDA-SE. Equal amounts of Lp and Lp labeled with cFDA-SE (Lp-cFDA-SE) were applied to detect the fluorescence by flow cytometry (CytoFLEX LX, Beckman).

2.9 Flow cytometry

Caco2 cells were grown in 35 mm cell culture dish and cultured to reach the 90% density and washed three times with MEM medium. Then, Caco2 cells were respectively

inoculated with the 500 μL of PBS, 500 μL 1.0×10^9 cfu/mL of Lp and Lp-cFDA-SE, and then were incubated for 30 min. The excess Lp and Lp-cFDA-SE were removed with sterile PBS, and the cells were collected to detect the fluorescence by flow cytometry (CytoFLEX LX, Beckman).

2.10 Mouse imaging in vivo

6-8-weeks-old female BALB/c mice were respectively intragastrical administrated with 500 μL 1.0×10^9 cfu/mL of Lp-cFDA-SE and Lp-mini-HA-SP-cFDA-SE, and the mice's intestines of each group were collected and dissected for fluorescence detection by IVIS spectrum *in vivo* imaging system (Pekinelmer, USA) at day 3, day 5, day7, and day 9 post administration. Meanwhile, the 6-8-weeks-old female C57BL/6J mice were intragastrical administrated with 500 μL 1.0×10^9 cfu/mL of Lp that integrated with *mCherry* (Lp-mCherry). The fur of the mice from each group were scraped, and the fluorescence was detected by IVIS spectrum *in vivo* imaging system (Pekinelmer, USA) at day 5, day 7, day 9, and day 11 post administration.

2.11 Protein expression, purification, and identification

Lp integrated with mini-HA-SP (Lp-mini-HA-SP) was cultured with 100 mL MRS medium at 37℃ for 12 h. The sample was centrifuged with 5000 rpm for 10 min at 4℃. The supernatant was collected and incubated with 500 μL of Flag resin at 4℃ for 3 h. Then, the Flag resin was collected and washed with 10 mL of TBST buffer (25 mM Tris-HCl, 150 mM NaCl, pH 7.4), and eluted with 500 μL of elution buffer (25 mM Tris-HCl, 150 mM NaCl, 200 μg/mL DYKDDDDK peptide, pH 7.4). The eluted sample

was added loading buffer (100 mM Tris-HCl, 200 mM DTT, 4% SDS, 0.1% BPB, 20% Glycerol, pH 6.8) and boiled at 100℃ for 10 min. Then, the eluted sample were subjected to SDS-PAGE and the protein expression was identified by western blot using anti-Flag and anti-His antibodies, respectively.

2.12 Mini-HA expression detection of Lp-mini-HA-SP in mice intestine

6-8-weeks-old female BALB/c mice were evenly divided into three groups, each group was intragastrically administrated with 500 μL 1.0×10^9 cfu/mL of Lp and Lp-mini-HA-SP, respectively. The mice were dissected on the 3rd day, and the obtained cecum was washed with sterile PBS until the contents were cleaned and subjected to be analyzed by immunohistochemistry with His antibody (CW0286, CWBIO).

2.13 Animal Experiment

6-8-weeks-old female BALB/c mice were evenly divided into four groups, including treated with MRS medium (NC), Lp, Lp-mini-HA-SP, and H1N1/H9N2 inactive vaccine. The inactivated vaccine group of mice were immunized by intramuscular injection, the first immunization was 5 weeks before the challenge, and the booster immunization was 2 weeks before the challenge. Apart from the inactive vaccine group, the other three groups were immunized twice by intragastric administration with 500 μL 1.0×10^9 cfu/mL of Lp and Lp-mini-HA-SP or equal volume of MRS medium. The cycles of first immunization and booster immunization were 14 consecutive days, and the interval between the two immunizations was 7 days. The mice were nasally challenged with 50 μL of 10 $TCID_{50}$ H1N1 or $10^{4.3}$ $TCID_{50}$ H9N2. Then, the weight change and survival rate of the mice were

recorded for 14 consecutive days. Meanwhile, on the 3rd and 5rd day after the challenge, HE staining was performed on lung tissues of mice in different groups to observe the lung lesions.

2.14 Enzyme-Linked Immunosorbent Assays (ELISA)

Blood was collected from the tail vein of 6-8-weeks-old female BALB/c mice at 3 days before the challenge and serum was isolated for ELISA detection. The protein of mini-HA was expressed in Sf9 cells after being cloned into pFastBac1 with strep tag. The mini-HA was purified with Strep-Tactin2 NUPharose Fast Flow (NRPB61S, NUPTEC) and diluted with coated solution and added to the high adsorption enzyme label plate at a dose of 100 ng per well, and incubated at 4℃ for 12 h. Discard the liquid in the plate and washed it 3 times with PBS. The plate was added 5% skim milk for 300 μL per well, and incubated at 37℃ for 2 h. The skim milk was discarded, and the plate was washed 3 times with PBS. The serum samples were diluted with PBS in double ratio and added to the well of the plate to incubate at 37℃ for 2 h. The plate was washed with PBST (0.5% Tween-20 in 500 mL PBS) for 3 times and incubated with HRP-conjugated anti-mouse for 1 h. The plate was washed with PBST for 3 times and added reaction solution A and B to incubate for 15 min. Then the stop reaction solution was added, and the absorption value was detected in $OD_{630\ nm}$.

