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Evaluation of the immunogenicity of auxotrophic *Lactobacillus* with CRISPR-Cas9D10A system-mediated chromosomal editing to express porcine rotavirus capsid protein VP4

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ABSTRACT

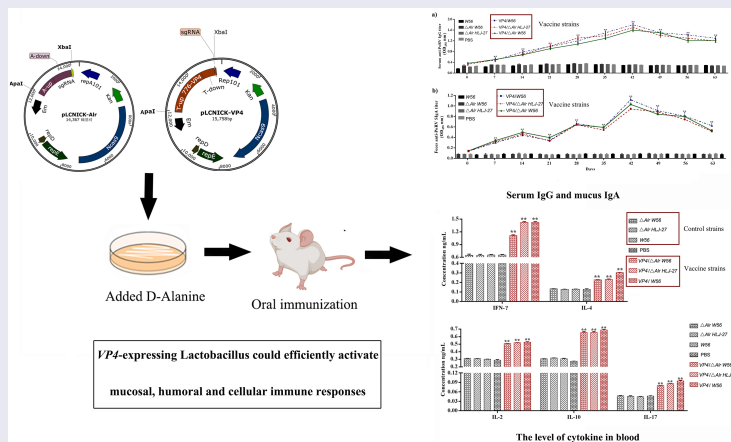
Porcine rotavirus (PoRV) is an important pathogen, leading to the occurrence of viral diarrhoea. As the infection displays obvious enterotropism, intestinal mucosal immunity is the significant line of defence against pathogen invasion. Moreover, as lactic acid bacteria (LAB) show acid resistance, bile salt resistance and immune regulation, it is of great significance to develop an oral vaccine. Most traditional plasmid delivery vectors use antibiotic genes as selective markers, easily leading to antibiotic accumulation. Therefore, to select a food-grade marker in genetically engineering food-grade microorganisms is vital. Based on the CRISPR-Cas9D10A system, we constructed a stable auxotrophic *Lactobacillus paracasei* HLJ-27 (*Lactobacillus* Δ Alr HLJ-27) strain. In addition, as many plasmids replicate in the host bacteria, resulting in internal gene deletions. In this study, we used a temperature-sensitive gene editing plasmid to insert the VP4 gene into the genome, yielding the insertion mutant strains VP4/ Δ Alr HLJ-27, VP4/ Δ Alr W56, and VP4/W56. This recombinant bacterium efficiently induced secretory immunoglobulin A (SIgA)-based mucosal and immunoglobulin G (IgG)-based humoral immune responses. These oral mucosal vaccines have the potential to act as an alternative to the application of antibiotics in the future and induce efficient immune responses against PEDV infection.

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Introduction

Rotavirus is one of the primary diarrhoea-causing viral pathogens found in animals worldwide. Its effects on livestock production often lead to substantial economic loss. There is currently no effective means to control and treat the diseases caused by this virus, and vaccination is still the only way to prevent infection [1].

Porcine rotavirus (PoRV) proliferates primarily in the villi of the small intestinal epithelium; this is, therefore, a key potential therapeutic target for oral vaccines. Nevertheless, it is challenging to deliver sufficient amounts of antigen to the mucosal surface by oral vaccination due to the stomach acidity [2]. Efforts are currently underway to develop a lactic acid bacteria (LAB) vaccine as an antigen delivery vehicle to

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overcome the limitations posed by the internal environment and adequately elicit a mucosal immune response [3]. LAB are acid and bile tolerant, which effectively prevents their degradation in the digestive tract and during immune tolerance reactions [4]. Furthermore, they are considered to be safe with beneficial effects on the health of humans and animals [5]. Their impacts range from maintaining intestinal flora balance, neutralizing food mutagens, and alleviating allergic reactions, to lowering serum cholesterol levels [6,7]. These microorganisms are ubiquitous in the intestine and widely utilized in food fermentation, vaccine production, health supplements, and feed additives [8–10]. There have been some studies regarding their effects on the immune cells of the gut. These probiotic bacteria can interact with gut-associated lymphoid tissue, Peyer's patches, and the intestinal lamina propria [11]. Cells involved in the innate immune response have been reported to be the main targets of LAB, inducing immune stimulation in the gut [12]. *Lactobacillus paracasei* (*L. paracasei*) is an example of one LAB strain with the advantage of being susceptible to heterologous delivery using a vector. It persists in intestinal and vaginal tissues, regulating the balance of flora and reducing the levels of *Escherichia coli* and *Shigella* spp. in the human gut microflora [13]. The researchers tested the probiotic effects of *L. paracasei* in mice, and the results showed that the strain could regulate the balance of intestinal flora and enhance the specific immune response in the body similar to antigens [14]. These studies indicate that *L. paracasei* strains show a powerful potential for application in vaccine delivery.

To date, most of the expression systems of LAB used plasmids as antigen delivery vehicles. However, there are several disadvantages to traditional plasmid expression as the recombinant bacteria generally require the selection pressure of an antibiotic [15,16]. The application of antibiotics is generally not accepted in genetically modified organisms, as it might cause the transmission of antibiotic resistance from one organism to another. Therefore, food-grade markers have been selected to replace antibiotic resistance markers in genetically engineering food-grade microorganisms, especially LAB. So far, there are many studies concentrated on complementary selection markers for LAB, the premise of which was to construct auxotrophic LAB [17,18]. The auxotrophic type of bacteria with certain genes deletion, such as housekeeping genes, which code for synthesizing substances to catalyze the essential metabolic reactions of bacteria. Mutation or deletion of these genes results in the absence of the corresponding products. Thus, bacterial strains in the external environment or basic media

cannot grow unless the corresponding substrate is supplemented. Alanine racemase (*Alr*), a key enzyme that participates in bacterial cell wall biosynthesis and catalyses the conversion of L-alanine (L-Ala) to D-alanine (D-Ala), is an important element for bacterial cell wall biosynthesis of peptidoglycan [19]. D-Ala does not exist naturally, so LAB lacking the *Alr* gene can only grow normally in MRS basal medium supplemented with D-Ala [18]. Recent studies have shown that deletion of the *Alr* gene affects the growth of strains such as *Lactobacillus plantarum*, *E. coli*, and *Bacillus subtilis* [20–22]. Therefore, the construction of *Alr*-deficient strains would provide new candidates for selecting food-grade screening markers.

In addition, many plasmids are replicated through the rolling circle mechanism in host bacteria, generating unstable single-stranded intermediates, resulting in internal deletions [4,23]. Therefore, as a way to remove the resistance selection pressure and guarantee stable gene expression, the integration of heterologous genes into the genome for expression has been proposed [4]. Currently, homologous recombination based on plasmids is utilized in LAB for genome editing. The disadvantages of this system include its time-consuming and complicated nature [24]. Single-stranded oligonucleotide-mediated recombineering has been applied to *Lactobacillus* to generate site-directed mutations [25], though the mutagenesis efficiency was lower [26]. Therefore, developing a faster and more accurate method to improve the editing efficiency of LAB is vital. The clustered regularly interspaced short palindromic repeat (CRISPR) system, an adaptive bacterial immunity system for prokaryotes to resist foreign gene invasion, has been successfully developed as a powerful tool for genome editing [27,28]. In CRISPR-Cas9 systems, the Cas9 nuclease is guided by CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA) to distinguish and break the target DNA based on Watson – Crick base pairing between the single-guide RNA (sgRNA) and protospacer of target DNA, with the feature of an adjacent protospacer adjacent motif (PAM) [29]. Chimeric sgRNA, an artificially designed, optimized form of the crRNA-tracrRNA complex, can simplify the design and operation process [30]. The CRISPR-Cas9-mediated genome editing system has been developed and widely applied in various organisms; yet there are currently few studies to apply it to gene editing of *Lactobacillus* [31]. The Cas9 protein causes DNA double-strand breaks (DSBs), which are repaired by nonhomologous end joining in eukaryotes; however, most bacterial strains lack this repair mechanism.

The occurrence of DSB could thus lead to the death of bacteria [32]. Song et al. utilized a Cas9^{D10A} nickase (Cas9^{D10A}) mutant that only triggered DNA single-strand breaks, as a replacement for the wild-type Cas9 protein, by optimizing the promoters of sgRNA and the Cas9^{D10A} protein to achieve fragment knock-out and site-specific insertion in the *Lactobacillus* genome, proving that the CRISPR-Cas9^{D10A} system was an effective tool to overcome the lethality induced by DSB [33].

