Oral or intranasal immunization with recombinant Lactobacillus plantarum displaying head domain of Swine Influenza A virus hemagglutinin protects mice from H1N1 virus

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Research Article

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**Abstract**

**Background**

Swine influenza A virus (swIAV) is a major concern for the swine industry owing to its highly contagious nature and acute viral disease. Currently, most commercial swIAV vaccines are traditional inactivated virus vaccines. The *Lactobacillus plantarum*-based vaccine platform is a promising approach for mucosal vaccine development. Oral and intranasal immunisations have the potential to induce a mucosal immune response, which confers protective immunity. The aim of this study was to evaluate the probiotic potential and adhesion ability of three *L. plantarum* strains. Furthermore, a recombinant *L. plantarum* strain expressing the head domain of swIAV antigen HA1 was constructed and evaluated for its ability to prevent swIAV infection.

**Results**

The three *L. plantarum* strains isolated from healthy pig faecal samples maintained the highest survival rate when incubated at pH 3 and at bile salt concentration of 0.3 %. They also showed high adherence to intestinal cells. All three *L. plantarum* strains were monitored in live mice, and no major differences in transit time were observed. Recombinant *L. plantarum* expressed swIAV HA1 protein (pSIP401-HA1-ZN-3) and conferred effective mucosal and systemic immune responses in the intestine as well as in the upper respiratory airways of mice. In conclusion, the oral and intranasal administration of *L. plantarum* strain pSIP401-HA1-ZN-3 in mice induced mucosal immunity and most importantly, provided protection against lethal influenza virus challenge.

**Conclusion**

In summary, these findings suggest that the engineered *L. plantarum* strain pSIP401-HA1-ZN-3 can be considered as an alternative approach for developing a novel vaccine during an influenza A pandemic.

**Introduction**

Swine influenza A virus (swIAV) is one of the most dominant respiratory pathogens in swine, which often results in a significant economic burden to the pork industry [1]. Additionally, swIAV poses public health concerns owing to its zoonotic potential [1]. Historically, the influenza A(H1N1) virus has triggered several human influenza pandemics [2–5]. More recently, two studies have reported the reassortment of novel influenza viruses in pigs. IAV surveillance of the pig population from 2011 to 2018 in China revealed a recently emerged genotype 4 reassortant Eurasian avian-like H1N1 virus, which contains internal genes from both the triple-reassortant H3N2 and the 2009 pandemic H1N1 viruses [1, 6].

Vaccination remains the most efficient and cost-effective strategy of protecting human and animal populations against IAV [7, 8]. Most currently marketed influenza vaccines are inactivated influenza A virus (IIV) vaccines administered parenterally. The following three types of IIV vaccines exist: whole
inactivated virus (WIV) vaccines consisting of formaldehyde- or β-propiolactone-inactivated whole virion, split virus vaccines, and subunit vaccines [8]. Currently, most commercial swIAV vaccines are traditional WIV vaccines, often with oil-in-water adjuvants [9, 10]. However, these commercial vaccines are not regularly updated and do not protect against the large diversity of swIAVs circulating in swine populations [9]. The limited strains currently available in commercial swine influenza vaccines highlight the urgent need for a universal swine influenza vaccine.

Mucosal immunisation is a promising approach for controlling and preventing influenza A virus infection, and has advantages over conventional injection routes [11, 12]. The upper respiratory tract mucosa is the site of influenza virus infection and local mucosal immune responses play an important role in preventing influenza A virus infection [12]. Therefore, mucosal delivery via the oral or intranasal route has more advantages than those with systemic administration. The next generation of mucosal vaccines should ideally be administered in a single, tolerable, and efficacious dose, which induces robust neutralising humoral and cellular immunity against specific microbial pathogens [11]. Food-grade lactobacilli have been safely consumed by humans as fermented foods since a long time. Safe and highly efficient lactobacilli-based vaccines are an attractive alternative for inducing mucosal immune responses to protect against infective agents. Recently, recombinant lactobacilli have been successfully engineered to effectively produce a wide variety of antigens from microorganisms [11–13]. Lactobacillus strains may be considered promising vehicles not only for antigens but also for biologically active compounds, such as immunomodulators, antibodies, enzymes, and peptides [11–13].

The aim of the present study was to evaluate the probiotic properties of *Lactobacillus plantarum* strains isolated and identified from the faecal samples of healthy pigs using *in vitro* assay methods; among them, one strain showing desirable probiotic properties was selected for further assay of vehicles for swIAV antigen effects in mice. In addition, cell wall-anchored *L. plantarum*-HA1 was delivered via oral and intranasal immunisation, and its immunogenicity was evaluated in a mouse model.

**Materials And Methods**

**Characterization of *Lactobacillus* isolates from swine intestine**

Twenty faecal samples were collected from 10 conventionally raised healthy pigs in Wuhan, China. The animals had no history of gastrointestinal disease and had not been administered antibiotics for at least 2 weeks before sampling. Samples were serially diluted in an isotonic sodium chloride solution (0.85 % w/v), streaked on de Man, Rogosa and Sharpe (MRS) plates, and incubated at 37 °C for 48 h under anaerobic conditions. Following this, approximately 10–15 randomly selected colonies were plated on MRS agar, to identify the pure culture by colony morphology. All isolates were identified based on their morphological and staining characteristics (gram-positive bacilli) and negative catalase reaction (3 % v/v H$_2$O$_2$). The pure colonies were stored in a stock medium (10 % skimmed milk and 20 % glycerol) at −80 °C for further testing.
Identification of the tested isolates was performed using 16S rDNA sequence analysis. Strain DNA was extracted from the bacterial colonies using a QIAamp DNA Mini Kit (Qiagen, Wuhan, China). The 16S rRNA gene was amplified using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Purified polymerase chain reaction (PCR) products (one product per isolate) were sent to Sangon Biotech (Shanghai, China) for sequencing. Species were identified by comparing the 16S sequence with bacterial sequences in Medline databases; a score higher than 99% was considered significant. Phylogenetic trees were reconstructed using neighbour-joining and maximum likelihood methods in the MEGA7 program, with bootstrap values based on 1000 replications.

**Evaluation of probiotic properties**

To determine the probiotic potential, the following properties were evaluated: tolerance to low pH and bile salts, antibiotic susceptibility, and antimicrobial activity.

**Resistance to low pH**

Bacterial culture (1 mL) was inoculated into five tubes, each containing 9 mL MRS broth adjusted to pH ranging from 2–6. After 16 h of incubation at 37 °C, the bacterial cells were harvested by centrifugation at 10000 ×g, 4 °C for 5 min and washed twice with phosphate-buffered saline (PBS) (pH 7.2). Resistance to low pH was assessed in terms of viable colony counts and enumerated on MRS agar plates after incubation at 37 °C.