3. Results

3.1 Lactobacillus plantarum exhibits acid and bile salt resistance

For the development of orally administrative vaccines based on the *Lactobacillus*, we screened a strain of *Lactobacillus*, and the purple rod-like structure of the strain was observed by gram staining (**Fig. 1A**). We further confirmed that the strain was a Lp (ATCC 14917) by high-throughput sequencing (**Fig. 1B**). Lp possesses the potential as an engineered strain for oral administration and immunization *in vivo*, so we investigated its acid and bile salt tolerance in simulated intestinal condition *in vitro*. We identified that the Lp was in the optimal growth state when the pH value of MRS was 6, whereas the strain growth hardly when the pH value was below 3 (**Fig. 1C-D**). Meanwhile, the Lp was sensitive to the bile salts. However, the strain could still proliferate in MRS containing 0.2% bile salt (**Fig. 1E-F**). Hence, as gram-positive bacteria, Lp could proliferate in weakly acidic and low bile salt environment.

3.2 Lactobacillus plantarum binds to Caco2 and colonizes the intestines of mice

In order to further explore the potential of *L. plantarum* used for continuous immunization in intestinal tract, we investigated the colonization of Lp in the intestinal tract of mice. We applied cFDA-SE fluorescent to label the Lp, and the labeling rate was 97.24% detected by flow cytometry (**Fig. 2A**). We first verified the binding of Lp to intestinal cells (Caco2) *in vitro*. cFDA-SE labeled Lp was subjected to incubate with the Caco2 cells, and it was found that the co-incubation group of cFDA-SE labeled Lp and Caco2 cells (Lp-cFDA-SE+Caco2) had obvious fluorescence signal compared with the

group of Lp and Caco2 cells (Lp+Caco2) (**Fig. 2B**), suggesting the ability of Lp in binding with intestinal cell. Then, 6-8-weeks-old female BALB/c mice were intragastrically administrated with Lp-cFDA-SE or Lp-mini-HA-SP-cFDA-SE, respectively. The intestines of the treated mice were collected and subjected for fluorescence imaging identification. It was showed that fluorescent signals were both detected in the Lp-cFDA-SE and Lp-mini-HA-SP-SE group on day 3 and day 5 (**Fig. 2C**). Taken together, the gene editing modification of Lp and the expression of mini-HA did not affect its colonization characteristics in the intestine of mice, and the colonization period was preliminarily determined to be at least 5 days.

3.3 Gene editing and colonization of Lactobacillus plantarum in vivo

To eliminate the impacts of exogenous fluorescent dye and further determine the exact colonized time of Lp in mouse intestine, we integrated *mCherry* into the genome of Lp by RecE/T gene editing system (**Fig. 3A**). Meanwhile, we found that mCherry could be detected by western blot in the Lp-mCherry group compared to wild type Lp group (**Fig. 3B**). Subsequently, 6-8-weeks-old female C57BL/6J mice were respectively intragastrically administrated with Lp or Lp-mCherry, and live imaging was performed to observe the colonization of Lp-mCherry with mice intestine. The fluorescence of Lp-mCherry could be detected until day 9 and became be undetectable on day 11 (**Fig. 3C**). Taken together, we determined that Lp could colonize with the mouse intestine for 9 days.

3.4 Gene editing and expression of Lp-mini-HA-SP

Given that the Lp could colonize with the mouse intestine, we expected the genetically integrated Lp could secrete antigens, thus providing continuous immune stimulation to the host. To provide broad-spectrum antiviral protection against IAV, the highly conserved mini-HA among different subtypes of IAV was selected as the targeting antigen. The conserved mini-HA sequence of H9N2 was synthesized and a secretory signaling peptide was located in the 5' end of the *mini-HA*, fused with the Flag and His tag. Then, we constructed the mini-HA-secreted Lp strain (Lp-mini-HA-SP) by RecE/T gene editing system (**Fig. 4A**). By measuring the growth curves of Lp and Lp-mini-HA-SP, we found that the growth of Lp-mini-HA-SP was similar to Lp, with the Lp-mini-HA-SP growing to reach the logarithmic growth phase slightly slower compared to the Lp (**Fig. 4B**). On this basis, Lp-mini-HA-SP was cultured and the supernatant was collected and purified. The expression of mini-HA was identified by western blot by using anti-Flag tagged and anti-His tagged antibodies (**Fig. 4C-D**). Hence, mini-HA was expressed and released secretively from the genetically integrated Lp-mini-HA-SP.

3.5 Prophylactic efficacy of Lp-mini-HA-SP against lethal H9N2 challenge

To verify the antiviral immune effects of Lp-mini-HA-SP in mice, the mini-HA expression of Lp-mini-HA-SP were firstly determined. 6-8 weeks-old female BALB/c mice were intragastrically administrated with the Lp or Lp-mini-HA-SP, and the caecum of mice at 3 days post administration were collected and subjected to detect the expression of mini-HA by immunohistochemical analysis. It was found that mini-HA

could be detected in the intestinal lumen of Lp-mini-HA-SP administered mice, while no mini-HA could be detected in Lp administered mice (**Fig. S1**), indicating that Lp-mini-HA-SP could secrete the mini-HA in the intestine of mice.