VP4, the dimeric spike protein of the PoRV, could induce the production of neutralizing antibodies [34]. Studies have shown that newborn mice born from female mice immunized with this protein can gain passive immunity [35]. This study aimed to use the CRISPR-Cas9^{D10A} system to integrate the coding sequence of the neutralizing epitopes of a fragment of PoRV VP4 and the promoter P776 of the pyruvate hydratase gene [4], a constitutively highly expressed gene, into *Lactobacillus* W56, auxotrophic *Lactobacillus* W56 (*Lactobacillus* Δ Alr W56), and auxotrophic *Lactobacillus paracasei* HLJ-27 (*Lactobacillus* Δ Alr HLJ-27) isolated from pig intestines, in order to observe the expression of the heterologous protein VP4. The immunogenicity of these strains were then analysed in mice through oral administration with the strains VP4/ Δ Alr HLJ-27, VP4/ Δ Alr W56, and VP4/W56. The immune response induced by oral immunization with recombinant strains in mice could provide a reference for the immunization of piglets. The immunity assessment of piglets is in progress.

Materials and methods

Bacteria, viruses, and plasmids

In the laboratory, *L. paracasei* HLJ-27 isolated from pig intestines [36] and wild-type *Lactobacillus* W56 were grown in de Man, Rogosa, and Sharpe broth (MRS; Sigma-Aldrich, St Louis, MO, USA). *Lactobacillus* Δ Alr W56, stored in our laboratory, and Δ Alr HLJ-27 were grown in MRS medium containing 200 ng/mL D-alanine (Sigma-Aldrich, St Louis, MO, USA) at 37°C without shaking. The PoRV JL94 strain was isolated in our laboratory and stored at -140°C. Erythromycin (EMC; Sigma-Aldrich, St Louis, MO, USA) was applied at a concentration of 10 ng/mL to strains containing the pLCNICK-Alr/VP4 plasmid. The gene-editing backbone plasmid pLCNICK was gifted by Yang Sheng from the Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. All *E. coli* strains containing the gene-editing plasmids were grown

anaerobically at 30°C in Luria – Bertani (LB) broth containing 50 mg/L kanamycin.

Construction of the gene-editing plasmids

The gene-editing plasmids were constructed as shown in Figure 1. Figure 1a shows the deletion plasmid, and Table 1 lists the primers used. Cloning was performed following the procedure described below. The Alr gene was designed as the auxotrophic site. The backbone pLCNICK, containing the Cas9 gene, LAB replicon, EMC, two homologous arms (Has), and sgRNA, is a temperature-sensitive plasmid only able to replicate at 30°C. The upper and lower homology arms (Alr-up and Alr-down, respectively) were amplified from the genome of *Lactobacillus* W56 and extracted with the bacterial Genome Extraction Kit (Novizan, Nanjing, China). sgRNA was acquired from the plasmid pUC57-sgRNA, designed using predecessors targeting the Alr site and synthesized by Kumei Biotechnology Co., Ltd. (Jilin, China). All polymerase chain reaction (PCR) fragments were gel purified and fused to one fragment with the homologous sequence, by fusion PCR in the order of Alr-up, Alr-down, and sgRNA sequences. The Alr-up-Alr-down-sgRNA fragment was inserted into the vector pLCNICK at the *Apa* I and *Xba* I restriction sites to generate the deletion gene-editing plasmid pLCNICK-Alr-up-Alr-down-sgRNA (pLCNICK-Alr). The plasmid pLCNICK-Alr was identified using PCR, enzyme digestion, and sequencing (data not shown). The plasmid pLCNICK-VP4 (sequence as shown in Table 2) was constructed to exchange the heterologous genes into the genome (Figure 1b). The thymidylate synthase gene (*thyA*) was selected as the insertion site, and the expression cassette was composed of the P776 promoter, RBS, and VP4-flag sequences. The plasmid was successfully constructed and subjected to enzyme digestion and sequence identification.

Electroporation

Many plasmids of pLCNICK-Alr and pLCNICK-VP4 were extracted and then electrotransformed into competent cells, as previously described with some modifications [37,38]. In brief, bacterial cells were inoculated into MRS medium with (special MRS medium) or without (basic MRS medium) D-Ala at a ratio of 1% and cultured at 37°C until the OD₆₀₀ value reached 0.4–0.8. The cells were placed in an ice bath for 30 min and then centrifuged at 3500 rpm for 5 min to acquire the bacterial pellet. They were then washed with ice-cold EPWB and EPB buffer twice and resuspended in 1 mL

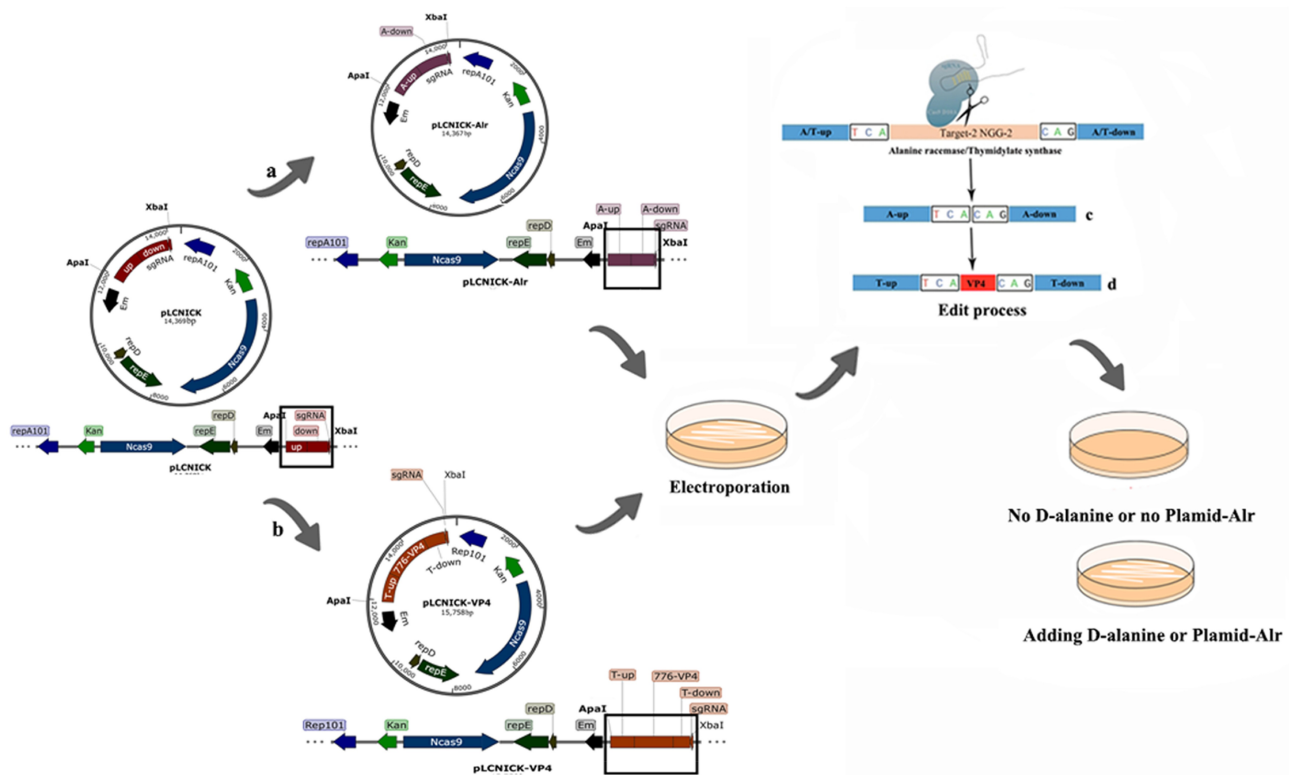


Figure 1. Schematic drawing of the construction of gene editing pLCNICK series plasmids (a,b) and the plasmids-mediated *Alr* knockout and *VP4* expression cassette insertion (replacement) in *Lactobacillus* chromosome (c,d). The black boxes shown in the figure are the replacement parts in different plasmids. a: the plasmid pLCNICK-Alr construction process. The PCR products of upstream (*Alr*-up) and downstream homologous arms (*Alr*-down) were acquired from the genome of *Lactobacillus W56* stored in our laboratory. The sgRNA sequence was designed by predecessors targeting the *Alr* site, synthesized by Kumei Biotechnology Co., Ltd (Jilin, China). The fragments were fused into a sequence with the repetitive sequence in the primers and inserted into the vector pLCNICK at *Apa* I and *Xba* I restriction sites, generating the gene editing plasmid pLCNICK-Alr. b: the plasmid pLCNICK-VP4 construction process. The genes coding the promoter P776 and the fragments RBS-VP4 were amplified with the plasmids pG-P776-EGFP and pMD19T-RBS-VP4, respectively, constructed in the laboratory. The upstream (*thyA*-up) and downstream homology arms (*thyA*-down) and sgRNA sequence are obtained as described earlier, yet the targeted site was *thyA*. The fragments were amplified by fusion PCR in the order *thyA*-up, P776, RBS-VP4, *thyA*-down, and sgRNA with the complementary sequence in the primers and inserted into the vector pLCNICK at *Apa* I and *Xba* I restriction sites, yielding the gene editing plasmid pLCNICK-VP4.

