Recombinant *Porcine Rotavirus* VP6 Proteins Expressed in *Lactobacillus plantarum* NC8 Strain Induces Specific Mucosal and Systemic Antibody Production in Experimental BALB/c mice

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Abstract

Background

Rotaviruses are the main cause of animal and infant diarrhea and are widely distributed worldwide. In the pig industry, *porcine rotavirus* infection is a significant cause of mortality and morbidity; therefore, the optimization and well-organized distribution of vaccines for infection prevention is needed. Because immune responses related to protection are mainly mucosal in nature, the induction of mucosal immunity is significant for preventing *porcine rotavirus* infection.

Methods

The major protective VP6 antigens against *porcine rotavirus* (PRV) expressed by *Lactobacillus plantarum* NC8-pSIP409-pgsA-VP6-Dcpep were used to orally immunize mice. Western blot analysis and SDS-PAGE were used to confirm the expression of recombinant NC8-pSIP409-pgsA-VP6-Dcpep, and immunofluorescence was also used to verify its surface expression on *L. plantarum* NC8.

Results

High levels of mucosal IgA and serum immunoglobulin G (IgG) were produced in mice that were orally immunized with recombinant *L. plantarum* NC8-pSIP409-pgsA-VP6-Dcpep. Mice immunized with NC8-pSIP409-pgsA-VP6-Dcpep had higher IgA titers than mice immunized with NC8-pSIP409-pgsA and PBS. The mice orally immunized with NC8-pSIP409-pgsA-VP6-Dcpep demonstrated a virus challenge-induced mean fever peak of 38.6°C in the NC8-pSIP409-pgsA-VP6-Dcpep group and 41.1°C in the NC8-pSIP409-pgsA group 4 to 6 days after the challenge, and 7 to 9 days following challenge.

Conclusion

Our results confirmed that with regard to stimulating mucosal immunity, the VP6 genes of the *porcine rotavirus* (PRV) Chinese PRV isolate DN30209 strain expressed by *L. plantarum* NC8 is effective and that Dcpep is superior in its ability to stimulate mucosal immunity, suggesting that this approach can be implanted in pigs.

Background

Rotaviruses are members of the family Reoviridae. Depending on their particular inner capsid protein sequences, porcine rotaviruses are categorized into groups A, B and C [1]. Rotaviruses have a nonenveloped, double-stranded RNA genome that is composed of 11 segments enclosed by a triple-layered icosahedral capsid, [2-5]. The main source of acute diarrhea in piglets is *porcine rotaviruses*, which can cause high rates of mortality and morbidity [6-11]. In both pre- and post-weaning pigs, rotavirus Group A is the main source of rotavirus-associated diarrhea and accounts for 54% and 45% of the diarrhea experienced in those populations of pigs, respectively [1]. Some research has reported that in commercial pig farms, 89% of all rotavirus-associated diarrhea can be attributed to group A rotavirus

infections [12]. Rotavirus outbreaks are difficult to prevent because they are transmitted via the fecal oral route and can survive in the environment for a long period of time. Replication of the virion takes place in the intestinal villi in epithelial cells and destroys enterocytes mainly in the ileum and jejunum, leading to villous atrophy [13-16]. In addition, in the affected regions, nutrients cannot be absorbed or digested, causing severe malabsorption [13-16]. The control of rotavirus infections requires a solid understanding of the epidemiology of rotaviruses, which will contribute to prevention programs and improve the current vaccines. Presently, the obtainable vaccines cannot provide adequate protection. To activate immunocompetence, repeated administrations and a large vaccine dose are generally needed. This repeatedly causes unwanted clinical signs. To overcome these weaknesses and deliver antigens to the mucosal immune system, possible improvements to lactic acid bacteria (LAB) have been proposed.

