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Recombinant *Lactobacillus acidophilus* expressing S_1 and S_2 domains of porcine epidemic diarrhea virus could improve the humoral and mucosal immune levels in mice and sows inoculated orally

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ABSTRACT

Porcine epidemic diarrhea (PED) is a highly contagious intestinal infectious disease caused by porcine epidemic diarrhea virus (PEDV), which is characterized by a high mortality rate in piglets. Since 2012, a remarkable growth in PED outbreaks occurred in many pig farms in China, landing a heavy blow on the pig industry. In order to develop a new effective vaccine for the current PEDV, oral vaccines were generated by transferring eukaryotic expression recombinant plasmids carrying the S_1 and S_2 (antigenic sites of the S protein) epitopes of PEDV into a swine-origin *Lactobacillus acidophilus* (*L. acidophilus*). After oral immunization of the BALB/c mice, higher levels of anti-PEDV specific IgG and SIgA antibodies and cellular immune responses were detected in mice orally administered with the recombinant *L. acidophilus*-S_1 compared to the *L. acidophilus*-S_2. Furthermore, *L. acidophilus*-S_1 was used to inoculate the pregnant sows orally and the results showed that the recombinant *L. acidophilus*-S_1 could elicit a specific systemic and mucosal immune response. In summary, our study demonstrated that oral immunization with *L. acidophilus*-S_1 could improve the humoral and mucosal immune levels in sows and would be a promising candidate vaccine against PEDV infection in piglets.

1. Introduction

Porcine epidemic diarrhea (PED), caused by PED virus (PEDV), is an acute enteric infectious disease characterized by severe vomiting, diarrhea, and dehydration (Debouck and Pensaert, 1980; Pensaert and De Bouck, 1978). The spike (S) protein of PEDV is a type 1 transmembrane envelope glycoprotein, which is responsible for the virus invading host cells through membrane fusion and mediating the production of neutralizing antibodies in infected hosts (Lee et al., 2010). Based on the homology of the coronavirus S protein, it can be divided into two domains: S_1 (1-789aa) and S_2 (790-1383aa) (Sun et al., 2006; Follis et al., 2006). There are four neutralizing epitope domains in the PEDV S protein: COE (499–638 aa), SS2 (748–755 aa), and SS6 (764–771 aa) are located in the S_1 domain, and 2C10 (1368–1374 aa) is in the S_2 domain (Chang et al., 2002; Cruz et al., 2008; Sun et al., 2008). S_1 is responsible for binding receptors and S_2 is responsible for membrane fusion (Gallagher and Buchmeier, 2001;Song and Park, 2012;Oh et al., 2014). Since 2012, outbreaks of PED have significantly increased in China, and the mortality rate of suckling piglets is as high as 90%–100%, which seriously hinders the healthy development of the pig industry (Zhang et al., 2017).

Due to immature immune system development in newborn piglets, vaccination with traditional vaccines does not produce protection in time, resulting in immune failure (Li et al., 2012; Zhao et al., 2012). To prevent intestinal infectious diseases in newborn piglets, an ideal strategy is for maternal antibodies to be produced in immunized pregnant sows and passively transferred to suckling piglets via colostrum (Wang et al., 2017).

Gut-associated lymphoid tissues (GALT) are distributed in the pig intestine. The immunity induced at mucosal sites in the pregnant sow and passively transferred to suckling piglets via colostrum and milk (lactogenic immunity) is crucial for immediate protection of neonates

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against enteric infections. As recognized in previous studies of passive immunity to transmissible gastroenteritis virus (TGEV), sows that recovered from TGEV infection protected their exposed litters against TGEV. This protection was associated with high levels of antibodies in the milk (lactogenic immunity), but not in the serum of the sows (Bohl et al., 1972; Bohl and Saif, 1975; Saif et al., 1972; Saif, 1999).