**Bile salt tolerance**

Tolerance to bile salts was tested as previously described. MRS broth was supplemented with different concentrations (0, 0.1, 0.2, and 0.3 %) of bile (Oxgall). These were inoculated with lactobacilli to investigate bile salt tolerance. Bacterial cells from overnight (16 h) cultures were harvested (10000 ×g, 5 min, 4 °C) and washed twice with PBS (pH 7.2). Resistance was assessed in terms of viable colony count and enumerated after incubation at 37 °C. All the values were obtained from three independent experiments.

**Antibiotic susceptibility test**

The agar well diffusion method was used to test antibiotic susceptibility to frequently used antibiotics. The antibiotics evaluated in this study against all ten isolates with the same concentrations are ampicillin (2 mg/L), gentamycin (16 mg/L), erythromycin (1 mg/L), kanamycin (64 mg/L), and clindamycin (4 mg/L). The antibiotic stock solutions were prepared in distilled water according to guidelines from the Clinical and Laboratory Standards Institute and the European Food Safety Authority. Distilled water was used as the control.

**Antibacterial activity**
The antimicrobial activity of the isolated strains was measured using a previously described method, with some modifications. Twenty pathogen strains preserved in our laboratory were used in antimicrobial assays to evaluate the antimicrobial activity of the isolated *L. plantarum* strains. The pathogenic strains included *Escherichia coli* ATCC35150, *Escherichia coli* XT-13, *Escherichia coli* AV006, *Escherichia coli* GS-1, *Salmonella* ST, *Salmonella* SH, *Salmonella* SS, *Salmonella* SE, *Salmonella* SO, *Salmonella* pullorum SA023, *Salmonella* enteritidis SA083, *Salmonella* typhimurium SA014, *Riemerella* anatipestifer 2020008, *Riemerella* anatipestifer 2020014, *Pasteurella* multocida 2018133, *Clostridium* perfringens 22, *Staphylococcus* sp 1, *Staphylococcus* sp 2, *Staphylococcus* aureus, and *Staphylococcus* epidermidis. Each pathogen was coated on a Luria-Bertani (LB) agar plate. Following this, the Oxford cup was carefully placed in the plate and 200 µL of overnight *L. plantarum* culture was placed inside the Oxford cup. The plate was incubated at 37 °C for 24 h, and the inhibition zone diameters were measured.

**Bacterial strains and growth conditions**

The bacterial strains and plasmids used in the present study are listed in Table 1. *L. plantarum* strains were cultured in MRS broth at 37 °C without shaking. *Escherichia coli* DH5α used in the transformation experiments involving the subcloning of DNA fragments was cultivated in LB broth at 37 °C with agitation. When appropriate, erythromycin was used at a concentration of 10 and 200 µg/mL for *L. plantarum* strains and *Escherichia coli*, respectively, both in broth and solid media.

**Table 1**

Strains, plasmids, and primers used in this study
| Strain, plasmid, or primer | Relevant characteristic                                                                 | Source or reference |
|---------------------------|----------------------------------------------------------------------------------------|---------------------|
| **Strain**                |                                                                                        |                     |
| *E. coli* DH5<sup>a</sup> | Cloning host                                                                           | TransGen Biotech    |
| *L. Plantarum* MQDR2      | Cloning host                                                                           | This study          |
| *L. Plantarum* A37        | Cloning host                                                                           | This study          |
| *L. Plantarum* M3         | Cloning host                                                                           | This study          |
| *L. Plantarum* 185362     | Cloning host                                                                           | This study          |
| *L. Plantarum* ZN-3       | Cloning host                                                                           | This study          |
| **plasmid**               |                                                                                        |                     |
| pSIP401                   | *spp*-based expression vector, Erm<sup>r</sup>                                         |                     |
| pSIP401-<i>mcherry</i>    | pSIP401 derivative, *mcherry* controlled by *P*<i>sppA</i>, Erm<sup>r</sup>            | This study          |
| PUC57-HA1                 | Gene synthesis                                                                         | This study          |
| PUC57-SP1216-2578         | Gene synthesis                                                                         | This study          |
| pSIP401-SP1216-2578       | pSIP401 derivative, CWA200 fused to the spLp<sub>2588</sub>; Erm<sup>r</sup>           | This study          |
| pSIP401-SP1216-HA1-2578   | pSIP401 derivative, HA1-CWA200 controlled by *P*<i>sppA</i>, Erm<sup>r</sup>           | This study          |
| **primer**                |                                                                                        |                     |
| 401-<i>mcherry</i>-<i>F</i> | CCGCCATGGATGGTATCAAAGAGAAGAAGA                                                            | Nco I               |
| 401-<i>mcherry</i>-<i>R</i> | CCCAAGCTTTTTATTTATATAATTCATCCATACCA                                                     | Hind III            |
| HA1-<i>F</i>              | CGCGGTACCTCCTTTAATGGTAAAAATTCCA                                                         | Kpn I               |
| HA1-<i>R</i>              | GCCGGATCCATGAACCTTGAGCATCTGA                                                            | BamH I              |
| PUC57-SP1216-HA1-2578-F   | taggagtatgattccatggTGAAGAGTTTAATTATGAGCAT                                               | Seamless Assembly   |
| PUC57-SP1216-HA1-2578-R   | cgtgctgtaaggctcggTGAAGAGTTTAATTATGAGCAT                                               | Seamless Assembly   |
| 401-SP1216-HA1-2578-F     | taggagtatgattccatggTGAAGAGTTTAATTATGAGCAT                                               | Seamless Assembly   |
| 401-SP1216-HA1-2578-R     | cgtgctgtaaggctcggTGAAGAGTTTAATTATGAGCAT                                               | Seamless Assembly   |

<sup>a</sup> DH5 is commonly used for the transformation of plasmids into *E. coli*.
DNA manipulations and plasmid construction

Primers used in this study are listed in Table 1. All plasmids used in this study for expression in *Lactobacillus* spp. are derivatives of the modular pSIP401 vector series, constructed and developed for inducible gene expression, secretion, and surface anchoring of proteins [14-18]. pSIP401-mcherry was constructed by amplifying the mCherry protein from pMcherry-C1 using the primer pair 401-mcherry-F/401-mcherry-R (Table 1), following which the resulting 0.7 kb PCR fragment was digested with Nco I/Hind III. The IRFP713 gene fragment was optimised for expression in *L. plantarum*, synthesised using GenScript (Nanjing, China), and cloned into a pSIP401 plasmid, yielding pSIP401-IRFP713.