EPB buffer (200 μ L per tube) for electrotransformation. The electroporation process was performed on ice at 2.2 kV with approximately 1 μ g of gene-editing plasmid DNA added to 200 μ L of competent cells in a 0.4-cm cuvette. Transformed cells were cultured statically at 30°C for 4 h. Subsequently, they were plated on 5 μ g/mL EMC special MRS medium or basic MRS medium.

Construction of deletion strain *Lactobacillus* Δ *alr* HLJ-27 and insertion mutation strains using the CRISPR-Cas9^{D10A} system

A schematic diagram of the gene-editing process of the plasmid in the host strain is shown in Figure 1 (c: deletion; d: insertion). The recombinant strains with the gene-editing plasmids were cultivated for 48 h at 30°C following electroporation. Single clones were

picked and cultured in the corresponding MRS medium (special MRS medium or basic MRS medium), and the chromosomes were extracted for identification by PCR and sequencing with a pair of primers that targets the genome. Agarose gel electrophoresis identification results were used to confirm the results of deletions and substitution, and the hybrid mutant strains were acquired and named Δ *Alr* HLJ-27, *VP4*/ Δ *Alr* W56, *VP4*/ Δ *Alr* HLJ-27, and *VP4*/W56.

In order to obtain mutant strains, a purification process was conducted as follows: the hybrid strains were streaked on the corresponding MRS without antibiotics. Then, the purified strains were obtained, and the editing plasmids were eliminated. The hereditary stability of the recombinant strains was evaluated by continuous inoculation (1%, 20 times) and culturing (at 37°C in corresponding MRS medium), with every two

Table 1. The sequence of primers.

Target	Primer Sequences	Primerssequences
Alr	Alr-F Alr-R	GACCAGACCCACTGAAATCG AACCACCAACAGCAGAAGAA
thyA	thyA-F thyA-R	CAAAGAAGAAAACAAAACCTGAC ACGAACACTACAAATGCACATAAC
Alr-up	Alr-up-F Alr-up-R	tctttttctaaactagggcccGACCAGACCCACTGAAATCGC aagaaggtcctgTGATGGTGGCTCGTATGCC
Alr-down	Alr-down-F Alr-down-R	caccatcaCAGGACCTCTTTTCTAAAATTACCT gtcgggtcttttttgagCCAAACGAAATCGAATATTGCA
Has-up(thyA)	Has-up-F Has-up-R	TctttttctaaactagggcccGGATCCCATTCAGATCGCCA tatgcTGCTCTTCCCTCCAGTGGG
Has-down(thyA)	Has-down-F Has-down-R	cgacgataagtagACGAAGCAGATGCTTGGGC aaggatgatcacctctagaAAGCCTGATCGCACGCATAC
sgRNA(Alr)	sgRNA-F sgRNA-R	ggCTCAAAAAAAGCACCGACTCG aaggatgatcacctctagaCAGCTTTTCGCTGTGGTCAGT
P776	P776-F P776-R	ggaggggaagaagacaGCATATTACAAAAAGTCTCTGCTC tatcgtcactcctAAGGCACGTCCTTCTTAATGG
RBS	RBS-F RBS-R	gtgccttAGGAGTGACGATAAAGATGAAATAAA agcgaagcTCCATCAGCTTAACTGTTGTGGC
VP4	VP4-F VP4-R	aaagctgatggaGCTTCGCTCATTTATAGACAACACTT gcttcgtCTACTTATCGTCATCCTTGTAAATC

Restriction enzyme recognition sites applied for cloning are shown with the red. Homologous sequence was shown in bold.

Table 2. The sequence of VP4.

Target	Primerssequences
VP4	GCTTCGCTCATTTATAGACAACACTTACTAATTCATACACAGTCAATCTTTCTGAC GAAATTC AAGAGATTGGATCGGCTAAGTACAGGATGTTACTATAAATCCTGGTC CATTCCGCACAAACAGGTTATGCACCAGTTAATTGGGGAGCAGGTGAGACTAATG ACTCCACAACGTGCGAGCCGTTATTAGATGGTCCATACCAACCAACCACTTTCAA TCCACCAACAAGCTATTGGGTACTCTTGGCCAACTGTAGAGGGCGTAATGTT CAAGGAACAAACAATACCGATAGATGGTGGCCACTATACTAATTGAACCAACG TACAACAACACTAACAGAATATACAATCTTTTGGTCAGCAAGTAACCTTTGCGGT GGAGAATACGTCACAGACACAATGGAAGTTCATTGATGTGAGTACAACACTACGCC AACAGGAAGTTATACGACGACGACGACCACTGTTCTCTACACCAAAATTATACGCT GTAATGAAATTCAGTGGTGAATATATACATATAATGGAACCAACCAACGCAAC AACAGGATACTATTCAACTATTAATTATGACACAGTAAATATGACATCATTTTGTGA TTTTTATATTATACCAAGAAATCAAGAAGAAAAATGACTGAGTATATCAATCATG GATTACCTCTATACAAATACAAGGAATGTTGTGCCAGTATCTTTATCGGCTAGA GAGATAGTGACACACAAGAGCTGATTACAAGGATGACGACGATAAGTAG

Red shows the sequence of flag.

generations' genome extracted for identification by PCR. These mutant strains were then streaked onto MRS agar plates to verify the degree of bacterial demand.

Protein expression

In an effort to analyse the expression of VP4 protein in mutant bacterial strains, *VP4/ΔAlr W56*, *VP4/ΔAlr HLJ-27*, and *VP4/W56* were inoculated in a corresponding MRS medium at 37°C without shaking, and the bacterial precipitation was acquired by centrifugation at 12,000 rpm for 2 min. After lysis with lysozyme, ultrasonic breaking, and centrifugation, the proteins in the supernatants were detected by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 15% gel, followed by

a western blot assay. The proteins were further electro-transferred onto polyvinylidene fluoride membranes (Millipore, Milford, MA, USA). Next, the proteins were incubated with mouse anti-flag monoclonal antibody (Sigma, St. Louis, MO, USA) at a dilution of 1:1500 and then with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Sigma, St. Louis, MO, USA) as a secondary antibody with a dilution of 1:8000. The colour development of immunoblots was carried out using an ECL reagent (Thermo Scientific, Durham, NC, USA).

Immunization of mice

Five-week-old female pathogen-free BALB/c mice were purchased from Harbin Veterinary Research Institute, China, and raised under specific pathogen-free

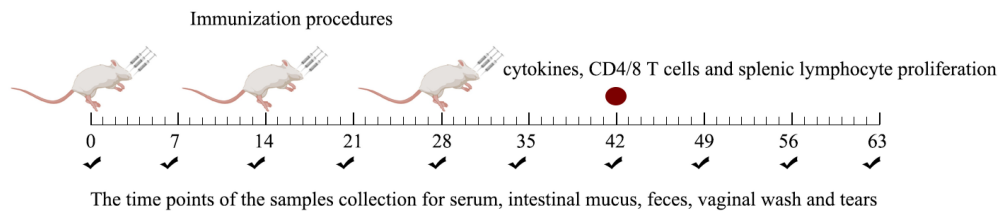


Figure 2. Immunization procedures of mice and the process of samples collection. Mice were immunized once a day for 3 consecutive days (days 0, 1, and 2) and boosted twice at 2-week intervals (days 14, 15, and 16 and days 28, 29, and 30). Feces, sera, vaginal fluid, and intestinal mucus were collected (black check marks) at different time points. The red circle means the time point to detect the level of cytokines and CD4/CD8 T-cell and lymphocyte proliferation. Sampling time points were calculated post the primary immunization.

conditions with free access to a standard chow diet and water. Prior to oral administration, the mutant insertion strains were inoculated into an MRS medium. The bacterial precipitate was collected by centrifugation at 4500 rpm for 5 min and resuspended in PBS to adjust the bacterial count to 2×10^{10} CFU mL⁻¹. A total of 240 mice were randomly divided into eight groups with 30 mice in each group: PBS, ΔAlr W56, ΔAlr HLJ-27, W56, VP4/ ΔAlr W56, VP4/ ΔAlr HLJ-27, and VP4/W56. The immunization doses and procedures are presented in Figure 2. All groups of mice were immunized for three consecutive days, with the first immunization on days 0, 1, and 2, booster immunization on days 14, 15, and 16, and second booster immunization on days 28, 29, and 30.