Oral immunization can induce more IgA-plasmablasts and T cells in the intestine. In addition to localized intestinal tracts, some of these cells migrate to the breast via the "intestinal-mammary-SIgA axis" to produce more SIgA and cytokinesin milk (Song et al., 2007). The presence of gastric acids and proteases in the gastrointestinal tract of animals affects the effectiveness of oral vaccines. In recent years, many scholars have studied how to improve the effect of oral immunization, and the application of antigen-presenting carriers is an effective strategy. As an important predominant bacterium in intestine, *Lactobacillus acidophilus* (*L. acidophilus*) can not only effectively antagonize pathogenic bacteria in the digestive tract and maintain the intestinal microecological balance, but also be used as an antigen-presenting carrier in practical production. *L. acidophilus* is the candidate with the greatest potential for oral vaccines with a series of advantages (Yu et al., 2013). In a previous report, tetanus toxin was successfully constructed on a *Lactobacillus* expression vector to be expressed as an immune antigen to induce the immune system to produce specific IgG and SIgA antibodies in mice inoculated by nasal cavity (Grangette et al., 2001). In China, scientists have compared the effectiveness of *Lactobacillus* carrier vaccines on piglets by using both oral and injection immunization routes. The results showed that oral administration of the *VP1* gene of foot-and-mouth disease virus using *Lactobacillus* as a carrier could produce a higher antibody titer than injection, and induce humoral and cellular immunity (Li et al., 2007).

In this study, eukaryotic recombinant expression plasmids pRc/CMV2-S_1-Rep.8014 and pRc/CMV2-S_2-Rep.8014 carrying the S_1 and S_2 epitopes of PEDV and replication gene Rep.8014 of *L. acidophilus* were constructed and transformed into swine-origin *L. acidophilus*. Subsequently, BALB/c mice and pregnant sows were orally immunized with the recombinant *L. acidophilus*. The results showed that the recombinant *L. acidophilus* vaccine pRc/CMV2-S_1-Rep.8014 could induce higher levels of humoral and mucosal immune response.

2. Materials and methods

2.1. Construction of recombinant transfer plasmid

The plasmid pRc/CMV2 (SIGMA, Germany) is a 5.5 kb vector designed for high-level stable and transient expression in mammalian hosts. The human cytomegalovirus (CMV) immediate-early promoter provides high-level expression in a wide range of mammalian cells. Nucleic acid manipulations and cloning procedures were performed according to standard procedures. Based on the spike gene of sequences deposited in GenBank (accession No. JQ282909), the primers for obtaining *S_1* gene (LS_1-F 5'-AGT AAG CTT ATG AAG TCT TTA ACC TAC TTC TGG-3' and LS_1-R: 5'-TAT AGC GGC CGC TCA CCT AAT ACT CAT ACT AAA GTT GGT G-3') and *S_2* gene (LS_2-F: 5'- AGT AAG CTT ATG TAT AGT GCG TCT CTC ATC-3' and LS_2-R: 5'- TAT AGC GGC CGC TCA CTG CAC GTG GAC CTT-3') were designed to amplify 2373 bp and 1305 bp fragments respectively. Two genes were amplified by RT-PCR from the cDNA of PEDV CH-SDBZ-1-2015 strain (KU133232, Shandong Provincial Center for Animal Disease Control and Prevention, China) (Zhang et al., 2017). The PCR products were identified and purified by gel electrophoresis. Purified *S_1*and *S_2* genes and plasmid pRc/CMV2 were subjected to a *Hind* III and *Not* I double digestion separately and ligated by T4 DNA ligase (TIANGEN, Beijing, China). The recombinants pRc/CMV2-S_1 and pRc/CMV2-S_2 were sequenced and analyzed. The plasmid of pGEM-T-Rep.8014 (Shandong Provincial Center for Animal Disease Control and Prevention, China) carrying replication gene Rep.8014 of *L. acidophilus* (Li et al., 2007) and two recombinants were then subjected to a *Not* I and *Apa* I double digestion separately and ligated by T4 DNA ligase. The resulting recombinants pRc/CMV2-S_1-Rep.8014 and pRc/CMV2-S_2-Rep.8014 were then sequenced and analyzed.