To test the antiviral immune effects of Lp-mini-HA-SP against IAV infection, 6-8 weeks-old female BALB/c mice were intragastrically administrated with the Lp-mini-HA-SP for two rounds of 14 days, with 7 days interval. Then, the mice were challenged with H9N2 on day 42, and the weight and survival rates were recorded continuously for the next 14 days (**Fig. 5A**). We detected the serum of mice on day 39 by ELISA, and found that the serum titers stimulated by the Lp-mini-HA-SP was higher than the Lp (**Fig. 5B**). Meanwhile, Lp-mini-HA-SP administrated mice exhibited slightly attenuated infection as measured by reduced weight loss and increased survival rates compared to the Lp and negative control administrated mice, with inactivated vaccine treated mice showed completely protective effects. (**Fig. 5C-D**). The lungs of the mice on 5-day post challenge were collected and subjected be histopathological analysis. It was found that the lungs of mice in NC group and Lp group had moderate to severe bronchiolar necrosis, pulmonary edema, and inflammatory cell infiltrates, while the lungs of mice for Lp-mini-HA-SP and H9N2 inactive vaccine were completely protected (**Fig. 5E**). In summary, Lp-mini-HA-SP treated mice showed immune protection against H9N2 influenza virus challenge.

3.6 Prophylactic efficacy of Lp-mini-HA-SP against lethal H1N1 challenge

To investigate the broad-spectrum immune protective effect of Lp-mini-HA-SP against

influenza virus, 6-8 weeks-old female BALB/c mice were also intragastrically administrated with the Lp-mini-HA-SP and challenged with the H1N1 influenza virus following the similar process as utilized in H9N2 challenge. (**Fig. 6A**). The serum titers of mice stimulated by the Lp-mini-HA-SP was higher than the Lp treated mice (**Fig. 6B**). Similarly, Lp-mini-HA-SP administrated mice experienced the most significant weight loss on day 8 at 11.69%, which was followed by a gradual weight regain. Meanwhile, Lp-mini-HA-SP administrated mice possessed a 50% higher increased survival rates compared to the Lp and negative control administrated mice, with inactivated vaccine treated mice exhibited completely protective effects. (**Fig. 6C-D**). The lungs of the mice on 5-day post challenge were subjected to histopathological analysis. The lungs of mice in NC group and Lp group had moderate to severe bronchiolar necrosis, pulmonary edema, and inflammatory cell infiltrates, while the lungs of mice in Lp-mini-HA-SP and H1N1 inactive vaccine group were completely protected (**Fig. 6E**). In summary, Lp-mini-HA-SP treated mice showed immune protection against H1N1 influenza virus challenge.

4. Discussion

Current vaccines exhibit limited antiviral activities due to the frequent antigenic shift and drift of the IAV. Developing a broad-spectrum vaccine against IAV infection has been a significant challenge. Meanwhile, traditional administrations of vaccines rely on the injection, which requires a complex process and extra cost. The oral administration of

potential vaccines based on the Lp simplify the processes and covers many advantages. However, the recombinant Lp-mini-HA-SP vaccine we developed could not provide complete immune protection in mice, and it was considered that the expression of mini-HA from Lp-mini-HA-SP was insufficient to provide immune stimulation to mice. The key factors that controlled the expression of mini-HA were the types of promoters [14-15] and signal peptides [16-19]. Therefore, it is required to screen suitable promoters and signal peptides to increase the expression of mini-HA. Meanwhile, it was showed that the colonization period of Lp-mini-HA-SP was determined to be at least 5 days in mice, but at last cleared by the host, indicating the stomach acid in the host possesses a great challenge to the activity of Lp. In that case, periodic or booster vaccinations are needed to provide long-term immune protection. Alternatively, the recombinant Lp vaccine could serve as a feed additive, and poultry could obtain long-term and effective immune protection through continuous diet. In addition, protecting the Lp from being inactivated by the stomach acid is another strategy for improvement. It has been reported that an electrostatic interaction-mediated bio-interface mineralization, and it formed an ultra-resistant and self-removable coating on *Bacteroide fragilis* (BF839) surface [20]. The coating could neutralize gastric acid, and the generated calcium ions could trigger micellar aggregation of bile acid, thus achieving unimpaired bacterial viability and could be applied in the Lp. Therefore, optimizing condition to increase the expression of mini-HA and improve the utilization rate of Lp would be beneficial for the immune protection effects against influenza virus challenge *in vivo*.

Our research identified that the mice administrated with the Lp-mini-HA-SP exhibited reduced weight loss and increased survival rates when challenged with H9N2 and H1N1 compared to the Lp treated group, but the protective effects of Lp-mini-HA-SP against other subtypes of influenza virus are not investigated. In order to improve the broad-spectrum protective effect of recombinant Lp vaccine, more conserved epitopes of influenza virus antigenic proteins could be jointly utilized to compensate the shortcomings of present recombinant Lp vaccine, such as the mini-HA, the ectodomain of the M2 ion channel (M2e), and nucleoproteins (NP) hold the potential to developing universal anti-influenza vaccines [21-26]. Therefore, we can introduce the M2e and NP into the genome of Lp in tandem form of Lp-(mini-HA+M2e+NP)-SP, and investigate the effects of broad-spectrum anti-influenza virus. Alternatively, the M2e and NP were respectively integrated into the genome of Lp in the form of Lp-M2e-SP and Lp-NP-SP, and identify the effects of immune protection against divergent influenza viruses by combination intragastric administration in mice and poultry. In that way, the recombinant Lp vaccine we developed will provide broad-spectrum and comprehensive effects against influenza virus.