The HA1(H1N1) and SP1261-2578 gene fragments were optimised for expression in *L. plantarum*, synthesised using GenScript (Nanjing, China), and cloned into a pUC57 plasmid, yielding pUC57-HA1 and pUC57-SP1216-2578, respectively. These plasmids contain N-terminal signal peptides (SP) derived from genes encoding Lp_1261 [19]. The total length of the Lp_1261 anchor is 75 residues, including 22 amino acids in the SP. The cell wall anchor sequence comprises 223 C-terminal residues from Lp_2578, of which 189 residues are the linker region, followed by the LPQTSE motif, which is followed by a hydrophobic stretch and a positively charged C-terminal [14, 19-21]. The plasmid for the intracellular production of SP1216-HA1-2578 was constructed by amplifying the HA1 fragment using the primer pair PUC57-SP1216-HA1-2578-F and PUC57-SP1216-HA1-2578-R (Table 1), using pUC57-HA1 as a template. The resulting PCR fragment was ligated into the KpnI/BamHI-digested vector PUC57-SP1216-2578 using an In-Fusion® HD Cloning Kit (Clontech, Dalian, China) to yield the plasmid PUC57-SP1216-HA1-2578. pSIP401-SP1216-HA1-2578 was constructed by amplifying the SP1216-HA1-2578 open reading frame from PUC57-SP1216-HA1-2578 using the primer pair 401-SP1216-HA1-2578-F/401-SP1216-HA1-2578-R (Table 1), following which the PCR fragment was ligated into the Ncol/HindIII-digested vector, pSIP401, using the In-Fusion® HD Cloning Kit.

Gene expression in *Lactobacillus plantarum*

To generate the four expression strains, pSIP401-mcherry, pSIP401-IRFP713, and pSIP401-SP1216-HA1-2578 were transformed into electro-competent *L. plantarum* strains and transformants were selected on MRS agar plates containing 5 µg/mL erythromycin. Gene expression was performed by diluting the overnight cultures of *L. plantarum* strains harbouring the plasmids in 100 mL of fresh pre-warm MRS broth (for erm-based systems, 5 µg/mL of erythromycin was added) at an OD<sub>600</sub> of approximately 0.1, and incubated at 37 °C without agitation. The cells were induced at OD<sub>600</sub> of 0.3 by adding the peptide pheromone IP-673 [22] to a final concentration of 25 ng/mL. The cultures were harvested 6 h after induction by centrifugation at 5000 x g for 5–10 min. The cells were washed once in PBS and stored at
−20 °C, before staining or sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

**Flow cytometry and indirect immunofluorescence microscopy**

Bacterial cultures were grown and induced as previously described. Cells from approximately 0.5 mL of culture were harvested once with PBS, washed four times with PBS, and resuspended in 1 mL PBS without bovine serum albumin (BSA) at 4 °C. The resulting bacterial suspensions were immediately analysed by flow cytometry using a MACSQuant analyser (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions.

For indirect immunofluorescence microscopy, the stained cells were resuspended in 100 µL of PBS and stained with monoclonal anti-c-Myc antibody and goat anti-mouse Alexa Fluor® 594 (IgG H&L) (Abcam; Cambridge, UK) and visualised under an Axio Observer.z1 microscope (Zeiss, Oberkochen, Germany) using excitation and emission wavelengths of 591 and 614 nm, respectively, and a bright field photomultiplier tube for transmitted light.

**Assay of adhesion to Caco-2 cells**

Caco-2 cells were routinely grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % foetal bovine serum, 100 IU/mL penicillin G, and 100 μg/mL streptomycin. For adhesion assays, 10⁵ Caco-2 cells were seeded in 24-well plates with glass cover slips and maintained at 37 °C under 5 % CO₂ for 3 days. Prior to the experiments, all bacterial cultures were harvested until the stationary phase or after induction, washed twice in PBS, and suspended in DMEM without antibiotics at 1.5 × 10⁸ colony forming units (CFU)/mL. Cells were co-incubated for 2 h (5 % CO₂ atmosphere at 37 °C). After co-incubation, non-adherent bacteria were washed twice with PBS. For microscopy, Caco-2 cells were seeded onto a glass coverslip and cultivated for 24 h. Subsequently, the cells were fixed with 4 % paraformaldehyde and stained with 0.1 % crystal violet staining solution. The experiments were performed in duplicates. The percentage of Caco-2 cells (1000 cells) adhering to at least one *Lactobacillus* species was counted.

**Transmission electron microscopy**

The bacteria were resuspended in fresh DMEM and added to the prepared Caco-2 monolayers to be cultured in 6-well plates at 37 °C with 5 % CO₂ for 2 h. The monolayers were then washed five times with PBS to remove unbound bacteria. The samples were then fixed with glutaraldehyde solution for 6 h. Caco-2 cells were stained with phosphotungstic acid and observed using H-7650 transmission electron microscopy (Hitachi, Tokyo, Japan).

**In vivo fluorescence imaging**
Fluorescence imaging was performed using a multimodal IVIS Lumina XR imaging system (PerkinElmer). The mice were euthanised by cervical dislocation, followed by exposure of the abdominal cavity and removal of the intestine from the stomach to the rectum [22]. The instrument background fluorescence was removed using an adaptive fluorescence background subtraction tool. Images were analysed using Living Image version 4.3.1[22].

**Colony detection of strains in mice intestines**

A total of 48 BALB/c female 6-week-old mice were received $10^9$ CFU/100 µL fluorescent recombinant *L. plantarum*. The intestines were harvested immediately after the animals were euthanized at eight time points (2, 4, 6, 12, 24, 48, 72, and 96 h; n = 6 mice in each group). At each time point, the gastrointestinal tract (GIT) of mice was collected and imaged using an IVIS Lumina XRMS Series III (PerkinElmer); the status of probiotics in the mouse intestine was observed at different time points. Simultaneously, the small intestine (the duodenum and jejunum) and the large intestine (the caecum and colon) of each mouse were segmented, PBS was used to wash the intestinal mucosa and the attached contents, and the abundance of probiotics was quantified using the plate coating method.

**Western blotting**

The protein extracts, in amounts that were adjusted based on the OD$_{600}$ at harvesting, were separated by SDS-PAGE using 10 % Mini-Protean TGX precast gels (Bio-Rad) and transferred to a nitrocellulose membrane. The monoclonal murine anti-Myc antibody (ABclonal, Wuhan, China) and used as recommended by the manufacturer. Protein bands were visualised using a polyclonal rabbit anti-mouse antibody conjugated with horseradish peroxidase (HRP) (ABclonal, Wuhan, China).