2.2. Indirect immunofluorescence assay (IFA)

For detection of the displayed S_1 and S_2 of recombinant plasmids, IFA was used as described previously (Xue et al., 2019). In brief, the BHK-21 cells were transfected with the recombinant plasmids pRc/CMV2-S_1-Rep.8014 and pRc/CMV2-S_2-Rep.8014, as well as the negative control plasmid pRc/CMV2 with Vigofect Transfection reagent (Vigorous Biotechnology, Beijing, China), respectively. After incubation for 48 h, the IFA was conducted with the swine PEDV-positive serum (Shandong Provincial Center for Animal Disease Control and Prevention, China) as the first antibody and DyLight 488-Goat Anti-Swine IgG (KPL, USA) as the second antibody. Samples were then washed three times with PBS and stained with 0.1% Evans Blue (TIANGEN, Beijing, China) for 20 s. The fluorescence was observed under fluorescence microscope.

2.3. Construction of recombinant L. acidophilus

Swine-origin *L. acidophilus* named SW1 was isolated from healthy pigs and kept in Shandong Provincial Center for Animal Disease Control and Prevention, China (Su et al., 2014). Electroporation assay was performed with minor modifications as described previously (Landete et al., 2014). In brief, *L. acidophilus* SW1 colony was inoculated into 3 mL of MRS broth (Hopebiol, Qingdao, China) for static cultivation at 37 °C with 5% CO_2 for 16 h and then further cultured for 2–3 h until the same situation was obtained (OD_{600} 0.1–0.2). Cells (OD_{600} 0.2–0.3, incubation for 1–1.5 h) were chilled on ice for 10 min and washed twice with ice-cold EPWB (5 mM sodium, 1 mM $MgCl_2$, pH 7.4). A total of 10 μL of plasmid DNA (pRc/CMV2-S_1-Rep.8014 or pRc/CMV2-S_2-Rep.8014, 200 ng/μL) was mixed with 100 μL of the ice-cold cell suspension in a 0.2 cm cuvette on ice for 10 min. Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA) was used for electroporation. Meanwhile, the non-electroporated bacterial cells were set as a negative control. Positive clones were confirmed with primers LS_1 (F and R) and LS_2 (F and R) by nucleotide sequencing. To detect the stability of the two recombinant plasmids in *L. acidophilus* SW1, the recombinant *L. acidophilus*-pRc/CMV2-S_1-Rep.8014 (*L. acidophilus*-S_1) and *L. acidophilus*-pRc/CMV2-S_2-Rep.8014 (*L. acidophilus*-S_2) were passaged five times in MRS broth without any antibiotics, and all the generations were detected by PCR with primers LS_1 and LS_2 respectively.

2.4. Protein expression and analysis

S_{PEDV} fragment concluding the COE (499-638 aa) and 2C10 (1368-1374 aa) epitopes, based on the nucleotide sequence of spike protein of the strain (GenBank Acc. No. JQ282909), was synthesized after codon-optimization by Genscript (Piscataway, NJ, USA). S_{PEDV} was cloned into the pET-30a (QIAGEN, Germany) plasmid DNA vector, and then positive recombinant plasmids were transformed into *Escherichia coli* (*E. coli*) BL21 (TIANGEN, Beijing, China) for protein expression. Briefly, the positive BL21 colony containing recombinant expression vector pET-30a-S_{PEDV} was cultured in LB broth containing 5 μg/mL of kanamycin for 8–10 h at 37 °C until the OD_{600} reached 0.6. IPTG was then added to a final concentration of 1 mmol/L to induce the expression at 37 °C for 6 h.

Recombinant protein was purified as described previously (Xue et al., 2019). After sonication, the lysate was centrifuged at 8000 rpm for 10 min. The supernatant and precipitate were collected and submitted to SDS-PAGE and Western blot as described previously (Zhang et al., 2015). SDS-PAGE was conducted on pre-made 12% polyacrylamide mini-gel run in a Mini-Protean electrophoresis system (Bio-Rad, CA, USA). The expressed soluble protein was purified according to the

procedure of the His·Bind® Purification Kit (Novagen, USA) and determined according to the manufacturer's instructions of the BCA Protein Assay Kit (Beyotime, Shanghai, China). Western blotting was conducted with swine PEDV-positive serum as the first antibody and HRP-conjugated goat anti-swine IgG antibody (BioDee, Beijing, China) as the second antibody.