Serum, faecal, intestinal mucus, and vaginal lavage samples were collected from seven days post-immunization for 63 d and stored at -40°C until analysis. The preparation of samples prior to detection was slightly modified from the previously described method [39]. In brief, 0.1 g of faecal pellet was mixed with 500 μL faecal lysate in PBS containing 0.05 mmol/L EDTANa₂ and incubated overnight at 4°C . Intestinal mucus was acquired with a glass slide by scraping the gut mucosa, and the mouse vagina was repeatedly rinsed with PBS to collect vaginal lavage, which was then directly used for detection.

Detection of the IgG and SIgA antibody levels

The detection of the sera IgG level and the SIgA level in the vaginal lavage, intestinal tract, and faecal pellets, was performed by indirect ELISA. PoRV propagated on MA104 cells were utilized as an antigen with which to coat polystyrene microtiter plates, which were then incubated overnight at 4°C . Cell cultures were used as negative controls for the antigen. After discarding the waste solution, the well plates were washed three times with PBS containing 0.05% Tween 20 (PBST) for 5 min and blocked with 5% skim milk in PBS at 37°C for 2 h.

After the well plates were washed three times with PBST, the collected samples were incubated for 2 h at 37°C , and each sample repeated three times. The serum was diluted 10 times with 5% skim milk, and the vaginal lavage, intestinal tract, and faecal lysate were centrifuged to collect supernatant for use. Subsequently, an HRP-conjugated goat anti-mouse IgG or IgA antibody (Invitrogen, USA) was added to wells with a dilution of 1:5000 and incubated for 1 h at 37°C . The colour development was carried out under dark conditions with a TMB solution at a ratio of 1:1, and the OD₄₅₀ value was determined.

Neutralizing activity of the sera

In order to detect the neutralizing activity of the sera IgG in mice with the mutant strains VP4/ ΔAlr W56, VP4/ ΔAlr HLJ-27, and VP4/W56, on day 42 post-immunization, the serum was collected through eyeball blood and then diluted 2 \times serially. It was mixed with an equal volume of $200 \times 50\%$ tissue culture infectious dose (TCID₅₀) of PoRV propagated on MA104 cells and incubated at 37°C for 1 h. Then, 100 μL of the mixture was added to MA104 monolayer cells grown to 80% confluence, with each dilution having five replicates. PoRV-specific cytopathies were observed after incubation for 60 h in a 37°C , 5% CO₂ incubator. Data statistics were calculated by Reed – Muench.

Lymphocyte proliferation

Three mice in each group were sacrificed by cervical dislocation on day 42 after the primary immunization. Splenic lymphocytes were obtained under sterile conditions for the proliferation assay as previously described with minor modifications [40]. Briefly, splenocytes at a density of 5×10^6 cells/mL were added to 96-well plates with eight replicates and incubated with RPMI-1640 plus containing 20% (v/v) of foetal calf serum at 37°C in a 5% CO₂ incubator. The cells were

stimulated for 66 h with 5 µg/mL concanavalin A (ConA) as a positive control, RPMI-1640 medium as a negative control, and 1, 5, or 25 µg/mL purified VP4 protein, prepared and stored in our laboratory, as the specific antigen stimulation [41]. By measuring the absorbance at 490 nm, lymphocyte proliferation was assessed using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA).

T cell analysis

Flow cytometry was used to analyse the proportion of CD4+ and CD8+ in mouse spleen T lymphocytes by adjusting the density to 10⁶ cells/mL, as previously described [42]. Three mice in each group were sacrificed under aseptic conditions by cervical dislocation 42 d after primary immunization to acquire splenic lymphocytes. T cells were suspended in complete RPMI 1640, and 0.5 µL P-phycoerythrin (PE)-conjugated mouse anti-CD4 antibody was added. Allophycocyanin (APC)-labelled mouse anti-CD8-allophycocyanin antibodies were used as primary antibodies and incubated with the cells under dark conditions at 37°C for 30 min. Cells were then washed and suspended in PBS (pH 7.2) for analysis using a fluorescence-activated cell sorting (FACS) calibre cytometer. Cells were gated based on side scatter/forward scatter (SSC/FSC) parameters and CD4+/CD8+ T cells were gated based on APC/PE-labelled fluorescence parameters.

Cytokine detection

For cytokine detection, mouse serum was collected 40 d after primary immunization. Interferon (IFN)-γ, interleukin (IL)-2, IL-4, IL-10, IL-12, and IL-17 levels were tested using OptEIA Set Mouse series ELISA Kits (Biosource International, USA). The cytokine content was calculated using a linear regression equation and by drawing a standard curve of the absorbance values of the samples [43].

Statistical analysis

Data are represented as the mean ± standard error of triplicates and were analysed with GraphPad Prism 7. A one-way analysis of variance and Tukey's multiple-comparison test was used to determine the significance between treatment and control groups. A single asterisk represents $P < 0.05$, and double asterisks represent $P < 0.01$.

Results

Construction of the deletion and VP4 insertion mutant strains

The CRISPR-Cas9^{D10A} series plasmid pLCNICK-Alr or pLCNICK-VP4 was electroporated into *Lactobacillus* competent cells *HLJ-27* or Δ *Alr* *W56*, Δ *Alr* *HLJ-27*, and *W56*, respectively, yielding the mutant strains Δ *Alr* *HLJ-27*, *VP4*/ Δ *Alr* *W56*, *VP4*/ Δ *Alr* *HLJ-27*, and *VP4*/*W56*. The results of the agarose gel electrophoresis are shown in Figure 3. In the deletion strains, the lengths of amplified wild-type and mutant-type genome using the primers *Alr*-F/R were 2700 bp and 1500 bp (Figure 3a), while in the insertion strains, the lengths were 2583 bp and 1893 bp (primers *thyA*-F/R; Figure 3(b-d)), respectively. These results were in line with the anticipated results. The precise gene insertion and deletion were confirmed by sequencing, and the deletion and insertion mutant strains Δ *Alr* *HLJ-27*, *VP4*/ Δ *Alr* *W56*, *VP4*/ Δ *Alr* *HLJ-27*, and *VP4*/*W56* were successfully constructed.

The stability of the deletion and insertion expression cassette was assessed by inoculating the strains Δ *Alr* *HLJ-27*, *VP4*/ Δ *Alr* *W56*, *VP4*/ Δ *Alr* *HLJ-27*, and *VP4*/*W56*, continuously for 20 generations at a ratio of 1% in the corresponding MRS medium and PCR amplifying every two generations. The results revealed that these strains were stably inherited over 20 generations, with each passage containing the same DNA sequences (Figure 3(F-i)). In order to verify the requirement for D-Ala by the deletion and insertion strains Δ *Alr* *HLJ-27*, *VP4*/ Δ *Alr* *W56*, and *VP4*/ Δ *Alr* *HLJ-27*, the strains were inoculated in the corresponding liquid MRS medium on MRS agar plates, and the results are shown in Figure 4(a,b). The mutant strains grew only in the presence of D-Ala medium and not in the normal MRS medium.

Expression of VP4 by insertion mutant strains

To detect the expression of VP4 protein, labelled with a flag tag in the mutant strains, the *Lactobacillus* was processed with lysozyme and sonication to prepare protein samples. The mouse anti-flag-labelled monoclonal antibody was applied as the primary antibody for Western blot analysis. As shown in Figure 3j, the predicted immunoblot bands of VP4 protein were approximately 35 kDa in *VP4*/ Δ *Alr* *W56*, *VP4*/ Δ *Alr* *HLJ-27*, and *VP4*/*W56*, but no bands were observed in the control strains Δ *Alr* *W56*, Δ *Alr* *HLJ-27*, *W56*, and *HLJ-27*. Our data indicated that the insertion mutant strains could efficiently express VP4 protein.