Mucosal immunity plays an important role in protective immunity because rotaviruses are enteric pathogens. In the gut, innate immune responses initiate acquired immune responses and offer the first line of protection against pathogenic microorganisms. In addition, the only appropriate way of eliciting gut immunity is to prompt the immune response via oral immunization because this oral route assists in gut-associated lymphoid tissue (GALT) stimulation, improving anti-viral IgA production [17, 18].

Live vaccines stimulate the most efficient defensive responses, unlike heat-killed or recombinant antigen formulations, because they elicit both mucosal and systemic immunity [17, 18]. Repeated vaccinations and large vaccination doses are needed due to the challenges posed by oral vaccination, such as the fact that the gut environment habitually deactivates and/or denatures potential vaccinogens, causing fever and diarrhea, with the live vaccine often being shed in the feces [17, 18]. With regard to the stimulation of mucosal immunity, lactic acid bacteria (LAB) can be used to overcome these challenges [19].

Furthermore, several LAB strains are capable of colonizing and surviving the intestinal tract, stimulating nonspecific immunoadjuvant consequences [19]. It is essential to boost the immunogenicity of genetically engineered vaccines by combining them with suitable adjuvants because they are poorly immunogenic and composed of a single recombinant antigen. The dendritic cells play a central role in targeting peptide by directing innate and regulating adaptive/acquired immunity. By phage display the three DC-binding 12 number of residues in the peptide 12-mer: pep3 FYPSYHSTPQRP, pep12 AYYKTASLAPAE and pep18SLSLLTMPGNAS was recognized in 2004. Out of the three peptide pep3 later was renamed Dcpep, for the reason that Dcpep bound to both immature and mature DCs in a saturable way, than pep12 or pep18 and had the fascinating property to bind both human Monocyte-Derived Dendritic Cells (mDCs) and mouse CD11c⁺ I-A⁺ DCs [20]

Herein, we constructed recombinant *L. plantarum* NC8 strains expressing porcine rotavirus VP6 to test the effectiveness of the expression of the VP6 porcine rotavirus protein by *L. plantarum* for oral vaccinations and the performance of *L. plantarum* as an antigen delivery system. Following the administration of live bacteria to mice, the immunogenic ability of these recombinant strains was analyzed. The oral administration of the recombinant strain NC8-pSIP409-pgsA-VP6-Dcpep stimulated specific anti-rotavirus systemic and mucosal immune responses. In mice immunized with *L. plantarum* NC8 expressing the VP6-Dcpep fusion, the effectiveness of the immune response evaluation was greater than in mice

immunized with NC8-pSIP409-pgsA, indicating the effectiveness of Dcpep as a mucosal adjuvant. Using standard molecular biology techniques, the recombinant NC8-pSIP409-pgsA-VP6-Dcpep was constructed to recognize DCs and the VP6-antigen more efficiently in the intestinal mucosa, and its immunogenicity was further investigated. The results indicated that the in vivo antibody levels were substantially improved after BALB/c mice were immunized by the lavage administration of our recombinant NC8-pSIP409-pgsA-VP6-Dcpep.

Results

Cloning, expression and purification of Rotaviral target genes VP6 in Prokaryotic Expression System

The recombinant plasmids were identified by restriction endonuclease digestion enzyme *Xba* I and *Hind* III digestion. As shown in Fig. 1, two distinct bands appeared which were in accordance with the expected sizes. The results of sequencing were compared by NCBI Blast, and the homology was 100%.

Flow cytometry detection of surface fluorescence intensity of *L. plantarum* NC8-pSIP409-pgsA-VP6-Dcpep.

To confirm VP6 expression, the recombinant NC8-pSIP409-pgsA-VP6-Dcpep was SpplP-induced and cultured. Anti-VP6 serum from rabbits was used as an antibody; IgG from goats labeled with FITC was used as the secondary fluorescent antibody. FlowJo 7.6.1 software was used for detection. The results showed that the peak value of NC8-pSIP409-pgsA-VP6-Dcpep group was 92.7%, compared with 57.3% count in the NC8-pSIP409-pgsA group, as shown in Fig. 2. The expression of VP6 protein on the surface of *L. plantarum* could be inferred from the obvious right shift.