**Oral and intranasal immunization with recombinant *Lactobacillus plantarum***

The wild-type and recombinant *L. plantarum* cultures grown in this study were resuspended to $10^9$ CFU/100 µL for oral immunisation. The immunisation procedure was divided into the following three phases: prime immunisation (days 1, 2, and 3), booster immunisation (days 14, 15, and 16), and the last immunisation (days 29, 30, and 31), with PBS containing $10^9$ CFU *L. plantarum* or 100 µL PBS (negative control) being administered by oral gavage.

Six- to eight-week-old female BALB/c mice (10 per group) received either recombinant bacteria expressing HA1 or the respective control bacteria harbouring the empty vector. An additional control group was administered isotonic sodium chloride solution. *L. plantarum* strains were grown until cultures reached an OD$_{600}$ value of 2.0, and the bacteria were collected by centrifugation (4000 x g, 20 min at 20 °C), washed with saline, and suspended at $10^7$ cells in 10 µL. For nasal immunisation, mice were anaesthetised with 2.5 % avertin (0.02 mL/g body weight) and 10 µL of an isotonic sodium chloride solution suspension containing $10^7$ cells was inoculated into the nostrils with the help of a micropipette on days 1, 14, and 29.
Enzyme-linked immunosorbent assay for the detection of HA-specific antibodies

Bronchoalveolar lavage fluid (BALF), intestinal lavage fluid (ILF), and serum samples were collected on day 7 post-immunization, as described previously [23-25]. Briefly, 1 mL sterile PBS was injected into the lungs via the trachea and following three rounds of flushing, the washes were collected and stored at −20 °C until analysis. Intestinal and faecal samples were added to 1 mL PBS containing 1 % BSA and 1 mM phenylmethylsulfonyl fluoride. After incubation at 4 °C for 12 h, the tubes were vortexed to disrupt all solid material, and then centrifuged at 16000 ×g for 10 min to collect the supernatants. Blood collected from mice was allowed to clot at 20 °C for 1 h, followed by centrifugation at 1000–2000 × g for 10 min at 4 °C. Serum was stored at −20 °C until analysis.

Antigen-specific serum immunoglobulin (Ig)G and faecal secretory IgA (sIgA) antibodies were detected using enzyme-linked immunosorbent assay (ELISA). In detail, whole inactivated virus (H1N1) was diluted to 10 μg/mL in carbonate–bicarbonate buffer (pH 9.6) and 100 μL per well was used to coat 96-well plates overnight at 4 °C. Free sites were blocked for 3 h with 250 µL of 2 % BSA in PBS at 37 °C. The serum and faecal samples were diluted 100 × and 20 ×, respectively. The diluted samples were added to the wells, incubated at 37 °C for 2 h, then washed thrice with PBS with tween-20. The plates were blotted in 100 µL goat-anti-mouse IgG-H&L (HRP) antibody (Abcam, Shanghai, China) and goat anti mouse IgA alpha chain (HRP) antibody (Abcam, Shanghai, China) at room temperature for 1 h. After incubation, the wells were washed thrice with 100 µL wash solution. Bound antibodies were detected with 3,3,5,5′-tetramethylbenzidine substrate solution in sterile water and incubated in dark at 37 °C for 15 min. The enzyme reaction was stopped by adding 100 μL of stop solution, and the absorbance of each microwell was recorded using a spectrophotometer at 450 nm as the primary wavelength. Two independent experiments were performed.

Viral challenge, clinical observation, and histopathological examination

Mice were anaesthetised intraperitoneally with 2.5 % avertin (0.02 mL/g body weight) and then inoculated intranasally with H1N1 (1×10⁵ TCID₅₀/0.1 mL). To assess the protective efficacy of recombinant L. plantarum, all mice were observed for general physical activity and pathophysiological parameters (body weight, fur ruffling, and conjunctivitis).

The animals were euthanised on day 7, and the lungs were collected. The lung tissues of mice in each group were fixed in 10 % neutral buffered formalin, embedded using established methods in paraffin, sectioned at 4 mm thickness, and stained with haematoxylin and eosin (H&E). The slides were then observed under a light microscope (Nikon, EX200) to detect histopathological lesions in the lungs. The scoring method was 2 points for inflammatory cell infiltration, 1 point for bleeding, and 1 point for alveolar incompleteness. They were rated blindly by three individuals.

Statistical analyses
All data in the experiment were obtained from at least three independent experiments and are expressed as the mean ± standard error of mean. Differences were tested using the GraphPad Prism software (version 5.0). Among experimental groups at different time intervals, one-way analysis of variance (ANOVA), with post hoc Bonferroni’s multiple comparison test, was used to determine the statistical significance of cytokine and sIgA levels and two-way ANOVA with post hoc Bonferroni’s multiple comparison test was used to determine significant differences in IgG levels. Survival percentages were analysed using the Kaplan–Meier method. A p-value less than 0.05 was considered statistically significant, and a p-value less than 0.01 was considered highly significant.

**Ethics statement**

Specific pathogen-free BALB/c mice were obtained from the Laboratory Animal Services Centre (Huazhong Agricultural University). All animal experiments were conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Research Ethics Committee, Huazhong Agricultural University, Hubei, China (approval number HZAUMO-2020-0007).

**Results**

**Lactobacilli were abundantly present in pig faeces**

Overall, 188 strains were isolated from the samples collected in our study. Isolates were identified by 16S RNA amplification. Among these strains, 103 isolates were characterised as lactic acid bacteria, including 80 *Lactobacillus* and 16 Coccus isolates. *Lactobacillus* strains included *Lactobacillus johnsonii* (27 isolates), *Lactobacillus reuteri* (17 isolates), *Lactobacillus zeae* (three isolates), *Lactobacillus rhamnosus* (five isolates), *Lactobacillus casei* (four isolates), *Lactobacillus paracasei* (five isolates), *Lactobacillus amylovorus* (one isolate), *Lactobacillus plantarum* (six isolates), *Lactobacillus harbinensis* (six isolates), *Lactobacillus perolens* (two isolates), *Lactobacillus coryniformis* (two isolates), and *Lactobacillus brevis* (two isolates). The Coci included *Pediococcus acidilactici* (five isolates), *Pediococcus pentosaceus* (six isolates), and *Enterococcus faecalis* (five isolates).

PCR amplification of the 16S rDNA of *L. plantarum* (six isolates) resulted in a single band of approximately 1500 bp, which corresponds to the expected size of the 16S rDNA gene. Both forward and reverse sequences were obtained and an assembly sequence was generated. The comparative analysis of *L. plantarum* (six isolates) sequences with the published sequences of different *Lactobacillus* species, using the MEGA7 program, demonstrated the phylogenetic distances in a generated neighbour-joining rooted tree (Fig. S1).