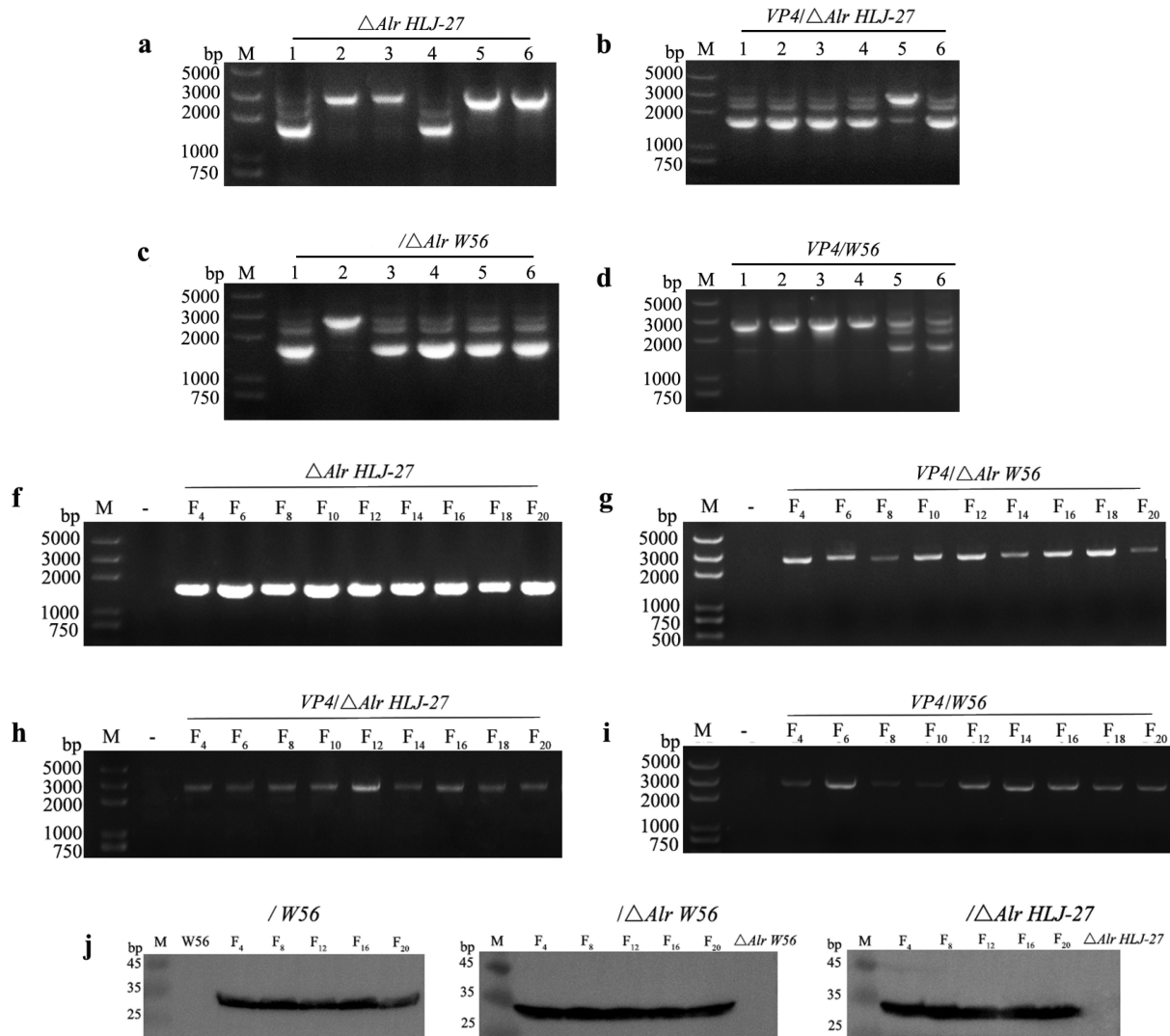


Figure 3. Identification and analysis of the stability *Lactobacillus* strains ΔAlr HLJ-27 and VP4/ ΔAlr W56, VP4/ ΔAlr HLJ-27 and VP4/W56. a: PCR amplification fragments of 1500 bp and 2700 bp correspond to the deletion mutant and wild type. b–d: PCR amplification fragments of 1893 bp and 2583 bp correspond to the insertion mutant and wild type. The stability was detected for every two generations using the primers Alr-F/R (a) and the A-F/R to identify PCR products of mutant strains genome DNA. f–i: Western blot analysis the protein stability of insertion mutant strain VP4/ ΔAlr W56, VP4/ ΔAlr HLJ-27 and VP4/W56. Expression of VP4 protein by the strains were detected with anti-flag tag monoclonal antibody every four generations for VP4/W56, VP4/ ΔAlr W56 and VP4/ ΔAlr HLJ-27, respectively (j).

Detection of IgG and SIgA antibody levels

The immunogenicity of insertion mutants VP4/ ΔAlr W56, VP4/ ΔAlr HLJ-27, and VP4/W56 was evaluated by measuring the levels of anti-PoRV IgG and SIgA antibodies. These antibodies were detected by indirect ELISA after oral immunization of mice as the standard to assess systemic and mucosal immune responses. Serum, faecal, intestinal mucus, and vaginal lavage samples to detect the anti-PoRV IgG and SIgA were collected on days 7, 14, 21, 28, 35, 42, 49, 56 and 63 post primary immunization. As shown in Figure 5a, there were no data differences observed between immune (VP4/ ΔAlr W56, VP4/ ΔAlr HLJ-27, and

VP4/W56) and control groups (ΔAlr W56, ΔAlr HLJ-27, W56, HLJ-27, and PBS; $P > 0.05$). A significant rise was observed in anti-PoRV-specific IgG antibodies in VP4/ ΔAlr W56, VP4/ ΔAlr HLJ-27, and VP4/W56 at 7 d post primary immunization, and their levels significantly increased after the booster immunization compared to that of the control groups (ΔAlr W56, ΔAlr HLJ-27, W56, HLJ-27, and PBS; $P < 0.01$). The levels peaked at 42 d post-immunization. The specific IgG level of VP4/W56 was the highest among the three groups, and the levels in the group VP4/ ΔAlr HLJ-27 was higher than that in VP4/ ΔAlr W56. There were no significant changes among the vaccine groups VP4/

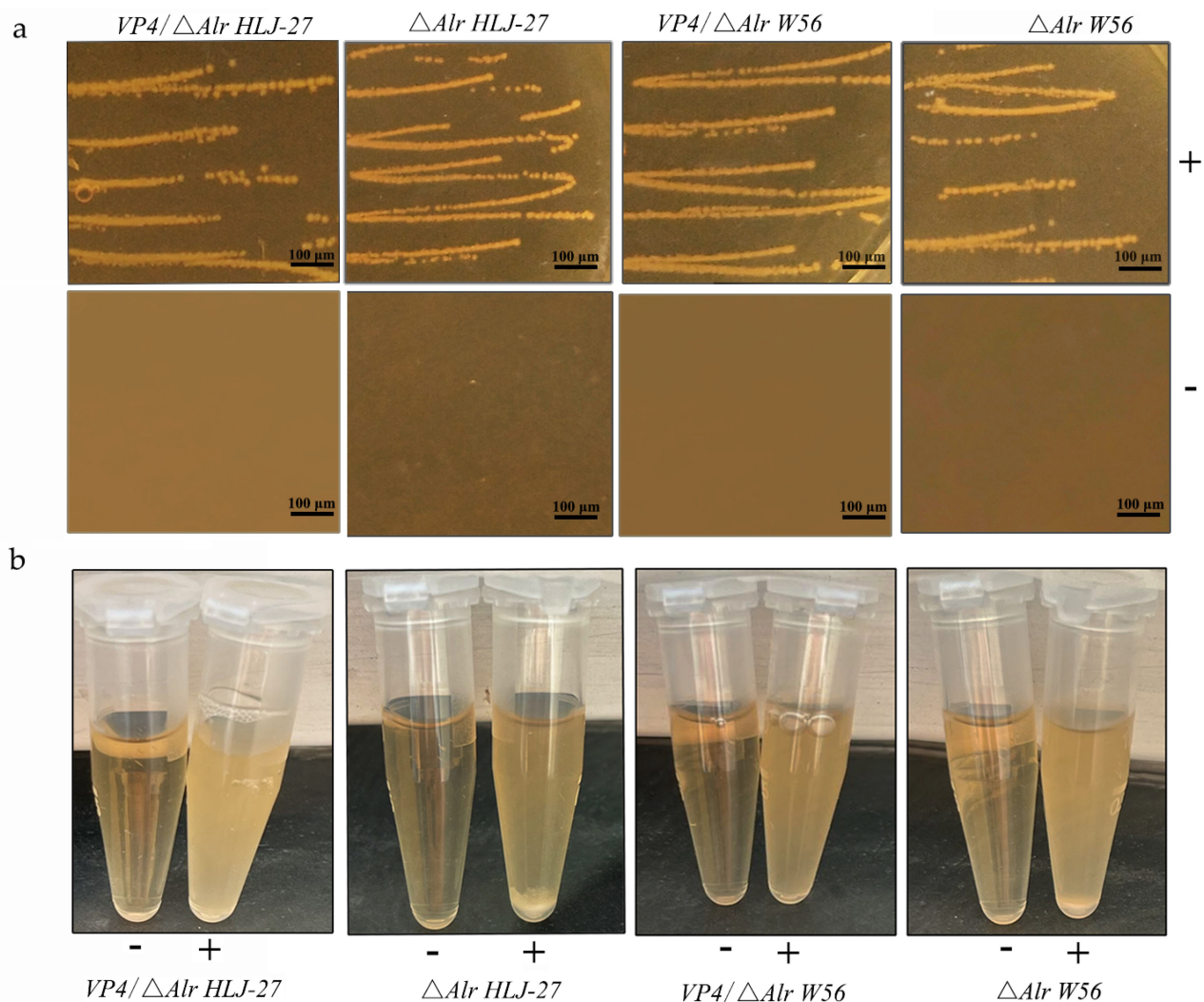


Figure 4. D-Alanine demand experiment of the mutant strains: “+” represents the MRS additional D-Alanine; “-” represent the basic MRS plates.