Expression of VP6-DCpep in *L. plantarum* NC8

NC8-pSIP409-pgsA-VP6-Dcpep supplemented with SPPIP was cultured in basal MRS medium. The SPPIP-induced NC8-pSIP409-pgsA-VP6-Dcpep was subjected to SDS-PAGE, and the corresponding VP6 recombinant proteins were confirmed at 48 kDa by Coomassie blue staining analysis (Fig 3 SDS-PAGE lanes 1-3). Then, the anti-VP6 antibodies were observed by Western blot analysis by running the gels in parallel and transferring them to nitrocellulose membranes. The results are shown in Fig 3, in the Western blot lanes 3 and 4. The SDS-PAGE results showed that there was a clear band at 48 kDa. The Western blot results showed a clear protein band at 48 kDa, which was consistent with the SDS-PAGE results. As a negative control, NC8-pSIP409 had no imprinting band after Western blot analysis, as shown in Figure 3 (lane 1). These results indicated that VP6 protein was expressed on the cell wall surface of *L. plantarum* NC8, which was reactive.

Immunofluorescence analysis

VP6 was detected on the surface of induced cells cultured with NC8-pSIP409-pgsA-VP6-Dcpep (Fig 4). No immunofluorescence was observed for non-induced NC8-pSIP409-pgsA, although it was incubated in a similar manner (Fig 4 NC8-pSIP409-pgsA). Immunofluorescence analysis of SpIP-induced *L. plantarum* NC8 indicated that there were green-yellow fluorescence reactions on the surface of SPPIP-induced recombinant NC8-pSIP409-pgsA-VP6-Dcpep.

Antibody responses following oral immunization

To assess the capability of the recombinant *L. plantarum* NC8-pSIP409-pgsA-VP6-Dcpep to induce systemic and mucosal immunity, the presence of anti-VP6 IgG and IgA antibodies was determined. After immunization, the serum, feces and bronchoalveolar lavage fluid of the mice were collected, and the expression levels of specific IgG and sIgA were detected by indirect ELISA. The results are shown in Fig 5 A. Compared with the empty vector group, the NC8-pSIP409-pgsA-VP6-Dcpep group had significantly increased serum IgG content ($P < 0.01$). The NC8-pSIP409-pgsA-VP6-Dcpep group feces and bronchoalveolar lavage fluid sIgA content was significantly increased compared with that of the empty vector group ($P < 0.05$, $P < 0.01$). There were no significant differences in the NC8-pSIP409-pgsA-VP6-Dcpep group, and the results are shown in Fig 5 B. The above results indicate that the NC8-pSIP409-pgsA-VP6-Dcpep group had significantly increased expression of specific IgG and sIgA and had good anti-microbial infection ability.

Effect of NC8-pSIP409-pgsA-VP6-Dcpep on Specific Antibody in Mice

To further evaluate the role of NC8-pSIP409-pgsA-VP6-Dcpep in humoral immunity, we used an ELISA kit to detect the levels of the cytokines IFN-gamma and IL-4 in the serum. The results showed that compared with the empty carrier group, the content of IL-4 in the NC8-pSIP409-pgsA-VP6-Dcpep group was significantly greater ($P < 0.001$). There was also significant difference ($P < 0.001$) in IFN-gamma between the NC8-pSIP409-pgsA-VP6-Dcpep group and the blank carrier group (Fig 6).

Effect of NC8-pSIP409-pgsA-VP6-Dcpep on Mouse B Cells

To further evaluate the effect of NC8-pSIP409-pgsA-VP6-Dcpep on mouse B cells, we used flow cytometry to detect the expression of intracellular IgA in B cells of mouse PP, as shown in Fig 7, compared with the empty vector group, the NC8-pSIP409-pgsA-VP6-Dcpep group. The expression of B220+IgA+ B cells in the PP node was significantly increased ($P < 0.05$).