**The amount of overexpression was estimated by comparing the expression level of exogenous proteins**

To determine the ability of the six *L. plantarum* isolates to express foreign proteins, we constructed recombinant *L. plantarum* expressing mCherry, the expression of which was analysed using fluorescence
microscopy and flow cytometry (Fig 1). The results showed that the expression of 185362, A37, m3, and mCherry were low, whereas the expression of ZN-3, 1.191, and MQDR2 mCherry were high. Thus, we subsequently evaluated the biological characteristics of ZN-3, 1.191, and MQDR2 and screened them for the most suitable mucosal immune vector.

Resistance to gastric juice and bile salts

The effects of simulated gastric juice on the survival rate of the isolated *L. plantarum* at an incubation time of 16 h are shown in Fig 2a. In this study, the isolates were evaluated for tolerance to varying pH (2.0, 3.0, 4.0, 5.0, and 6.0). *L. plantarum* isolates showed negligible growth up to pH 4. However, at pH 5–6, an increase in growth rate was observed, above which the growth rate declined. The MQDR2 isolates showed better tolerance to pH values lower than those tolerated by 1.191 or ZN-3. Among all the isolates, MQDR2 showed the most significant results.

Bile salt tolerance is an essential factor determining the stability of probiotics in the intestine. Bile salts present in the intestinal tract disrupt the bacterial cell membrane and prevent them from entering the stomach. The maximum ZN-3 growth was observed in the presence of bile salts up to 0.3 % (Fig 2b). The isolates, however, were less tolerant to high concentrations, such as 0.6 and 1 % (data not shown). Overall, in the presence of bile salts up to 0.3 %, ZN-3 produced significantly higher biomass (p < 0.05) than that obtained with 1.191 or MQDR2.

Antibiotic Susceptibility

All isolates were analysed for their tolerance to antibiotics owing to safety considerations regarding the threat of antibiotic resistance in bacteria. The antibiotic susceptibility test was performed using the disc diffusion method by measuring the zone of inhibition for various antibiotics (Fig 2c). The *L. plantarum* isolates were sensitive to cefazolin, tetracycline, neomycin, erythromycin, and chloramphenicol; among all the isolates, strain 1.191 showed the maximum diameter for the zone of inhibition against cefazolin (35.1 mm). It was concluded that isolates 1.191 and MQDR2 were resistant to norfloxacin. Antibiotic susceptibility tests showed that, compared to 1.191 and MQDR2, Z N-3 had better probiotic properties and was sensitive to all antibiotics used in this study.

Antibacterial activities of *Lactobacillus* strains against gram-positive and gram-negative pathogens

The isolated *Lactobacillus* strains showed notable antibacterial activity against all target bacteria commonly associated with gastrointestinal diseases. All three isolates successfully inhibited the growth of the clinical isolates *Salmonella* ST, *Salmonella* SH, *Salmonella* SS, *Salmonella* SE, *Salmonella* SO, *Salmonella* typhimurium SA014, *Salmonella enteritidis* SA083, *Salmonella* pullorum SA023, *Escherichia coli* (ATCC 35150), *Escherichia coli* XT-13, *Escherichia coli* AV006, *Escherichia coli* GS-1, *Riemerella anatipestifer* 2020008, *Riemerella anatipestifer* 2020014, *Pasteurella multocida* 2018133, *Clostridium perfringens* 22, *Staphylococcus* sp 1, *Staphylococcus epidermidis*, *Staphylococcus aureus*, and
Staphylococcus sp 2 (Fig. 2 D). The diameters of the growth inhibition zones were similar among all the isolates.

*L. plantarum* adheres to Caco-2 cells

The most important feature of any potential probiotic strain is its ability to adhere, which is referred to as the gold standard for identifying probiotic bacteria. The adherence ability of the isolated ZN-3, 1.191, and MQDR2 to the Caco-2 cell line was determined by direct microscopic examination using Giemsa staining. All isolates were categorised as strongly adhesive (Fig 3a). The adherent bacteria were further quantitated using a classical culture-dependent method. The adhesion efficiency was calculated as the percentage of adhesion values compared to the initial bacteria seeded in each well and assumed to be equal to 100 %, confirming the high colonisation efficiency previously observed using microscopy. All *L. plantarum* strains isolated from pig faeces showed adhesion efficiency to Caco-2 cells, with an adhesion percentage ranging from 68–95 % (Fig 3b).

These isolates were further analysed using scanning electron microscopy (SEM), to understand the morphology of the isolates that adhered to Caco-2 cells. Both untreated and bacteria-treated Caco-2 cells were observed under an SEM (Fig 3c). Fig 3c, at 2000× magnification, shows the presence of rod-shaped *Lactobacillus* adhering to the surface of Caco-2 cells. Among the three strains, ZN-3 exhibited significantly increased adherence to Caco-2 cells than those of the other strains (bacterial counts/cell, Fig 3d). Immunofluorescence microscopy was used to visualise the adhesion ability of red fluorescent protein-expressing *L. plantarum* (ZN-3-mcherry, 1.191-mcherry, and MQDR2-mcherry). ZN-3-mcherry was the most adherent strain (Fig 3e), and isolates 1.191 and MQDR2 exhibited moderate binding (Fig 3e).

**Ex vivo** epifluorescence time-course imaging of mouse intestine following the oral administration of IRFP713-expressing bacteria

To determine the spatial and temporal transit of ZN-3-IRFP713, 1.191-IRFP713, and MQDR2-IRFP713 in the GIT of mice after a single oral administration, the intestines of three mice were removed at different time points and imaged *ex vivo*. The results showed that 180 min after the administration of the bacterial strains, ZN-3-IRFP713, 1.191-IRFP713, and MQDR2-IRFP713 survived passage through the stomach and IRFP713 signal cells were observed throughout the small intestine (Fig 4a). After approximately 6 h, majority of the ZN-3-IRFP713, 1.191-IRFP713, and MQDR2-IRFP713 isolates had travelled through the small intestine and were located exclusively in the caecum and colon. Some of the viable cells of both strains remained in the small intestine after 6 h but emitted weak or no epifluorescence signals. They were retained in the caecum for several hours. Following this, they gradually cleared to the large intestine, from which more than 90 % were secreted in 10 h, as observed by the decrease in both, CFU number and radiant efficiency. After 24 h, IRFP713 signal cells remained localised in the caecum and colon.