ΔAlr W56, *VP4/ΔAlr HLJ-27*, and *VP4/W56*. Moreover, at 42 d post-immunization, the PoRV-neutralizing activity of the mouse sera was detected using a virus neutralization assay, as shown in Figure 6. The results indicated that the sera IgG antibody activity in mice activated by *VP4/ΔAlr W56*, *VP4/ΔAlr HLJ-27*, and *VP4/W56* exhibited anti-PoRV-neutralizing activity significantly different from that obtained from the control groups (*ΔAlr W56*, *ΔAlr HLJ-27*, *W56*, *HLJ-27*, and PBS; $P < 0.01$). The antibody activity in the group *VP4/W56* was slightly higher than that in the *VP4/ΔAlr HLJ-27* and *VP4/ΔAlr W56* groups. However, there were no statistical differences among the three groups. As expected, the mutant strains groups *VP4/ΔAlr W56*, *VP4/ΔAlr HLJ-27*, and *VP4/W56* could elicit an anti-PEDV immune response.

Moreover, to determine the production of anti-PoRV-specific SIgA in faeces, vaginal lavage fluid, and intestinal mucus of immunized mice, an ELISA analysis was performed. As shown in Figure 5b, anti-PoRV-specific mucosal SIgA was detected on day 7 post primary immunization. The SIgA antibody levels were significantly increased in the faeces, vaginal lavage, and intestinal mucus of mice orally immunized with *VP4/ΔAlr W56*, *VP4/ΔAlr HLJ-27*, and *VP4/W56* compared to that in the control groups ($P < 0.01$). The order of the levels of SIgA antibodies was $VP4/W56 > VP4/ΔAlr HLJ-27 > VP4/ΔAlr W56$, and there were no significant differences among the three groups. After booster immunization on day 14, the levels of SIgA antibody increased quickly and reached the highest point on day 42. Our results revealed that the application of the insertion mutant strains could significantly induce systemic and mucosal antibody responses.

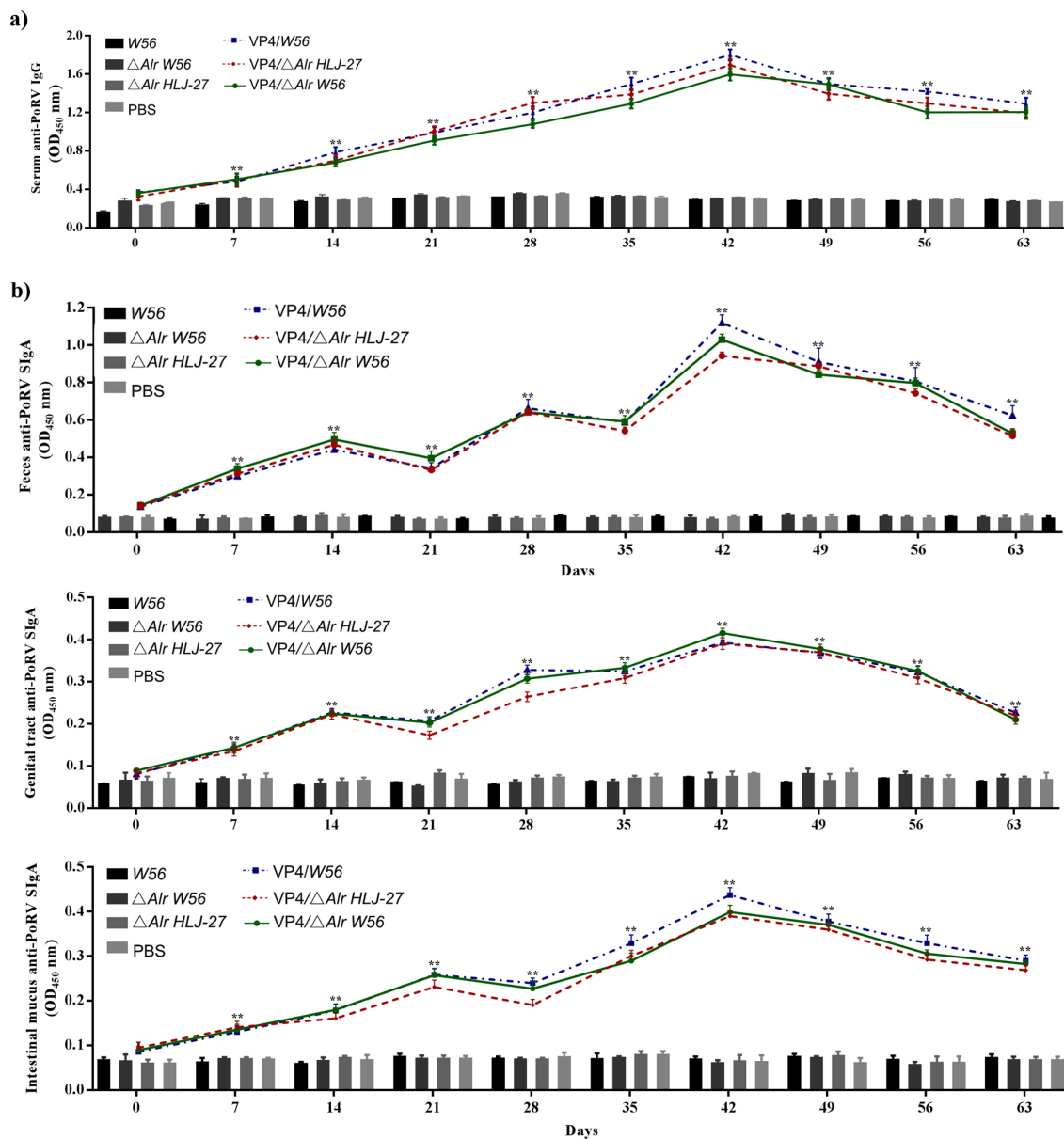


Figure 5. Anti-PoRV-Specific IgG antibody levels in the sera (a) and mucosal SIgA antibody in genital tract, intestinal mucus, and feces (b) by ELISA using PoRV as the coating antigen. Bars represent the mean SE in each group (** $P < 0.01$ compared to the control groups: Δ Alr HLJ-27, Δ Alr W56, W56 and PBS; Vaccine group strains: VP4/ Δ Alr HLJ-27, VP4/ Δ Alr W56 and VP4/W56).

Lymphocyte proliferation and T-cell analysis

To analyse the proliferation of splenic lymphocytes by the MTT assay, mice were sacrificed to obtain the cells and stimulated with the VP4 protein. As shown in Figure 7a, splenocyte stimulation index markedly increased in mice orally immunized with the insertion mutant strains VP4/ Δ Alr W56, VP4/ Δ Alr HLJ-27, and VP4/W56, but not in mice immunized with Δ Alr W56, Δ Alr HLJ-27, W56, HLJ-27, or PBS ($P < 0.01$). A significant change was noted in the insertion mutant strain groups stimulated with increasing

concentrations of purified VP4. These results indicated that the test bacterial strains could induce cellular immunity in mice. The effect of the cellular immune response of insertion mutant strains VP4/ Δ Alr W56, VP4/ Δ Alr HLJ-27, and VP4/W56 post immunization was determined by analysing the levels of CD4⁺ and CD8⁺ cells using a FACS calibre cytometer. The results indicated significant changes in the percentage of CD4⁺ and CD8⁺ in mice immunized with mutant strains VP4/ Δ Alr W56, VP4/ Δ Alr HLJ-27, and VP4/W56 than those in the control groups ($P < 0.01$), with no statistical difference among the mutant strains (Figure 7b-

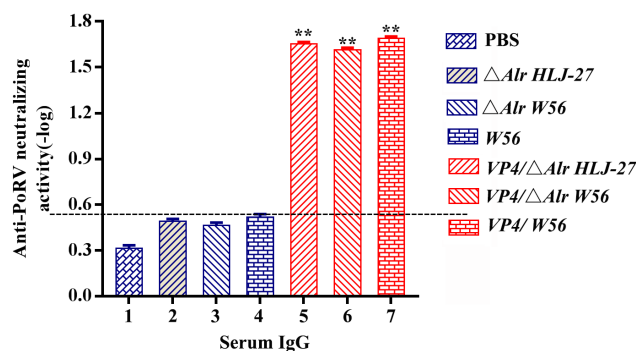


Figure 6. Levels of the anti-PoRV-specific neutralizing antibody titer in mice postimmunization serum. The results were determined by measuring the anti-PEDV IgG antibody and the anti-PEDV-neutralizing antibody titer with serial dilution in sera from immunized mice, ** $P < 0.01$ compared to the control groups.