Vaccinated mice against PRV challenge

To assess the protective effect of NC8-pSIP409-pgsA-VP6-Dcpep, a viral challenge experiment was performed in mice against PRV infection postvaccination, we infected mice with rotavirus after second booster immunization. As shown in Figures 8, NC8-pSIP409-pgsA-VP6-Dcpep groups were able to produce immunity in mice. The results demonstrated that an effectual defense rate of 81% against PRV challenge was detected in mice immunized with the NC8-pSIP409-pgsA-VP6-Dcpep, which was higher than the 59.2% in the mice immunized with NC8-pSIP409-pgsA; the control group of mice that received only PBS displayed severe clinical symptoms of infection after the viral challenge (Fig 8). The mice orally immunized with NC8-pSIP409-pgsA-VP6-Dcpep demonstrated a virus challenge-induced mean fever peak of 38.6°C (result not shown) in the NC8-pSIP409-pgsA-VP6-Dcpep group and 41.1°C in the NC8-pSIP409-pgsA group 2 to 5 days after the challenge, and 6 to 9 days following challenge, all mice were survived and recovered gradually from the clinical signs of PRV infection. A longer fever period with a mean fever peak of 42.1°C (result not shown) was observed in the PBS group of mice following viral infection until euthanasia (Fig 8).

Because the lactic acid bacteria expressing rotavirus mainly invade the intestinal epithelial cells, we selected the duodenum, liver, lung and spleen for pathological analysis. Compared with the control, the NC8-pSIP409-pgsA-VP6-Dcpep group had intact intestinal villi, clear liver structure, clear alveolar margin in the lung, clear spleen red pulp and white pulp, and no pathological changes (Fig 9). The pathological changes in PBS administered mice group were vivid such as congestion of intestinal wall, edema, vacuolar degeneration and integrity destruction of enteric epithelium, compared to the NC8-pSIP409-pgsA-VP6-Dcpep and NC8-pSIP409-pgsA groups. In the NC8-pSIP409-pgsA group the border of villi was still clear, but was less vacuolar degeneration and edema of intestinal wall occurred compared to NC8-pSIP409-pgsA-VP6-Dcpep which had intact intestinal villi.

Discussion

The main sources of acute diarrhea in piglets are porcine rotaviruses, and infection with these viruses can lead to mild to severe diarrhea with high mortality and morbidity rates. Porcine rotavirus infection has been an economic concern worldwide among pig breeders. The major prophylactic method for the prevention and control of porcine rotavirus infections is vaccination. There are a number of advantages offered by mucosal immunization that are not offered by other routes of antigen delivery, such as the ability to stimulate both the systemic and local immune responses, cost effectiveness and the ease of administration [21-23].

Specific antibodies are particularly important for humoral immunity therefore specific IgA anti-VP6 protein levels were observed to induce mucosal immune responses from different mucosal surfaces. The oral administration of recombinant *L. plantarum* NC8-pSIP409-pgsA-VP6-Dcpep stimulated both mucosal sIgA and systemic IgG immune responses. Compared to the control group the NC8-pSIP409-pgsA-VP6-Dcpep-immunized mice the expression level of specific IgG in serum and sIgA in intestinal lavage fluid

were significantly higher than those in empty vector group the specific sIgA for VP6 could be isolated from the gastrointestinal tract or faeces. This study recommended protecting pigs from porcine rotavirus infections using vaccination with recombinant *L. plantarum* NC8-pSIP409-pgsA-VP6-Dcpep because this recombinant effectively provoked a significant and specific anti-VP6 IgA response.