Owing to the fact that recombinant lactic acid bacteria (LAB) vaccine could be immunized orally, it had significant advantages in the field of animal husbandry. The premise of developing a recombinant vaccine was to determine the colonization cycle of LAB in the host intestine. We have identified a strain of Lp and it was able to colonize the intestines of mice for 9 days. However, it remains unknown whether the Lp that we

identified could be colonized in other species. Therefore, the colonization characteristics of Lp in poultry could be determined, and the immune protection effect of Lp-mini-HA-SP on poultry could be explored. Meanwhile, to better adapt to the poultry's intestine environment, the screening of endogenous LAB that colonized in the intestines of poultry is necessary. The development of recombinant vaccine based on the poultry intestine LAB would contribute to the poultry farming against avian influenza virus challenge.

In summary, we established a RecE/T gene editing system for Lp, and constructed a mini-HA-secreted Lp gene-edited strain. The Lp-mini-HA-SP colonized the mice intestines and showed protective effect against H9N2 and H1N1 influenza virus challenge. Our study provides a novel strategy for development of orally administrated broad-spectrum vaccines against IAV, which contributes to the prevention of IAV infection.

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CRediT authorship contribution statement

Yuanbao Zhou: Writing-original draft, Validation, Methodology, Investigation. **Zhipeng Lin:** Validation, Data curation. **Jiaqing Fang:** Validation, Data curation. **Zhihao Wang:** Validation, Data curation, Investigation. **Jinli Guo:** Investigation, Data curation. **Guohong Li:** Investigation, Data curation. **Qiaoxia Xu:** Investigation, Data curation. **Meilin Jin:** Formal analysis, Supervision. **Huanchun Chen:** Formal analysis, Supervision. **Jiahui Zou:** Writing-review & editing, Validation, Methodology, Investigation. **Hongbo Zhou:** Writing-review & editing, Supervision, Resources, Funding acquisition.