2.5. Indirect enzyme-linked immunosorbent assay (ELISA)

To detect the IgG and SIgA antibodies specific to S_{PEDV} epitope, indirect ELISA methods were developed referring to published protocols with minor modifications (Liu et al., 2010). Briefly, the optimal concentrations of serum and coating antigen were determined by a checkerboard titration; 96-well microtiter plates coated with two-fold diluted S_{PEDV} protein antigen (from 12 μg/well to 0.325 μg/well, 100 μL/well) were incubated at 37 °C for 1 h and then 4 °C overnight in carbonate buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.6). After washing, the wells were incubated with two-fold diluted mouse/pig PEDV positive sera and negative sera (from 1:25 to 1:400, 100 μL/well) at 37 °C for 1 h. A total of 100 μL HRP-conjugated goat anti-mouse (pig) IgG (IgA)-specific antibodies (BioDee, Beijing, China) was added and incubated at 37 °C for 1 h after washing with PBST. O-phenylenediamine dihydrochloride substrate (100 μL/well) (BioDee, Beijing, China) was added and further incubated for 15 min. The reaction was terminated with stop solution (2 M H_2SO_4, 50 μL/well) and the optical density (OD) was read at 450 nm. The optimum sera titer and concentration of coating antigen were established with the chessboard test.

2.6. Mouse immunization and detection of antibody and cytokines

A total of 24 six-week-old female BALB/c mice (Experimental Animal Center of Shandong University, China) were randomly separated into 4 groups, and 6 ones in every group. The first group was orally immunized with 10^9 CFU recombinant *L. acidophilus*-pRc/CMV2-S_1-Rep.8014 (*L. acidophilus*-S_1) (100 μL of the suspension). The second group was orally immunized with 10^9 CFU recombinant *L. acidophilus*-pRc/CMV2-S_2-Rep.8014 (*L. acidophilus*-S_2) (100 μL of the suspension). The third group were subcutaneously immunized with 100 μL PEDV commercial inactivated vaccine (Lanzhou Pharmaceutical Factory of Biology, China). The last group was orally administered with 100 μL sterile phosphate buffer saline (PBS).

The induction of antigen specific serum IgG and SIgA levels were measured by ELISA. The assay procedures were the same as described in the indirect ELISA section. All mice were boosted at 2 weeks post first immunization by the same strategy. Sera for the detection of PEDV specific antibody were collected via tail-bleeding at 7, 14, 21, 28, 35, and 42 days post immunization (dpi) and stored at −20 °C until used. Fecal samples used for detecting SIgA antibodies were collected in the same periods and treated according to methods described previously with slight modification (Liu et al., 2011).

Cytokines IFN-γ and IL-4 levels were measured by ELISA according to the manufacturer's instructions (R&D Systems, Shanghai, China) (Ma et al., 2018). Data were acquired on an automated ELISA plate reader at OD 450 nm immediately. The most effective vaccination group was used to test protective efficacy in the pig vaccination.

2.7. Pig immunization and detection of antibody production

PEDV-seronegative, crossbred, pregnant sows were obtained from a local farm and all the sows were confirmed to be negative for PEDV, from both pathogenic and serological tests by PCR (Vipotion, Guangzhou, China) and ELISA (Boyang, Guangzhou, China), respectively. Nine sows were randomly divided into three groups (3 ones in every group) and housed under similar conditions in different stables in order to avoid probiotic cross-contamination. The first group was orally dosed with 2 mL of *L. acidophilus*-S_1 (1×10^9 CFU/mL). The control groups of sows were orally dosed with 2 mL of PBS and subcutaneously immunized with an equal volume of PEDV commercial inactivated vaccine. All pigs were first immunized at 0 dpi and received booster immunization at 21 dpi under the same conditions.

Sera samples were collected at 0, 7, 14, 21, 28, and 35 dpi. Farrowing was induced at 16 days after the last vaccination. Colostrum samples were collected on the day of farrowing and treated as described previously (Adams and Marteau, 1995). The levels of anti-PEDV specific IgG antibody in the serum and SIgA antibody in the colostrum were determined by indirect ELISA. Piglets were allowed to suckle their dams and their sera samples collected on the 4th day after birth were tested by anti-PEDV IgG ELISA.