The serum from mice immunized with *L. plantarum* NC8-pSIP409-pgsA-VP6-Dcpep had higher titers of anti-VP6 IgG than the control group. *L. plantarum* NC8-pSIP409-pgsA-VP6-Dcpep even stimulated elevated IgA levels specific for VP6 compared to the serum from mice immunized with the control NC8-pSIP409-pgsA because of the Dcpep mucosal adjuvant. The use of the specific mucosal adjuvanticity of Dcpep was safe, and to stimulate specific anti-porcine rotavirus immunity, an efficient mucosal adjuvant can be used in combination with VP6. When activated in vivo, DCs will stimulate T cells to differentiate into helper T cells, such as Th1 and Th2, which play an important role in secreting IFN-gamma and IL-4 cytokines, respectively. NC8-pSIP409-pgsA-VP6-Dcpep stimulated T cells to secrete more IFN-gamma and IL-4, which resulted in T cells differentiating into Th1 and Th2. This result was consistent with the previous results that Pereira et al. [24].

On the other hand, we also evaluated the humoral immune response, namely the activation of B cells and the expression level of intracellular IgA antibodies. The results confirmed a significant increase in the percentage of IgA+B220+ B cells, indicating a certain immunoregulatory function on the host immune system. The mice orally immunized with NC8-pSIP409-pgsA-VP6-Dcpep demonstrated a virus challenge-induced mean fever peak of 38.6°C in the NC8-pSIP409-pgsA-VP6-Dcpep group and 41.1°C in the NC8-pSIP409-pgsA group 4 to 6 days after the challenge, and 7 to 9 days following challenge, the surviving mice recovered gradually from the clinical signs of PRV infection. Compared with the control, the NC8-pSIP409-pgsA-VP6-Dcpep group had intact intestinal villi, clear liver structure, clear alveolar margin in the lung, clear spleen red pulp and white pulp, and no pathological changes.

Conclusion

The VP6 genes of the porcine rotavirus (PRV) Chinese PRV isolate DN30209 strain expressed by *L. plantarum* NC8 can stimulate antibodies in mice that may be used for the development of vaccines against rotavirus-induced diarrhea in piglets. Rotavirus genes of other serotypes can also be expressed by the *L. plantarum* NC8 expression system for vaccines or other purposes.

Methods

Chemicals and enzymes

All chemicals for the enzyme assay; the cloning, expression, and purification of recombinants; all restriction enzymes such as (*Hind* III and *Xba* I), DNA polymerase, RNase-A and proteinase were purchased from Beijing Kang Century Biotechnology Co., Ltd and media components; kits; reagents/adjuvants; and proteins were purchased from Beijing Soleboard Technology Co., Ltd. and were

provided by the **Laboratory of Jilin Provincial Engineering Research Center of Animal Probiotics**, in the **Jilin Agricultural University of China**.

Strains and culture conditions

L. plantarum NC8 was kindly provided by the **Laboratory of Jilin Provincial Engineering Research Center of Animal Probiotics**. *L. plantarum* was grown anaerobically in MRS broth at 37°C without shaking; erythromycin (EM) was added when appropriate to the culture medium at a 10 µg/ml final concentration, and growth was monitored for 12 h. Then, single colonies were grown in MRS liquid medium according to the same procedure as described by [25, 26]. In the case of interventions with *L. plantarum*, cells were harvested by centrifugation and washed three times with a physiological salt solution (buffer solutions). *Porcine rotavirus* isolate DN30209 VP6 gene, complete cds (**GenBank: JN977137.1**) strains were purchased from HaiGene China Co. and provided by the Laboratory of Jilin Provincial Engineering Research Center of Animal Probiotics at the Jilin Agricultural University of China. The expression vector pSIP409-pgsA was obtained from previous work [27] in which it was used for the heterologous expression of target genes (VP6 and NC8) in a prokaryotic expression system.

Mice

In this model, we used *Rotavirus* antibody-free adult female Balb/C mice that were 7 weeks of age (weighing 25-30 g) obtained from Beijing Huafukang Biotechnology Co., Ltd. China; these mice are used to investigate the immune responses to the recombinant NC8-pSIP409-pgsA-VP6-Dcpep strain. Forty-five (45) mice were randomly divided into three groups and were housed under standard conditions with free access to food and water. This study was carried out in agreement with the principles established by Jilin Agriculture University Changchun China and guide for the use of laboratory and care animals and all experimental protocols were approved by a Jilin Agriculture University (No. JLAU08201007). The mice were euthanized by using cervical dislocation method after finishing the experiment.