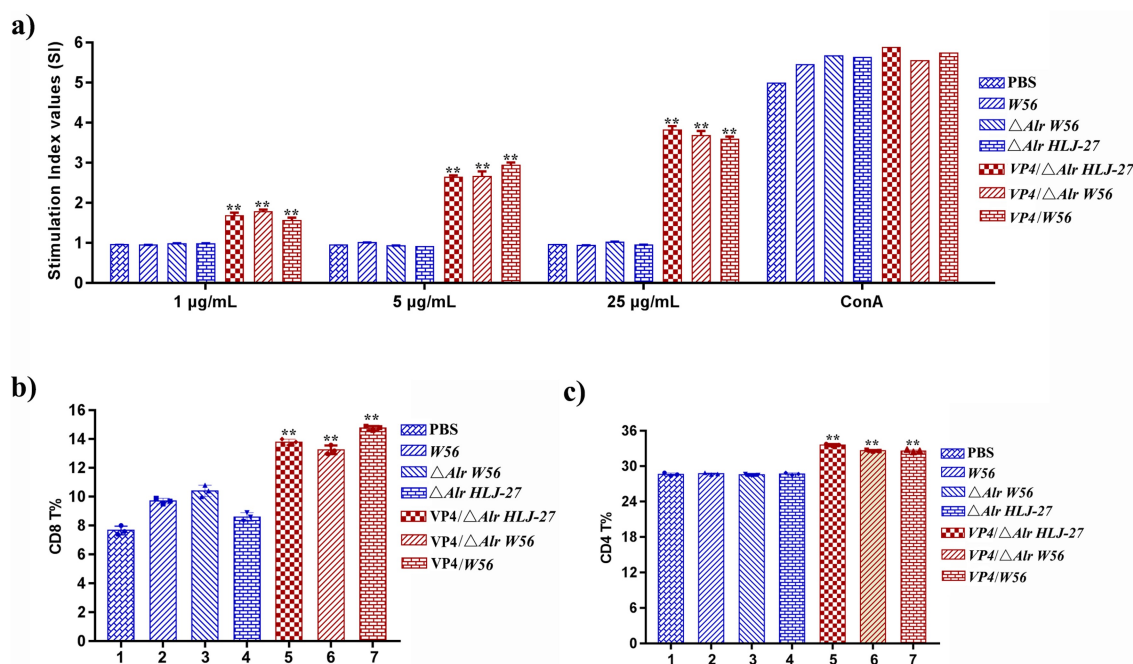


Figure 7. Determination of the level of lymphocyte proliferation (a) and specific T cell, CD8+% (b) and CD4+% (c) in splenic lymphocytes in immunized mice. The lymphocyte proliferation in immunized mice responding to PoRV VP4 antigen restimulation was determined by an MTT assay with ConA as a positive control. ** $P < 0.01$ compared to the control groups: Δ Alr HLJ-27, Δ Alr W56, W56 and PBS.

c). These results revealed that strains VP4/ Δ Alr W56, VP4/ Δ Alr HLJ-27, and VP4/W56 could induce cellular immunity in mice.

Analysis of cytokine responses

The cellular immune responses were evaluated by measuring the levels of IFN- γ , IL-2, IL-4, IL-10, IL-12, and IL-17 induced by the administered mutant strains VP4/ Δ Alr W56, VP4/ Δ Alr HLJ-27, and VP4/W56 using mouse series ELISA kits. As shown in Figure 8, a significant increase was noted in the levels of IFN- γ , IL-2, IL-4, IL-10, IL-12, and IL-17 at 42 d post-immunization in the serum of mice immunized with

VP4/ Δ Alr W56, VP4/ Δ AlrHLJ-27, and VP4/W56, compared to those in the control groups (Δ Alr W56, Δ Alr HLJ-27, W56, HLJ-27, and PBS; $P < 0.01$). There were no significant changes in levels between the strains VP4/ Δ Alr W56, VP4/ Δ Alr HLJ-27, and VP4/W56.

Discussion

Lactobacillus, a food-grade microorganism with adjuvant properties and weak immunogenicity, is widely used as a mucosal live vector vaccine. However, traditional expression systems are mostly plasmid-based with antibiotics as selection markers; these systems

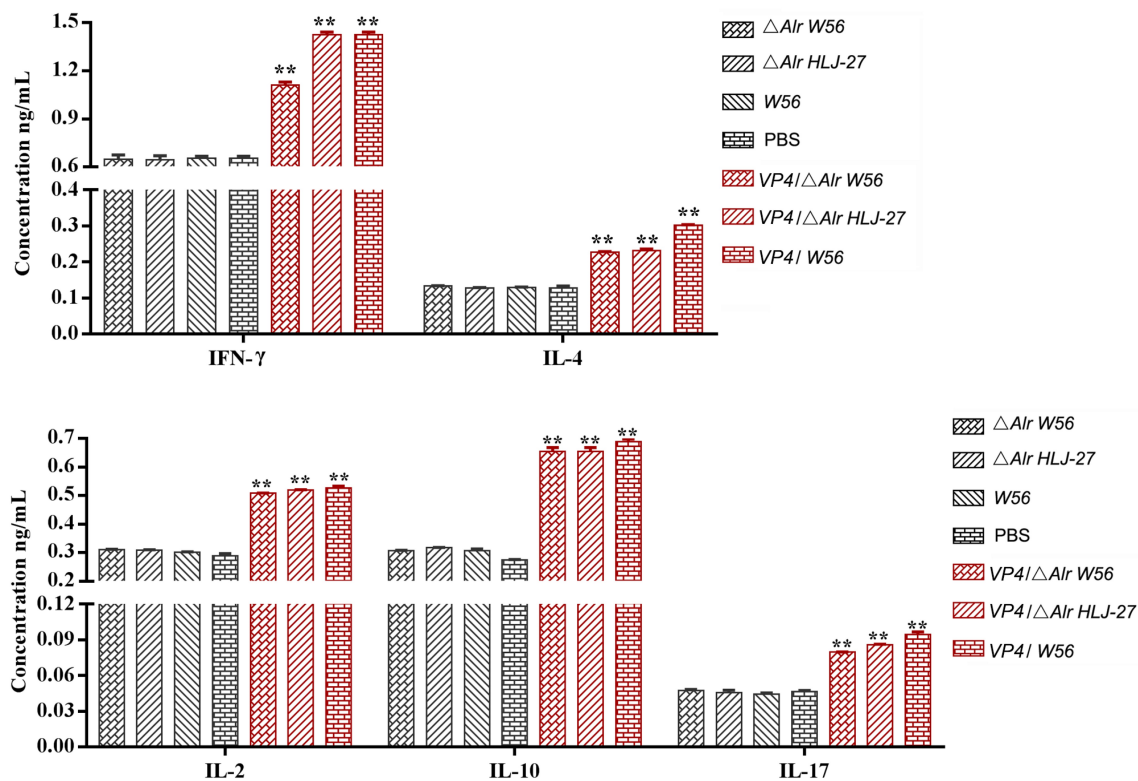


Figure 8. Determination the level of cytokine in blood in immunized mice with recombinant strains and PBS. The results are represented as the mean \pm SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared to the control groups: Δalr HLJ-27, Δalr W56, W56 and PBS.

typically exhibit unstable expression of heterologous proteins [4]. To solve these problems, an efficient genome editing tool was developed for chromosomal deletion and insertion with a site of interest. The rapid development of the CRISPR-Cas9 system for genetic editing has made modifying various organisms increasingly convenient, particularly for use in cases that are lacking in effective genetic tools for gene manipulation. The applications of CRISPR are mainly dependent on DSB induction by Cas9. However, DSBs have been observed to induce a high mortality rate in certain bacteria [44,45]. In this study, Cas9^{D10A}, a Cas9 protein mutant, was utilized to cause only DNA single-strand breaks to solve this problem. Song et al. revealed that the CPISPR-Ca9^{D10A} system could rapidly and efficiently manipulate chromosomal deletions and site-specific insertions [33].