2.8. Statistical analysis and Ethics statement

All of the data were analyzed a paired samples t-test in SPSS software. Comparison of antibody titers and cytokine levels at each time point were conducted by a paired t-test and the values are presented as mean ± standard deviation (SD), with $p < 0.05$ and $p < 0.01$ considered as statistically significant and highly significant, respectively.

All animal experiments were carried out in accordance with guidelines issued by the Shandong Agricultural University Animal Care and Use Committee (approval number, SDAUA-2018-054).

3. Results

3.1. Generation and characterization of the recombinant

The pRc/CMV2-S_1 and pRc/CMV2-S_2 plasmids were obtained by amplifying and sub-cloning the $S_1$and S_2fragments into the pRc/CMV2 vector, and the replication gene Rep.8014 of *L. acidophilus* frompGEM-T-Rep.8014 was then subcloned into pRc/CMV2-S_1 and pRc/CMV2-S_2, resulting in pRc/CMV2-S_1-Rep.8014 and pRc/CMV2-S_2-Rep.8014. The expression levels of recombinants were assessed in vitro by IFA. The results showed that positive fluorescence signals were observed in the cytoplasm under confocal microscope, while no fluorescence was observed in the mock cells, indicating that theS_1 and S_2epitopes were expressed successfully in BHK-21 cells (Fig. 1A).

3.2. Identification of the recombinant L. acidophilus

Recombinant plasmids pRc/CMV2-S_1-Rep.8014 and pRc/CMV2-S_2-Rep.8014 were transformed into swine-origin *L. acidophilus* SW1 by electroporation assay. To determine whether the bacterial strains were carrying the recombinant plasmids after electroporation assay, the specific primers of S_1 and S_2 genes were used for colony identification. In order to test if pRc/CMV2-S_1-Rep.8014 and pRc/CMV2-S_2-Rep.8014 could be carried in recombinant *L. acidophilus* steadily, the positive *L. acidophilus* were cultured for five generations and analyzed through PCR, and the genes were detected as expected (data not shown). The 2300 bp and 1300 bp bands were observed by agarose gel electrophoresis and the results showed that recombinant plasmids from all five passages were carried in recombinant *L. acidophilus* steadily without any antibiotics in the media.

3.3. Establishment of S_{PEDV}-based indirect ELISA

To establish S_{PEDV}-based indirect ELISA, the protein was expressed and purified in BL21 (DE3) *E. coli*. As shown in Fig. 3, the expression of the S_{PEDV} epitope protein was assessed via SDS-PAGE; a band with the expected molecular mass of 16.9 kDa was observed upon staining with Coomassie brilliant blue (Fig. 1e). The S_{PEDV} protein was purified successfully with the His·Bind Purification Kit (Fig. 1f), and the concentration of the purified protein was 0.2 mg/mL referring to BCA Protein Assay. The purified S_{PEDV} protein was further identified by Western blot using PEDV positive swine serum (Fig. 1g). A checkerboard titration was