Mouse anti-VP6 antibodies

The detection of the surface fluorescence intensity of NC8-pSIP409-pgsA-VP6-Dcpep by flow cytometry was performed as follows: NC8-pSIP409 and NC8-pSIP409-pgsA-VP6-Dcpep were induced in overnight culture. The bacterial solution was centrifuged for 1 minute at 12 000 rpm and then precipitated and washed three times with PBS containing 0.5% BSA. The sediment was collected and rabbit anti-VP6 serum (1:2000 dilutions) was added and mixed well. Then the solution was incubated on a 60 rpm shaking bed at room temperature for 1 hour. After washing three times with PBS containing 0.2% Tween 20, the sediment was collected and FITC-labeled goat anti-rabbit IgG fluorescent antibody (1:100 dilution) was added; the solution was incubated at room temperature for 1 hour at 60 rpm. After 3 more washes with PBS containing 0.2% Tween 20, 300 L PBS was added, and the solution was moved into the flow

tube after passing through the membrane. Flow cytometry was used to detect the results. The anti-porcine *rotavirus* VP6 (anti-VP6) serogroup C antibody used in the immunofluorescence and Western blot analyses was purchased from Arigo Biolaboratories.

Expression plasmid construction

Polymerase chain reaction (PCR) of VP6 gene proteins

A gene fragment obtained from the genome of PRV strain DN30209 encoding the main structural polypeptide of VP6 that was 1356 bp in length was amplified by polymerase chain reaction (PCR) (porcine rotavirus isolate DN30209 VP6 gene, complete cds **GenBank: JN977137.1**). Specific primers were designed with Primers Premier 5.0 to amplify the full length of the 48 kDa VP6 protein gene based on the sequence data given for the VP6 gene fragment, which was 1356bp of the porcine rotavirus genome. The restriction sites were as follows: 5' GGATCCCAAGACAACACCTGGTATG 3' (forward) containing a *Hind* III site and CTCGAG 3'GCTGACACCTTCGACTTTC 5' (reverse) containing an *Xba* I site. The PCR was carried out in a standard 50 ml reaction for the amplification of the VP6 gene using the primer sets following standard protocols. The tubes were then placed in a thermocycler, and the PCR conditions for amplification were as follows: predenaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 cycles and extension at 72°C for 10 minutes. After the reaction was finished, the 2 L PCR product was electrophoresed on a 0.8% agarose gel.

Electroporation of *L. plantarum* was carried out as follows: 10 µl of plasmid was gently added to 100 µl of *L. plantarum* NC8, mixed gently for 5 min at 4°C and subjected to an electric pulse. The mixture of plasmid DNA and *L. plantarum* NC8 was then anaerobically incubated without EM in MRS medium for 2 h at 37°C. Recombinant NC8-pSIP409-pgsA-VP6-Dcpep was selected on MRS agar medium including EM. The transformants with *L. plantarum* NC8 sequences were verified by plasmid DNA sequencing.

Flow cytometry

The cells were carefully washed with PBS, centrifuged at 12000 rpm for 5 min at 4°C, and washed twice with PBS; then, 10 µL of cells were added to each flow cytometry tube and incubated at room temperature for 20 min in the dark. The cells were washed twice with cold PBS, centrifuged at 4°C and 2000 rpm for 5 min; the cell suspension was centrifuged, and approximately 300 µL was transferred to a flow tube. The effect of lactic acid bacteria on the activation B cells was detected by flow cytometry. The data were analyzed using FlowJo 7.6.1 software [28].