Conflict of interest statement

The authors declare no conflict of interest

Data availability statement

Data are contained within the article

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Figures and figure legends

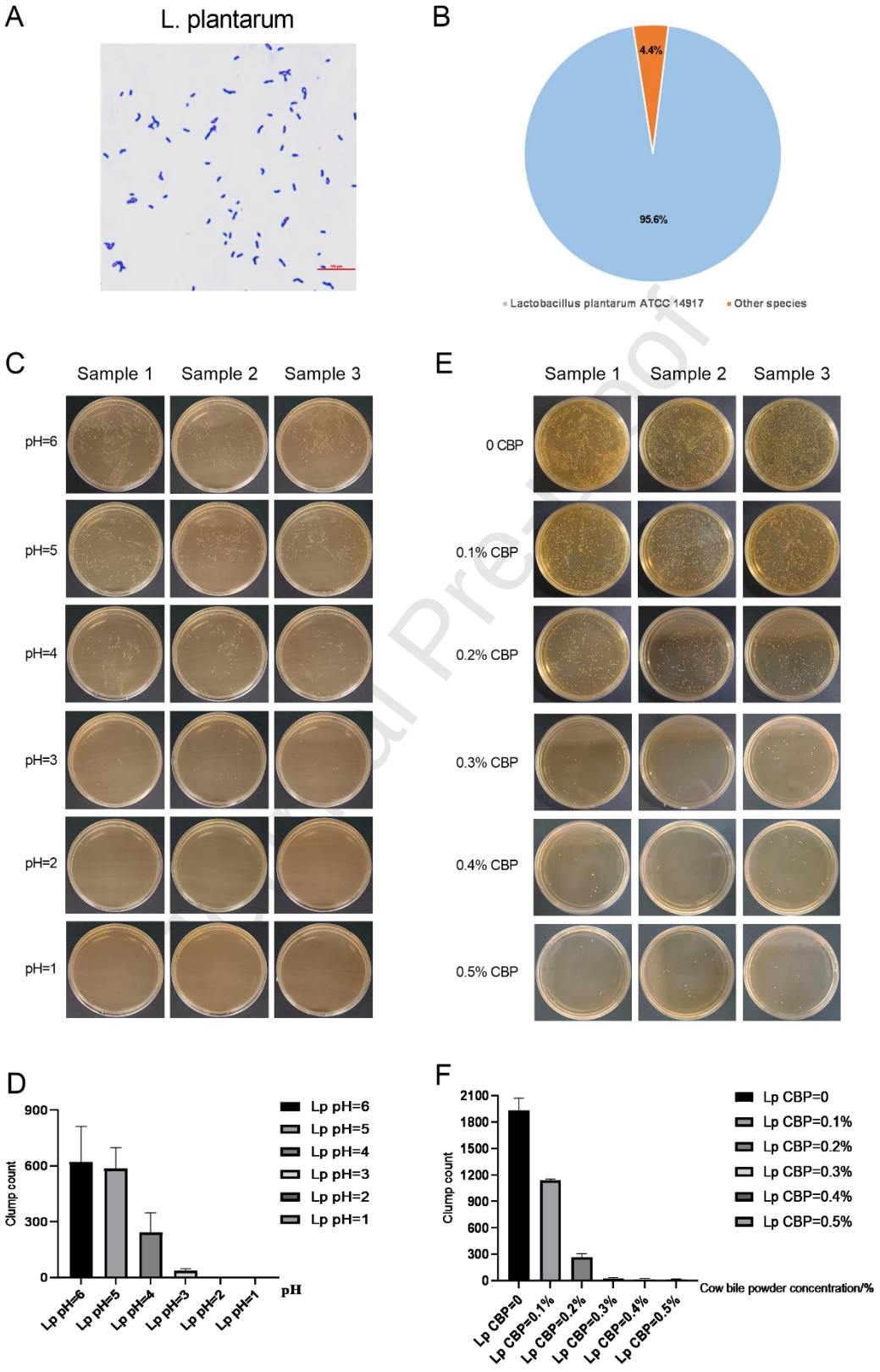

Figure 1. *Lactobacillus plantarum* exhibits acid and bile salt resistance.

(A) The Lp was cultured overnight and subjected for gram staining. scale bars = 200 μm. (B)The high-throughput sequencing for the Lp. (C) The growth state of Lp at different pH values. (D) Statistical analysis of *L. plantarum* growth state at different pH value. (E) The growth state of Lp at different bile salt concentrations. (F) Statistical analysis of Lp growth state at different bile salt concentrations.

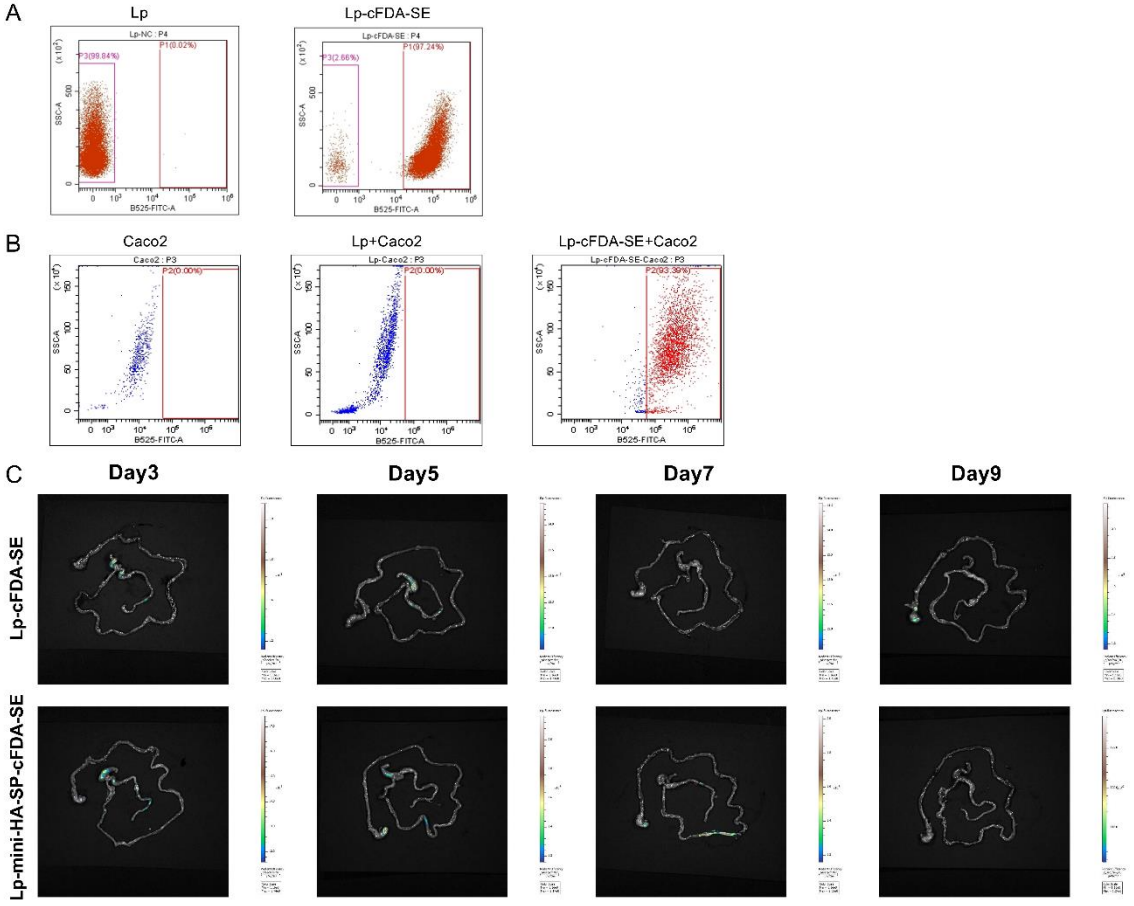

Figure 2. *Lactobacillus plantarum* binds to Caco2 and colonizes the intestines of mice

(A) Lp can be stained by cFDA-SE. The Lp was stained with cFDA-SE or not, and the staining efficiency was detected by flow cytometry. (B) Lp bind to Caco2 cells. Caco2 cells were respectively incubated with Lp-cFDA-SE or Lp for 1 hour, and the binding efficiencies of Lp-cFDA-SE to Caco2 cells were detected by flow cytometry. (C) Lp colonizes the intestines of mice. The mice were administrated with Lp-cFDA-SE or Lp-mini-HA-SP-cFDA-SE, respectively, and the colonization of Lp-cFDA-SE and Lp-mini-HA-SP-cFDA-SE in mice intestine at different timepoints post administration was detected by *in vivo* imaging system.