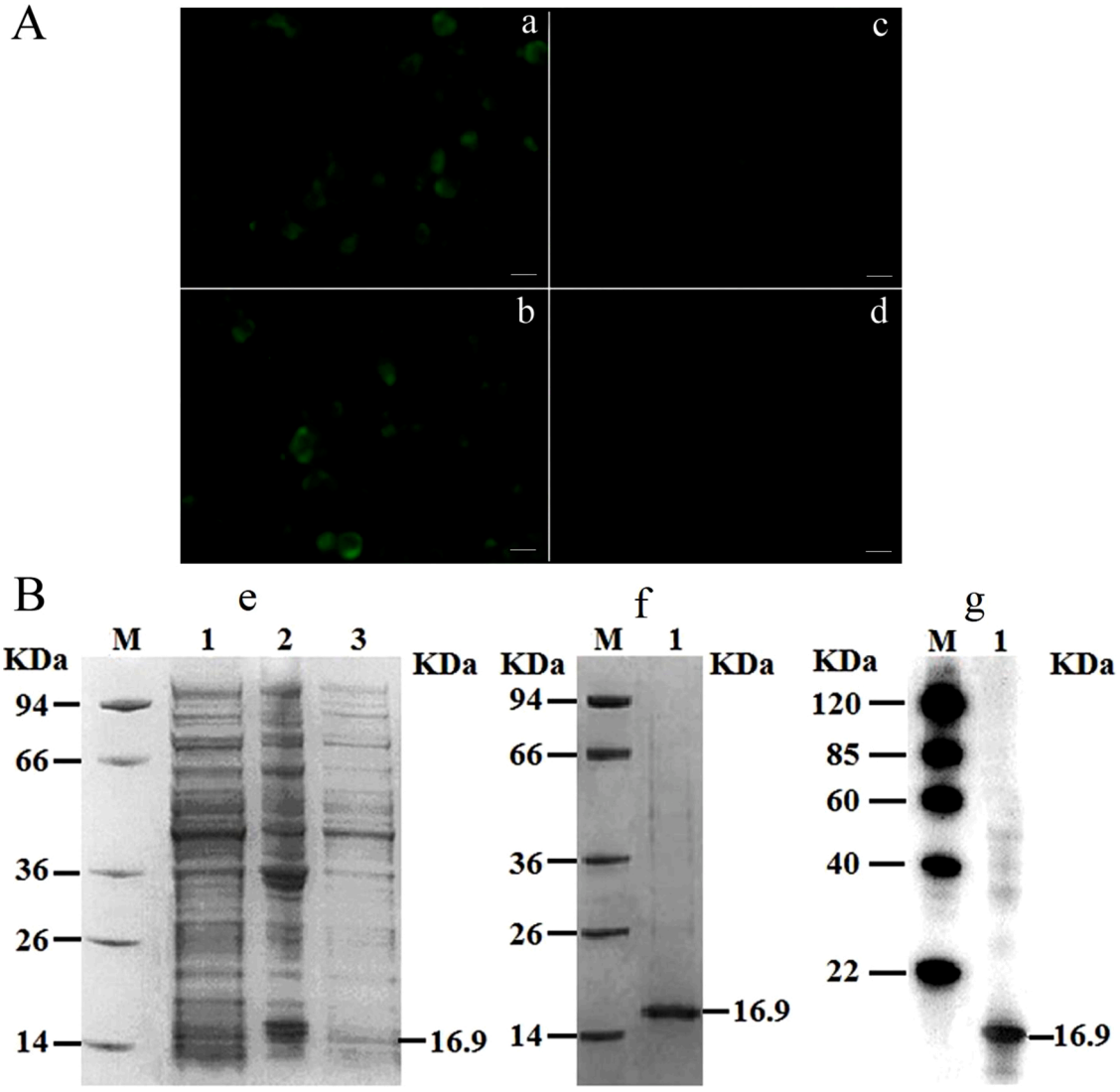


Fig. 1. Expression of the S_1 and S_2 protein in BHK21 cells (A) and S_{PEDV} fusion protein in BL21 *E. coli* (B).(a) BHK21 was transfected with the eukaryotic plasmid pRc/CMV2-S_1-Rep.8014; (b) BHK21 was transfected with the eukaryotic plasmid pRc/CMV2-S_2-Rep.8014; (c) BHK21 was transfected with pRc/CMV2; (d) BHK21; (e) SDS-PAGE analysis of S_{PEDV} fusion protein (M. Protein Maker; 1. BL21 control; 2. Pellets of induced product; 3. Supernatant of induced product); (f) SDS-PAGE analysis of the purified S_{PEDV} fusion protein (M. Protein Marker; 1. Purified of S_{PEDV} Protein); (g) Western Blotting result of S_{PEDV} fusion protein using PEDV positive swine serum as first antibody (M. Easy Western marker; 1. Purified of S_{PEDV} Protein). Bars represent 10 μm.