Protein expression and sodium dodecyl sulfate polyacrylamide gel electrophoresis, Western blot analysis

NC8-pSIP409 and NC8-pSIP409-pgsA-VP6-Dcpep frozen at - 80°C were subcultured. The second generation of bacterial liquid was added to 50 mL of MRS liquid medium (Em+) at a 1:100 ratio. Anaerobic culture was carried out in a 30°C incubator until the OD value was 0.3. SpplP was added at a ratio of 1:400 for overnight induction. The overnight culture solution was centrifuged for 10 minutes at 4°C and 5000 g, and the supernatant was discarded. The supernatant was washed three times with cold PBS. The precipitation was suspended in 500 µL of pyrolysis buffer. The supernatant was digested at 120 rpm for 30 minutes in a shaker at 37°C and centrifuged at 10000 g for 30 minutes. When the supernatant was collected, the soluble cell wall component, VP6, was contained in the supernatant. A total of 160 µL was removed from the supernatant and added to 40 µL of the 5 x SDS buffer. The protein was denatured by boiling at 100°C for 5 minutes. The supernatant was centrifuged for 2 min at 12 000 rpm. SDS-PAGE was performed.

SDS-PAGE

Two plates of glue were allocated for SDS-PAGE, one of which was dyed and decolorized according to the above method and observed on the machine; the other was carried out according to the following method.

Transmembrane

After the SDS-PAGE was finished, the gel was removed, and transfer filter paper was soaked in transfer buffer for 30 min. The PVDF membrane was first activated for 30 s with methanol and then soaked in the transfer buffer. According to the size of the gel, the filter paper and PVDF film were cut and then placed in the transfer buffer. The order was sponge, 3 layers of filter paper, PVDF film, gel, 3 layers of filter paper and sponge. The bubbles generated between each layer were removed by glass rods and then the gel was placed into the transfer clamp. The gel side was connected with the negative electrode. The PVDF film was connected with the positive pole at a constant current of 200 mA and transference occurred on ice for approximately 1 hour.

Immunofluorescence analysis

To examine the surface expression of VP6 protein in NC8-pSIP409-pgsA-VP6-Dcpep, we used immunofluorescence as follows: Briefly, 1 mL of the SPPIP-induced cultures were harvested when they reached an OD of 600 and then resuspended in 1 ml of sterile PBS 3%; then, goat serum albumin with anti-VP6 antibodies was added and incubated at -4°C overnight. Then, the cells were pelleted and washed three times with sterile PBS 3% Tween 20. Next, the cell antibody mixtures were incubated at room

temperature in the dark with fluorescein isothiocyanate (FITC)-conjugated mouse anti-porcine IgG containing 1% Evans blue. We washed the cells three times with PBS 3% Tween 20 and then transferred them to a glass slide for air drying. Noninduced recombinant NC8-pSIP409-pgsA was used as a negative control. Confocal microscopy was used to perform the analysis.

Immunizations

The induced culture of NC8-pSIP409-pgsA-VP6-Dcpep was centrifuged as described above. Then, we washed the cell pellets once with sterile PBS and resuspended them in 1 ml of PBS pH 7.4. Fifteen Balb/C mice from each group were orally administered 0.2 ml of 10^9 colony forming units/ml (c.f.u./ml) of the recombinant NC8-pSIP409-pgsA-VP6-Dcpep, NC8-pSIP409-pgsA and normal saline. All groups of mice were immunized nine times, that is, on days 0, 1 and 2, and they received boosters on days 14, 15 and 16 and again on days 28, 29 and 30.