In this study, *Alr* was selected as the auxotrophic site for deletion with CPISPR-Ca9^{D10A}. *Alr* participates in converting L-Ala to D-Ala, and D-Ala, a peptidoglycan component of the bacterial cell wall, is essential for bacterial growth. Studies have shown that only the double mutant of the *dadX* gene is responsible for *Alr* activity, and the *Alr* gene in *E. coli* is auxotrophic to D-Ala [46,47]. LAB, such as *Lactococcus lactis* and

Lactobacillus plantarum, encode *Alr* to catalyse the conversion of D-Ala and L-Ala [18,48]. Due to the stable expression of the *Alr* gene and the absence of D-Ala in most industrial fermentation media, the *Alr* gene of LAB is an important candidate marker for food-grade LAB that helps assess the biosafety of these microbes and prevent various side effects caused by antibiotic resistance in animals. Bron et al. utilized the gene, encoding *Alr* as a promoter-screening tool to clone a random fragment of the genome of *Lactobacillus WCFS1* upstream of the *Alr* gene without the promoter. The library was set up by introducing the plasmid into the *Lactobacillus* ΔAlr strain, and the effectiveness of the *Alr* complementary screening method in identifying the condition or compositional activity of the promoter was confirmed [48]. Nguyen et al. used the *Alr* gene from *Lactobacillus* to replace erythromycin in the pSIP expression vector and successfully overexpressed the β -galactosidase gene of *Lactobacillus reuteri* L103. This new selection marker provides a reference for and suggests the potential for its use as a replacement for antibiotics [49].

In this study, the CRISPR-Cas9^{D10A} gene-editing system was utilized to acquire an auxotrophic mutant, ΔAlr HLJ-27, by cutting a single strand of

the genome of *Lactobacillus* HLJ-27, isolated from piglet intestines. After the plasmid pLCNICK-Alr was electrotransformed into *L. paracasei* HLJ-27 to obtain the deficient strain, agarose gel analysis revealed the appearance of two bands, indicating that the cultured bacteria were a mixture of the mutants and wild type, consistent with the results of Song et al. [33]. The D-Ala demand experiment is an essential screening indicator for constructing *Alr* auxotrophic *Lactobacillus*. The demand experiment was performed with the deletion strain successfully constructed. The results showed that D-Ala played a vital role in the screening methods by replacing antibiotic screening of defective strains, making it possible to meet the requirements of safety standards for genetically modified biological products. Furthermore, the results revealed that the *VP4* gene could exist stably in the genome.

PoRV is a common virus that causes diarrhoea in pigs worldwide. It primarily infects the mucosal surfaces, especially the intestinal mucosal epithelium. *VP4*, an outer capsid protein of PoRV, could effectively stimulate the production of neutralizing antibodies, to provide protective immunity for animals [45,50]. For expressing the PoRV *VP4* protein, *Lactobacillus* was chosen as an oral vaccination vector, as it can elicit specific humoral and mucosal immunity safely [51]. Qiao et al. exploited the heat-labile B subunit of *E. coli* (LTB) and *VP4* protein for fusion expression using SDS-PAGE and western blotting analysis in *L. casei*. The mice were immunized orally with recombinant strains, and they observed higher levels of IgG and SIgA production [41]. Yin et al. expressed the *VP4* gene at the pyruvate hydratase site in the genome of *Lactobacillus*, and the results indicated that the heterologous gene could be inherited stably and induce an effective immune response [4]. In this study, in the laboratory-preserved *Lactobacillus* W56, the auxotrophic strains Δ *Alr* W56 and Δ *Alr* HLJ-27, were chosen as the host strains. The heterologous protein gene *VP4* was combined with the promoter *P776* of the constitutively expressed gene pyruvate hydratase from LAB to constitute the expression cassette for genome expression. The gene-editing plasmid was successfully constructed and then electrotransformed into the three competent cells. Two bands were present in the genome PCR amplification, in line with the principle of CRISPR-Cas9^{D10A} to cut one chain. The demand experiment results indicated that the insertion mutant strains *VP4*/ Δ *Alr* W56 and *VP4*/ Δ *Alr* HLJ-27 could only grow with exogenously added D-Ala. Genetic stability results also showed that these strains could be stably inherited. The insertion mutant strains *VP4*/W56, *VP4*/

Δ *Alr* W56 and *VP4*/ Δ *Alr* HLJ-27 could effectively induce the production of mucosal and humoral immune responses to fight against PoRV, as evidenced by significantly higher levels of serum IgG and mucosal SIgA. Moreover, genetically-engineered vaccines are safe, and the mode of administration is practical and convenient.

SIgA play a key role in protecting piglets from pathogens invading the gastrointestinal tract, and the production of SIgA reflects the capability of the body to fight viral invasion and the degree of a vaccine's protection [52]. In the present study, levels of anti-PoRV SIgA antibodies were observed in the faecal pellets, intestinal mucus, and genital lavage of the mice immunized with these insertion mutant strains. We observed that levels of SIgA antibodies increased and reached their peak at 42 d post primary immunization. The local production of SIgA could prevent pathogens from binding to small intestinal cells and deter the virus from crossing the mucosal barrier [53]. Although SIgA antibody levels reflect the primary mechanism of mucosal surface defence, serum-derived IgG also plays a vital role in immune defence. It impairs the ability of the virus to pass through the intestinal mucosa and blocks the spread of pathogens throughout the body. It also plays a synergistic protective role with SIgA [54]. In this study, the serum IgG level increased significantly after booster immunization, with a peak at six weeks, indicating that the inserted mutant strain could stimulate a systemic immune response and further prevent the invasion of antigens.

Studies have shown that protective immunity could be triggered by activated B cells secreting sIgA and activated T cells differentiation [55,56]. The research of Gao et. al revealed that the application of LAB as a delivery vehicle for oral vaccines could induce the occurrence of cellular immune responses in oral immunization experimental with mice [44]. In this study, to analyse the specific T-cell immune responses stimulated by the mutant strains in mice, T cell differentiation and cytokine production were tested. Flow cytometry data showed that the CD4+ and CD8+ T cells levels were significantly increased, and the cellular immunity of orally immunized mice was activated. This is a key method to assess the ability of vaccines to induce cellular immune responses [57]. Studies have shown that an appropriate dose of LAB promotes the differentiation of Th cells and the production of sIgA by B lymphocytes [58]. For analysing the ability of recombinant strains to trigger helper T cell differentiation (Th1 or Th2), the levels of cytokines induced by the mutant strains in mice were assessed [57]. CD4+ Th lymphocytes secrete cytokines that could participate in the regulation of

antibody production and cellular immune responses. In this study, we evaluated cytokine levels, including IFN- γ , IL-4, IL-17, IL-2, IL-10, and IL-12. We observed that oral immunization with *VP4/ Δ Alr W56*, *VP4/ Δ Alr W56*, and *VP4/ Δ Alr HLJ-27* could significantly induce cytokine production, revealing that these mutant strains could arouse Th1, Th2, and Th17-type cellular responses in vivo. The production of SIgA to fight pathogenic bacteria or foreign immunogens was thus via a T cell-dependent immune response [59]. In this study, the heterologous protein VP4 expressed by the *Lactobacillus*-induced Th1 and Th2-type immune responses, indicating that T cell-mediated immunity is key. Furthermore, these mutant strains could elicit an efficient lymphocyte proliferation response against the PoRV VP4 antigen. Our results suggest that *Lactobacillus* expressing the PoRV VP4 antigen might serve as candidate vaccine vectors and provide a novel construction method to induce potent immune responses against viral infections. However, these findings need to be confirmed via further challenge-protection experiments in piglets.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

Y.L. and X.W. conceived and designed the study. F.L., Z.M., L.S., X.F., X.L., Y.J., and W.C. performed the experiments. H. Z., L.W., X.Q., and L.T. analysed and interpreted the data. F. L. wrote the paper. All authors have read and agreed to the published version of the manuscript.

Ethical statements

Animal experiments were carried out in accordance with the recommendations in the institutional and national guidelines for animal care and use. The protocol was approved by the Committee on the Ethics of Animal Experiments of

Northeast Agricultural University, Harbin, China (2016NEFU-315, 13 April 2017). All procedures were performed under ether anaesthesia to minimize suffering.

Data availability statement

The data that support the findings of this study are available from the corresponding author, Yijing Li, upon reasonable request.

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