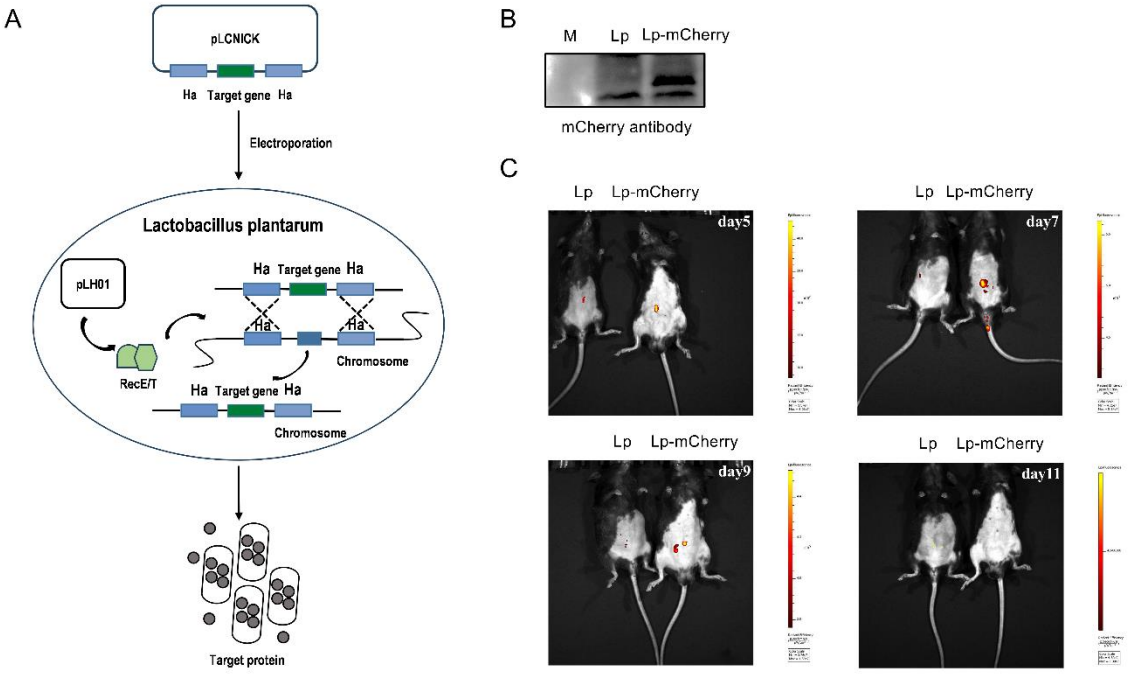

Figure 3. Gene editing and colonization of *Lactobacillus plantarum in vivo*

(A) The flow chart of the target gene was integrated into the genome of Lp by RecE/T gene editing. Ha, homologous arms. (B) The expression of mCherry was detected by western blot analysis of Lp-mCherry, and the Lp served as loading control. (C) Lp-mCherry colonizes the intestines of mice. The mice were administrated with Lp-mCherry or Lp, and the colonization of Lp-mCherry in mice intestine was detected by *in vivo* imaging system at different timepoints post Lp-mCherry administration.