We monitored the number of ZN-3-IRFP713, 1.191-IRFP713, and MQDR2-IRFP713 bacteria, as well as their respective IRFP713 signals in the mice small and large intestines at different time points after the oral administration of bacteria (Fig 4b and c). The number of viable bacteria increased with time in the
small and large intestine, reaching its maximum level after 4 h with approximately $10^9$ CFU/100 mg intestinal content for each strain (Fig 4b and c). The abundance of ZN-3-IRFP713, 1.191-IRFP713, and MQDR2-IRFP713 in mice small and large intestine plateaued for approximately 4 h and then declined. After 24 h, this number reached approximately $10^5$ CFU/100 mg intestinal content. After 96 h, no L. plantarum-IRFP713 was found in the intestinal contents, whereas the number of L. plantarum-IRFP713 organisms was still approximately $10^2$ CFU/100 mg intestinal contents. In summary, these results indicate that the three Lactobacillus strains are capable of colonising the intestines of mice, and the ZN-3 strain had the strongest ability to colonise and persist in the intestines of mice.

**L. plantarum displayed H1N1 HA1 protein on the cell surface**

To improve vaccination efficiency, an oral DC-targeted and M-targeted mucosal vaccine using L. plantarum as a vector delivering the HA1 protein fused to DCpep and the M cell-targeting peptide was constructed. The gene sequences encoding recombinant HA1 proteins were successfully cloned into the pSIP401 expression vector (Fig 5a). Sequencing of the plasmids confirmed the presence of the corresponding gene sequences in the frame with the SP1216 and 2578 domains. Subsequently, L. plantarum strain ZN-3 cells were transformed with recombinant plasmids (pSIP401-SP1216-HA1-2578) to engineer L. plantarum strains (pSIP401-HA1-ZN-3) expressing recombinant HA1 proteins. Western blot analysis of cell fractions showed the presence of specific immunoblots in the cell wall fraction at positions corresponding to the sizes of the recombinant fusion proteins (Fig 5b). Immunostaining with FITC-labelled secondary antibody resulted in strong immunofluorescence of the engineered L. plantarum cells induced with the peptide pheromone IP-673, indicating the specific binding ability of the antibody to the recombinant HA1 protein surface displayed by the engineered L. plantarum cells only. No detectable signal was observed in uninduced L. plantarum cells (Fig 5c). These results indicated the precise expression and location of the antigen proteins.

**HA-specific IgG and slgA responses after oral administration and intranasal immunization**

We tested whether the recombinant HA1 proteins could increase the Ag-specific antibody responses induced by oral administration and intranasal immunisation of engineered L. plantarum strains pSIP401-HA1-ZN-3. BALB/c mice were orally and intranasally immunised with pSIP401-HA1-ZN-3 or pSIP401-ZN-3 using three bi-weekly inoculations, each consisting of three daily doses (Fig 6a). The development of anti-HA1 antibody responses in the gut and circulation was monitored over time as HA1-specific ILF IgA and serum IgG, respectively. The ILF HA1-specific IgA was significantly induced by day 21 in mice administered pSIP401-HA1-ZN-3 (Fig 6b). The ELISA results showed that oral immunisation with pSIP401-HA1-ZN-3 did not produce a significantly higher slgA level in BALF than that in the pSIP401-ZN-3 and PBS groups throughout the experiment (Fig 6c). Serum nuclease-specific IgG levels increased by day 22 in mice administered pSIP401-HA1-ZN-3, and these levels were further elevated by day 42 (Fig 6d).

Although intranasal immunisation with pSIP401-HA1-ZN-3 increased the slgA titre in ILF compared to that in the control mice, no significant difference was observed in the groups administered only pSIP401-
ZN-3 or PBS (Fig 6e). The mean ELISA titre of sIgA in the BALF collected from mice belonging to the intranasal immunisation groups suggests a significant increase in sIgA levels in the mice administered induced engineered \textit{L. plantarum} cells expressing HA1 than those in the control mice (P < 0.0001) (Fig 6f). In the case of serum IgG response, antibody titres were found only at the basal level. Consistent with BALF, the mean serum IgG titre was significantly higher in the mice intranasally administered with pSIP401-HA1-ZN-3 cells than that in the control mice (P < 0.0001) (Fig 6g). These results indicated that, pSIP401-HA1-ZN-3 is more effective in activating local mucosal immune response.

**Protection against lethal H1N1 virus challenge**

A week after the final immunisation, the mice were intranasally challenged with lethal doses of highly pathogenic H1N1 virus and closely monitored for 14 days for weight loss and mortality. After viral challenge, all mice experienced certain levels of body weight loss (Fig 7a and b); however, mice orally immunised with pSIP401-HA1-ZN-3 gradually recovered after 6 days with 60 % survival (Fig 7c). In addition, mice intranasally immunised with pSIP401-HA1-ZN-3 gradually recovered after 4 days with 100 % survival (Fig 7d). In contrast, control mice (PBS-treated) and mice orally immunised with the empty plasmid vector (pSIP401-ZN-3) died within 10 days of challenge. Interestingly, the protective efficacy was 50 % for the mice intranasally immunized with pSIP401-ZN-3. The mice were challenged with H1N1 and the viral titres in the lungs of infected mice (n = 3/group) were determined 4 days post-challenge (DPC) (Fig 7E). Mock-vaccinated (PBS) mice orally immunised with pSIP401-ZN-3 exhibited lung viral titres of approximately $5 \times 10^3$ TCID$_{50}$/mL on day 4 post-challenge, whereas mice intranasally immunised with pSIP401-HA1-ZN-3 showed no detectable virus in the lungs. Furthermore, at DPC 6, the challenge virus titre in the lungs was reduced in both pSIP401-ZN-3 and pSIP401-HA1-ZN-3 intranasally immunised mice groups compared to the mock-vaccinated mice, suggesting less replication/shedding in the lungs of vaccinated mice (Fig 7f).

To determine whether the antigen-specific mucosal immunity in mice induced by pSIP401-HA1-ZN-3 resulted in pulmonary tissue damage upon H1N1 infection, we challenged vaccinated mice with H1N1 and performed histopathological analysis 7 DPC (Fig 8). The H&E-stained lungs (Fig 8a) showed significantly greater inflammation at 7 DPC in the PBS-inoculated group or oral immunisation with pSIP401-ZN-3 group than that with oral immunisation with pSIP401-HA1-ZN-3. In addition, at 7 DPC, inflammation was highest in PBS-treated mice, followed by intranasal immunisation with pSIP401-ZN-3, and lowest in intranasal immunisation with pSIP401-HA1-ZN-3 (Fig 8b). These observations indicate that oral immunisation of mice with pSIP401-HA1-ZN-3 or intranasal immunisation with pSIP401-HA1-ZN-3 or pSIP401-ZN-3 provided protection against a lethal dose of the influenza virus.