Enzyme-linked immunosorbent assay (ELISA)

To examine specific anti-VP6 antibodies, mouse serum was collected on days 14, 35 and 42 after immunization and centrifuged at 4000 rpm for 15 min at 4°C to obtain the serum, which was stored at -80°C and then analyzed by ELISA as described previously [29]. ELISA was performed to determine the levels of specific IgG and sIgA antibodies [30]. Feces samples were also collected at 14, 35 and 42 days after immunization. The serum samples (1:100) and fecal samples (1:10) were added separately after dilution and, following incubation for 2 h at 37°C, the wells were rinsed 5 times. Then, goat anti-mouse IgA-Biotin and goat anti-mouse IgG (H+L)-Biotin (Southern Biotechnology, Birmingham, AL) were added separately, and the plate was incubated at 37°C for one hour, after which streptavidin-HRP (Southern Biotechnology, Birmingham, AL) secondary antibody was added and the plate was incubated at 37°C for 10 min. Finally, chromogen solution A and B and stop solution were added, after which the color intensity was measured at 450 nm. The end-point titers ($\log_2$) were defined as the highest dilution yielding an absorbance that was two and three times higher than the background for the serum and fecal samples, respectively [31].

Detection of cytokines

The serum samples were examined for specific cytokines, including IFN- γ and IL-4, using an ELISA kit (LIUHEBIO, Wuhan, China) according to the manufacturer's instructions.

Response of Vaccinated Mice to PRV Challenge

A virus challenge experiment was performed to evaluate the protective effect of NC8-pSIP409-pgsA-VP6-Dcpep against PRV infection in mice post vaccination. Three weeks after the last immunization, the

immunized mice were challenged (by oral gavage) with 100 ID₅₀ of a Chinese porcine rotavirus isolate, strain DN30209,3 weeks after the final immunization.

Statistical analysis

Flowjo 7.6.1 software was used for flow cytometry analysis, GraphPad Prism 5 software was used for mapping and statistical data, and one-way ANOVA was used for statistical analysis of the differences between groups (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

Abbreviations And Acronynys

BALB/c	albino laboratory bred strain of house mouse
CD	Cluster of differentiation
DCpep	Dendritic cell targeting peptide
ELISA	Enzyme-linked immuno sorbent assay
IFN	interferon
IgA	immunogloblin A
IgG	immunogloblin G
IL	interleukin
IFN-γ	interferon gamma
<i>L.</i>	<i>Lactobacillus</i>
MLN	Mesenteric lymph nodes
PP	Peyer's patch
PBS	Phosphate buffered saline
slgA	immunoglobulin A

Declarations

Ethics approval and consent to participate:

This study was carried out in agreement with the principles established by Jilin Agriculture University Changchun China and guide for the use of laboratory and care animals and all experimental

protocols were approved by a Jilin Agriculture University (No. JLAU08201007).

Consent for publication: Not applicable

Availability of data and materials: will be provided after acceptance

Competing Interests:

Author1 declares that she has no conflict of interest.

Author 2 declares that he has no conflict of interest.

Author 3 declares that she has no conflict of interest.

Author 4 declares that he has no conflict of interest

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Authors' contributions Author 1 SMS -searching data, wrote manuscript and acted as corresponding author

Author 2 WY -editing manuscript

Author 3 GY- editing the manuscript and supervision of the manuscript

Author 4 CW- editing the manuscript and supervision of the manuscript

All authors have read and approved the manuscript

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Figures

Figure 1

Agarose Gel extraction of VP6 gene segment and vector

NC8-pSIP409-pgsA-VP6-DCpep

Figure 2

Flow cytometry analysis of VP6 surface expression in *L. plantarum* NC8

Figure 3

Expression analysis of NC8-pSIP409-pgsA-VP6-Dcpep by SDS-PAGE and Western blot

Figure 4

immunofluorescence assay analysis of VP6 surface expression in *L. plantarum* NC8

Figure 5

(A) Changes of specific IgG content in serum and (B) Changes of specific IgA content in fecal

Figure 6

the contents of two cytokines in serum were determined by ELISA

Figure 7

the expression of B220+IgA+ B cells in mouse PPs

Figure 8
 Effects on survival percentages (A) indexes and body weights (B) after immunization

Figure 9

Histopathological changes in mice infected with Chinese porcine rotavirus isolate DN30209 strains following immunization

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