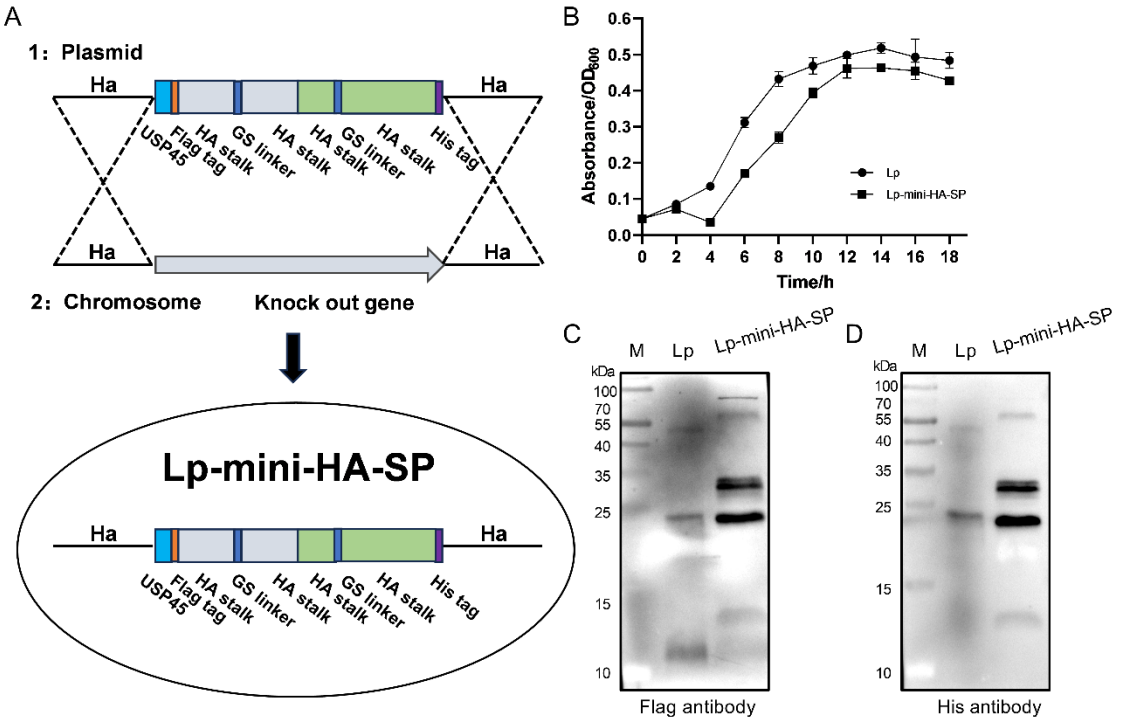

Figure 4. Gene editing and expression of Lp-mini-HA-SP

(A) Gene editing construction model diagram of Lp-mini-HA-SP. HA stalk, stalk region of influenza HA protein; USP45, signal peptide; Ha, homologous arms. (B) Growth curves of Lp and Lp-mini-HA-SP were measured. (C-D) The expression of mini-HA was detected by western blot. Lp-mini-HA-SP was cultured and the supernatants were collected and subjected for western blot by using (C) anti-Flag or (D) anti-His antibodies. The Lp served as loading control.

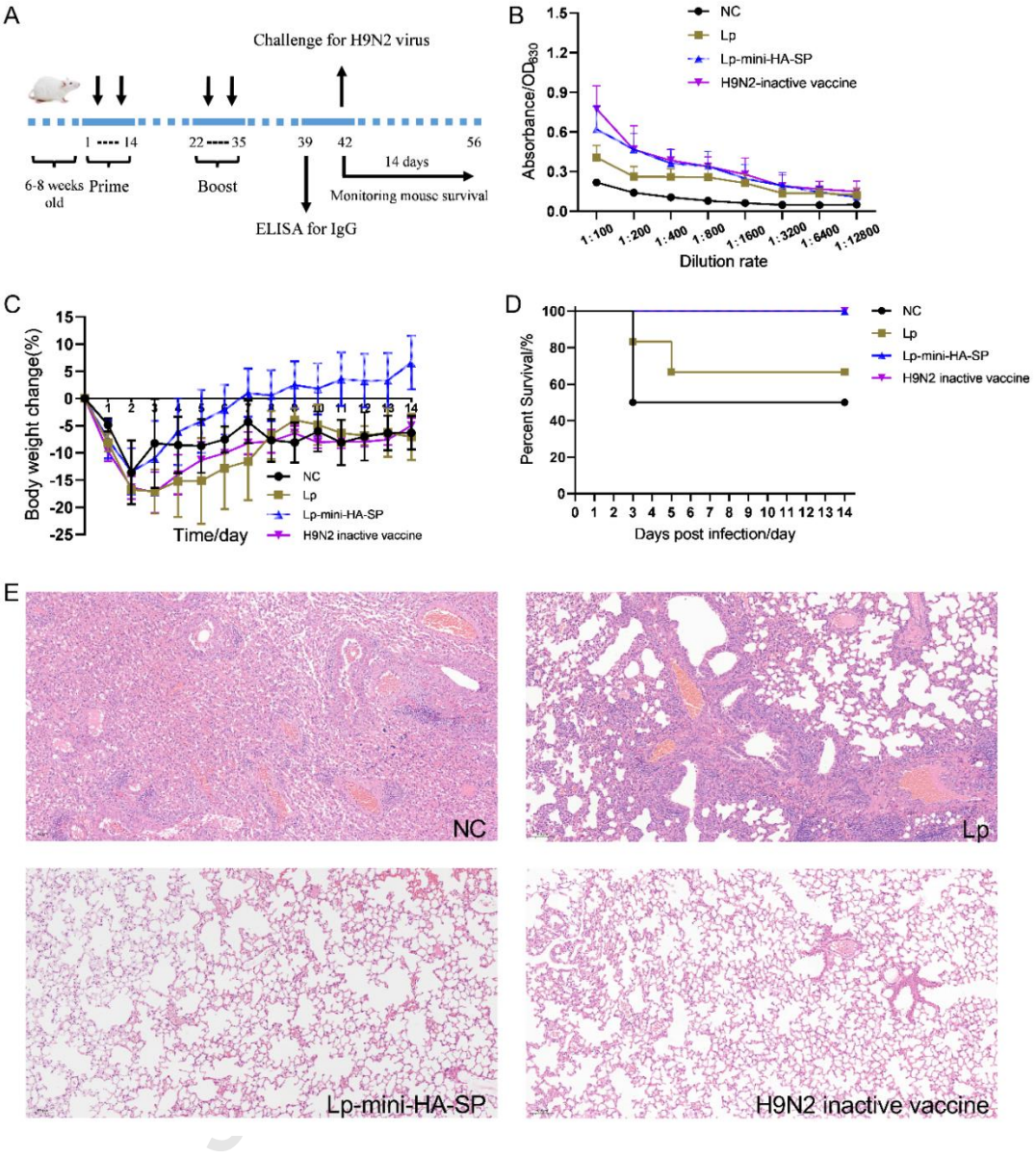

Figure 5. Prophylactic efficacy of Lp-mini-HA-SP against lethal H9N2 challenge

(A) Schematic of Lp-mini-HA-SP administration and H9N2 strain challenge *in vivo*. (B) The serums of mice were collected and subjected for ELISA determination. (C) Body weight change of mice following virus challenge. (D) Survival rate of mice after virus challenge. (E) Histopathological examination of the mouse lungs at 5 dpi. The slices were visualized using hematoxylin and eosin (H&E) staining, scale bars = 200 μm.