**Discussion**

In recent years, probiotics have been widely used as therapeutic agents for the management of various disorders, primarily in humans. Additionally, probiotics have become increasingly popular in the veterinary field as an alternative to limit the use of antibiotics for treating and preventing infections and for growth
promotion as feed components for farming and companion animals [26]. Previous studies have shown that the direct effects of probiotics such as Lactobacillus, Bifidobacterium, Streptococcus, and Enterococcus include the upregulation of immunoglobulins such as IgA, downregulation of inflammatory cytokines, and enhancement of the gut barrier function [27, 28]. However, as each strain is characteristically different, it is essential to select and identify probiotics with the desired characteristics.

In this study, six L. plantarum strains isolated from faecal samples of healthy pigs and strains ZN-3, 1.191, and MQDR2 were confirmed to achieve high expression levels of target genes and proteins. Thus, ZN-3, 1.191, and MQDR2 are promising carriers for gene transfer and vaccine delivery. One of the most important criteria for identifying a potential probiotic is its ability to cope with acidic conditions and bile salts, which dictates its survival in the extreme environment of the GIT [29]. The acid and bile tolerance results indicate that the three L. plantarum strains of ZN-3, 1.191, and MQDR2 could survive the study conditions, suggesting that the three strains could pass through the stomach and function effectively. In addition, all three strains showed good resistance to different bile concentrations, probably due to the expression of bile resistance-related proteins in the bacterial cells [30, 31]. Our results are consistent with those reported earlier by our research team, wherein all studied Lactobacillus strains were able to resist both acidic and bile conditions [29, 32].

Inhibiting the growth of harmful bacteria is a major property of probiotics. In this study, we found that strains ZN-3, 1.191, and MQDR2 inhibited the growth of a common intestinal pathogen in a commercial pig farm based on inhibition zone assays. This antagonistic activity has mainly been ascribed to the production of antimicrobial substances or to the different metabolites produced by probiotic strains [29]. Although it was unclear in this study if the antimicrobial mechanism of the L. plantarum strains against gram-negative and gram-positive bacteria was due to bacteriocins or other metabolic products, it is highly likely that metabolic products, such as lactic acid, are primarily responsible for antimicrobial activity.

According to the defined priority scale for probiotic selection [33], a key evaluation criterion is the adhesion efficiency to the intestinal epithelium aimed at ensuring persistence in the gut environment and thus conferring beneficial effects to human and animal health [34]. This criterion, together with the ability to endure gastrointestinal conditions, was used to select strains with confirmed in vivo probiotic traits [35], which are currently active ingredients of probiotic products [34]. Many probiotic Lactobacillus strains have been shown to be adhesive, including the well-known probiotic L. johnsonii La1, LGG, L. casei Shirota, and L. casei Imunitass [31, 36, 37]. In this study, Caco-2 cells, which have been used as an in vitro model for the intestinal epithelium, were used to assess the adhesion ability of the isolated strains. The adherence of ZN-3 was almost twice that of 1.191 and MQDR2. Similar results were obtained using SEM analysis, to determine the in vitro epithelial cell adherence effect.

Different imaging modalities have been employed to monitor the fate of bacteria after administration in mice [22]. The current study was designed to investigate the colonisation (spatial and temporal) dynamics of L. plantarum ZN-3-IRFP713, 1.191-IRFP713, and MQDR2-IRFP713 orally inoculated once over a period of 24 h. IRFP713 enables excitation/emission in the near-infrared region (690/713 nm),
which minimises the autofluorescence of background tissue [22]. In practice, however, the
autofluorescence in this spectral range is still considerable and prevents the exact localisation of the
signal source and estimation of its strength [22]. The exact positioning of all the three species of
fluorescent bacteria was achieved using epifluorescence ex vivo imaging of the isolated intestines where
tissue thickness was minimal [22]. After administration, *L. plantarum* ZN-3-IRFP713, 1.191-IRFP713, and
MQDR2-IRFP713 showed similar GIT transit dynamics initially, although the bioluminescent signal
remained higher for *L. plantarum* ZN-3-IRFP713 than that for the other two recombinant strains.
Differences between the three strains were observed 24 h post-feeding, with a higher bioluminescent
signal in whole animals for *L. plantarum* ZN-3-IRFP713, associated with a significantly higher number of
bacteria and bioluminescent signals in the faeces of mice than that observed for the other two strains.
Faecal bacteria were enumerated after 48, 72, and 96 h, and *L. plantarum* 1.191-IRFP713 and MQDR2-
IRFP713 were eliminated more rapidly than *L. plantarum* ZN-3-IRFP713. In conclusion, the caecum and
colon were the predominant sites for persistent *L. plantarum* infection in mice. The application of
IRFP713-labelled bacteria has significant potential to allow further study of the interactions between
lactic acid bacteria and mammalian hosts. This system may be used to analyse gene expression during
transit and persistence in the digestive tract for in situ real-time investigation of promoter activities both
in vitro and in vivo, or for studying the impact of gene mutations on the in vivo course of transit and
persistence of lactobacilli [38].

The influenza virus, a respiratory pathogen, contributes to high rates of morbidity and mortality in
humans globally [39]. Although antiviral therapy is available, vaccination is still the most effective
strategy for the prevention and control of influenza [39–42]. In addition, since influenza viruses naturally
infect the mucosa, it is highly desirable to develop vaccines that can induce mucosal as well as systemic
immune responses [41]. The mucosa is the first physical barrier restricting the entry of pathogenic
microorganisms into animals, and the mucosal immune system provides primary protection against
microbial infection. SIgA is an important component of mucosal immunity. It is the most abundant
immunoglobulin isotype in human secretions [43] and effectively protects against influenza virus
infections [40]. It has been reported to be more important than IgG in protecting the upper respiratory
tract, specifically the nose and trachea [40, 44], primarily by reducing viral attachment and preventing
virus internalisation at the mucosal surfaces, thereby preventing infection [40, 45–47]. In this study, *L.
plantarum* ZN-3 was used as a vehicle for the delivery of truncated H1N1 HA1 antigen.