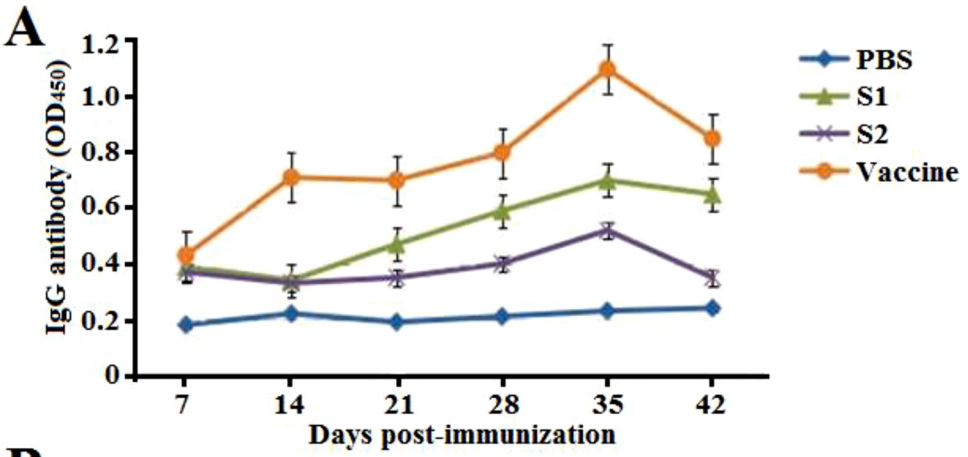
used to determine the optimal dilutions of antigen and serum. The optimal antigen concentration and serum sample dilution were set at 0.75 μg/well and 1:100, respectively.

3.4. Antibody and cytokine level detection of oral immunized mice

The levels of anti-PEDV IgG antibodies in mice induced by the recombinant *L. acidophilus* were determined by indirect ELISA. Compared to the PBS mock group, from the 14th day, significant levels of anti-PEDV IgG antibody ($p < 0.01$) were induced in mice that were orally administered *L. acidophilus*-S_1 and *L. acidophilus*-S_2 groups, and subcutaneously immunized with commercial inactivated vaccine group (Fig. 2A). Moreover, the IgG antibody levels of the commercial inactivated vaccine group were obviously higher ($p < 0.01$) than those of oral vaccine groups between 14 dpi to 42 dpi, and the IgG antibody level of the *L. acidophilus*-S_1 group were higher ($p < 0.05$) than that of the *L. acidophilus*-S_2 group.

Notably, compared to the PBS mock group and commercial inactivated vaccine group, the mucosal SIgA levels increased significantly ($p < 0.01$) after first immunization with PEDV *L. acidophilus*-S_1 and *L. acidophilus*-S_2 vaccines (Fig. 2B). The PEDV specific SIgA level of the *L. acidophilus*-S_1group was higher ($p < 0.05$) than that of the *L. acidophilus*-S_2 group after 14 dpi, suggesting the *L. acidophilus*-S_1 vaccine could efficiently induce mucosal immunity in mice. These results indicated that the oral recombinant *L. acidophilus* vaccines induced both humoral and mucosal immunity, and the oral recombinant vaccines induced much greater mucosal immunity and significantly less humoral immunity than that of the commercial inactivated vaccine (Fig. 2).

The serum IFN-γ and IL-4 levels of the three vaccine immunized groups began to increase after immunization and reached the peak at 35 dpi (Table 1 and Table 2). The serum IFN-γ level of the three vaccine groups was obviously higher than that of the PBS mock group ($p < 0.01$) between 14 dpi and 42 dpi (Table 1, Figure S1), while the IL-4 level of the two oral vaccine groups was obviously higher than that of the PBS mock group ($p < 0.01$) between 28 dpi and 35 dpi (Table 2). Though the serum IFN-γ and IL-4 levels of the *L. acidophilus*-S_1 group were higher than those of the *L. acidophilus*-S_2 group during the whole test period, the differences between the two orally immunized groups were not significant ($p > 0.05$). The serum IFN-γ and IL-4 levels of the commercial