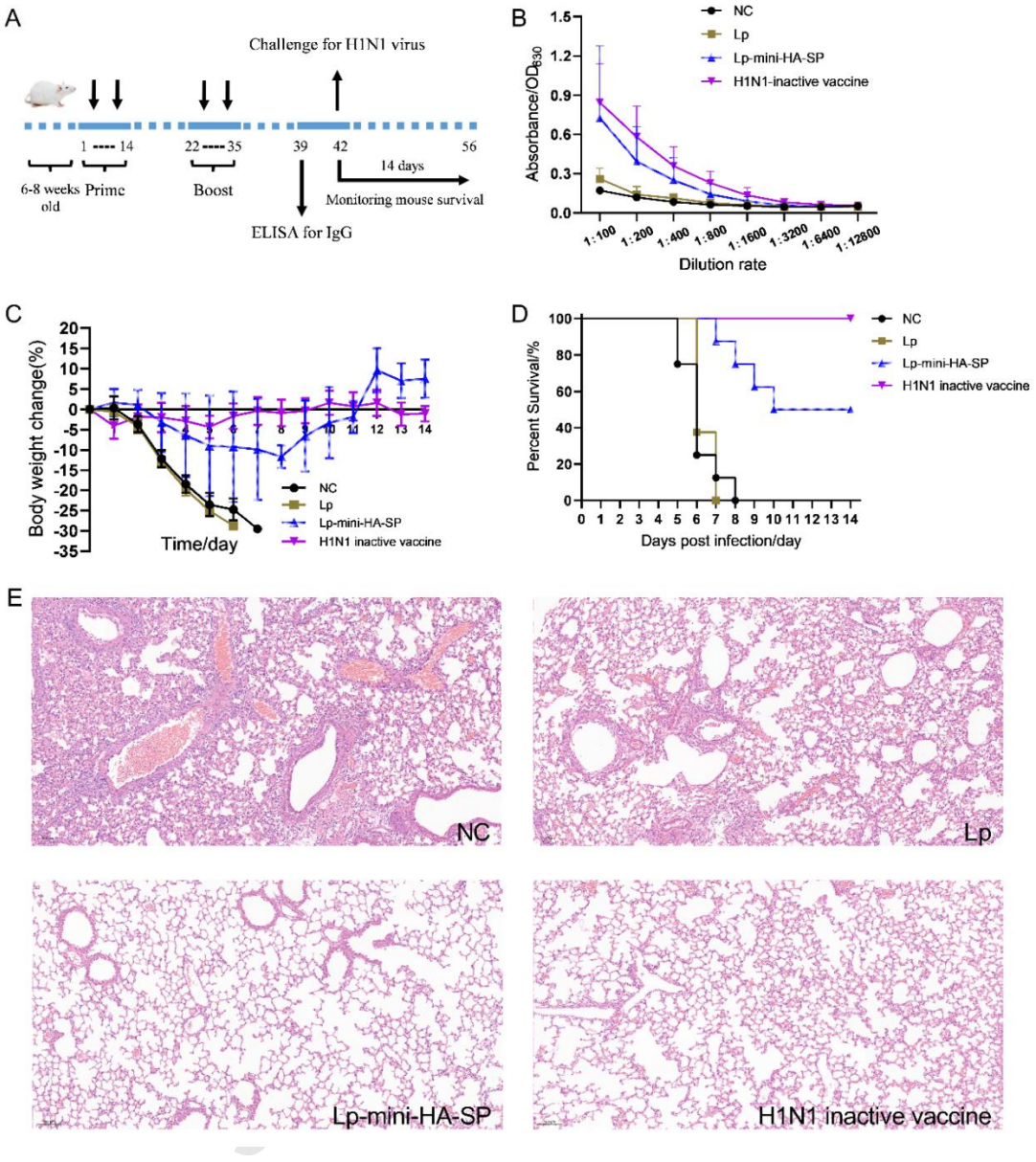

Figure 6. Prophylactic efficacy of Lp-mini-HA-SP against lethal H1N1 challenge

(A) Schematic of Lp-mini-HA-SP administration and H1N1 strain challenge *in vivo*. (B) The serums of mice were collected and subjected for ELISA determination. (C) Body weight change of mice following virus challenge. (D) Survival rate of mice after virus challenge. (E) Histopathological examination of the mouse lungs at 5 dpi. The slices were visualized using hematoxylin and eosin (H&E) staining, scale bars = 200 μm.

Declaration of Interest Statement:

The authors declare no potential conflicts of interest.

Highlights

- *L. plantarum* could proliferate in weakly acidic and low bile salt environment.
- *L. plantarum* could colonize with the mouse intestine for 9 days.
- *Mini-HA* was integrated into the genome of *L. plantarum* by RecE/T gene editing.
- Lp-mini-HA-SP showed immune protection against for H9N2 and H1N1 influenza virus.