In the present study, immunization with pSIP401-HA1-ZN-3 significantly increased the specific slgA
response than that in the non-immunised group. Furthermore, HA1-specific slgA in faecal extracts
demonstrated that oral immunisation with pSIP401-HA1-ZN-3 in mice elicited significant mucosal
immunity in the GIT. Therefore, in this study, slgA was not detected in tracheal mucosal; however, 60%
mice were protected against viral challenge. One advantage of intranasal immunisation is its potential to
induce a mucosal immune response. In the present study, mice vaccinated intranasally with pSIP401-
HA1-ZN-3 elicited strong mucosal immune responses, such as the secretion of IgA in the respiratory
mucosa. Final immune protection was assessed using the homologous H1N1 virus challenge monitoring
after post-challenge indicated no significant decrease in the body weight of mice vaccinated with
pSIP401-HA1-ZN-3, and the mortality rate results revealed that pSIP401-HA1-ZN-3 could provide 100% protection efficacy against homologous H1N1 virus. Pathological analysis revealed that the *L. plantarum* ZN-3 expressing H1N1 HA1 protein provided protection against pathological injury of influenza virus target organs.

**Conclusions**

This study shows the feasibility of inducing protective humoral and mucosal immunity after oral and intranasal administration of pSIP401-HA1-ZN-3 without the use of an adjuvant. In summary, the presented oral and intranasal mucosal vaccination strategies using *L. plantarum* offer tremendous potential for the development of new influenza vaccines.

**Abbreviations**

BSA Bovine serum albumin

DMEM Dulbecco's modified Eagle medium

DPC Days post-challenge

ELISA Enzyme-linked immunosorbent assay

GIT Gastrointestinal tract

H&E Haematoxylin and eosin

H1N1 Influenza A

HRP Horseradish peroxidase

IIV Inactivated influenza A virus

ILF Intestinal lavage uid

LB Luria-Bertani

MRS de Man, Rogosa and Sharpe

PBS Phosphate-buffered saline

PCR Polymerase chain reaction

SDS-PAGE Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

sIgA Secretory IgA
swIAV Swine influenza A virus
WIV Whole inactivated virus

Declarations

Ethics approval and consent to participate

The Authorization of the Institutional Animal Care and Use Committee of Huazhong Agricultural University, China, authorized all protocols utilized in this work.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The authors will make available the raw data supporting the conclusions of this article upon reasonable request.

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Authors’ contributions

YFZ and MLJ conceived and designed the study. LY, JLZ, KH, XMS, YY, TW, and ZZ performed the experiments and analysed the data. MLJ and QZ contributed technical knowledge. YFZ wrote the paper. JLZ and LY were involved in the interpretation of the results and critical reading of the manuscript. All authors have read and approved the final manuscript.

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**Figures**

**Figure 1**

Immunofluorescence microscopy and flow cytometry analysis of expression of mCherry in six strains of *Lactobacillus plantarum*.

**Figure 2**

Probiotic properties of the three strains of *L. plantarum* isolated from the faecal samples of healthy pigs.
a, Effect of pH on the survival of the *L. plantarum* strains GG (log CFU/mL); b, survival of the *L. plantarum* strains in the presence of different concentrations of oxgall (log CFU/mL); c, minimum inhibitory concentration (μg/mL) of antibiotics for the *L. plantarum* strains; d, the antimicrobial activity of the *L. plantarum* strains against potential pathogenic bacteria.

**Figure 3**

Adhesion efficiency of three *L. plantarum* strains to Caco-2 Cells

a, Adhesion of representative *L. plantarum* strains on Caco-2 cell cultures observed under a microscope (magnification: 400×); b, adhesion efficiency of *L. plantarum* strains in Caco-2 cells. Values are expressed as mean ± standard error of mean; c, examination of the adherence of *L. plantarum* strains to Caco-2 cells using scanning electron microscopy (SEM); d, computational adhesion ability of the *L. plantarum* strains to Caco-2 cells using SEM; e, Epifluorescence microscopy (magnification: 400×) analysis of the adhesion efficiency of Mcherry-tagged *L. plantarum* strains to Caco-2 cells.

**Figure 4**

Transit and survival of IRFP713-expressing three *L. plantarum* strains in mice.

Mice were administered 5.0 × 10^{11} cells. a, The intestines were extracted at different time points and analysed using epifluorescence imaging; b and c, The number of viable bacteria (CFU/mg; c) in different parts of the intestine were determined as a function of time.

**Figure 5**

Expression of HA1 presented on the *L. plantarum* ZN-3 surface.

a, Schematic of pSIP401-SP1216-HA1-2578. A GS linker was inserted between DCpep and M cell-targeting peptide; b, the protein of interest was identified on western blot. A relevant immunoreactive band was produced by the cell precipitation of strain pSIP401-HA1-ZN-3 (lane 1) and total cell supernatant of strain pSIP401-HA1-ZN-3 (lane 3), but not strain pSIP401-ZN-3 (lanes 2 and 4). M: protein molecular weight marker; c Immunofluorescence microscopy pSIP401-ZN-3 (a1, a2, and a3) and pSIP401-HA1-ZN-3 (b1, b2, and b3) (magnification: 1000×).

**Figure 6**
Specific anti-HA1 antibody levels in mice orally and intranasally immunized with the engineered *L. plantarum* strains.

a, Experimental protocol for oral and intranasal immunisation of mice with pSIP401-ZN-3 and pSIP401-HA1-ZN-3; b, Specific secretory immunoglobulin A (sIgA) levels in intestinal lavage fluid from mice in each group after immunisation with recombinant *L. plantarum* strains; c, Specific sIgA levels in bronchoalveolar lavage fluid from mice in each group after immunisation with recombinant *L. plantarum* strains; d, Specific serum immunoglobulin G (IgG) levels in mice from each group after immunisation with recombinant *L. plantarum* strains.

**Figure 7**

Changes in body weight and survival rate of mice following a lethal challenge with influenza virus.

Seven days after the last immunisation, all mice in the study group were intranasally challenged with H1N1 virus. (a) Body weight and (c) survival of mice orally immunised with PBS, pSIP401-ZN-3, or pSIP401-HA1-ZN-3 were monitored daily for 15 days following virus challenge; (b) Body weight and (d) survival of mice intranasally immunised with PBS, pSIP401-ZN-3, or pSIP401-HA1-ZN-3 were monitored daily for 14 days following virus challenge. Virus titres in (e) the lungs of mice orally immunised with PBS, pSIP401-ZN-3, and pSIP401-HA1-ZN-3 at DPC 6, and in (f) the lungs of mice orally immunised with PBS, pSIP401-ZN-3, and pSIP401-HA1-ZN-3 at DPC 6 are shown. Asterisks indicate statistically significant differences compared with the PBS-treated group (*p<0.05, **p<0.01, ***p<0.001). PBS: phosphate-buffered saline

**Figure 8**

Histopathological lesions in the lungs of H1N1-challenged mice stained with haematoxylin and eosin (H&E).

a, H&E for oral administration group; b, H&E for intranasal immunization group; Scale bar (black) 200 μm; (red) 50 μm. PBS: phosphate buffered saline

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.
• SupplementaryFigure1.pdf