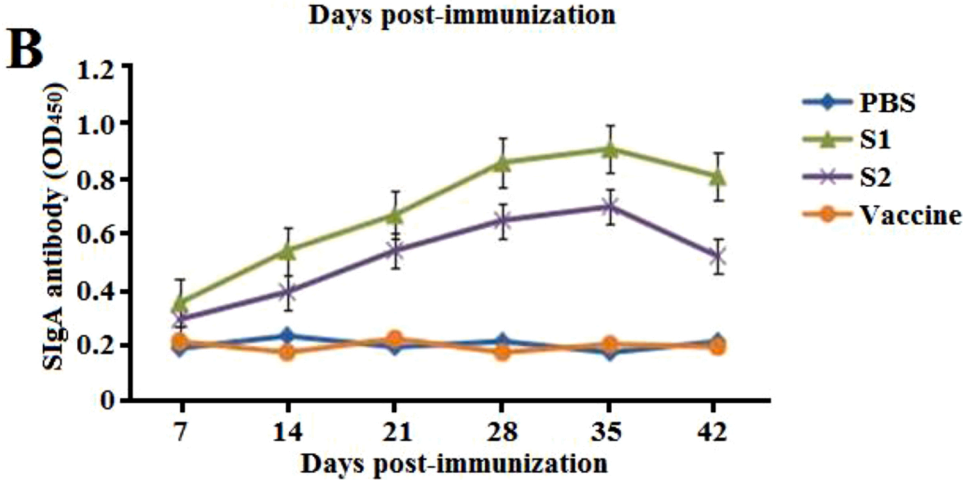


Fig. 2. The IgG and SIgA antibody levels of the immunized mice. (A) Anti-PEDV IgG antibody levels of the mice groups immunized with PBS, inactivated vaccine, oral vaccine *L. acidophilus*-S_1 and *L. acidophilus*-S_2. The IgG levels were monitored at 7, 14, 21, 28, 35, and 42 dpi; (B) Anti-PEDV SIgA antibody levels of the mice groups immunized with PBS, inactivated vaccine, oral vaccine *L. acidophilus*-S_1 and *L. acidophilus*-S_2. The SIgA levels were monitored at 7, 14, 28, 35 and 42 dpi. Error bars represent the standard deviation of the mean.

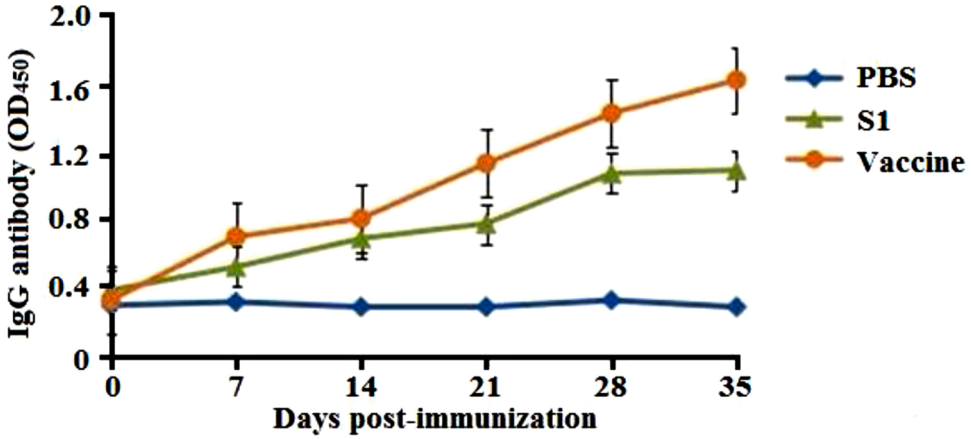


Fig. 3. Anti-PEDV IgG antibody levels of the sow groups immunized with PBS, inactivated vaccine and *L. acidophilus*-S_1. The IgG levels were monitored at 0, 7, 14, 21, 28 and 35 dpi. Error bars represent the standard deviation of the mean.

Table 1
Serum IFN-γ levels of the immunized mice (pg/mL).

Group	Days post immunization						
	0	7	14	21	28	35	42
PBS	19.95 ± 4.50^{a}	23.48 ± 3.52^{Bb}	25.76 ± 4.78^{Bb}	24.17 ± 3.19^{Bb}	26.00 ± 5.78^{Bc}	20.03 ± 5.89^{Cc}	19.00 ± 5.76^{Cc}
Inactivated vaccine	18.70 ± 4.20^{a}	33.49 ± 3.61^{ABab}	40.75 ± 3.60^{Aa}	73.54 ± 3.89^{Aa}	95.00 ± 6.71^{Aa}	125.04 ± 4.57^{Aa}	100.87 ± 4.32^{Aa}
L. acidophilus-S_1	19.07 ± 4.83^{a}	37.67 ± 6.32^{Aa}	44.69 ± 6.98^{Aa}	71.64 ± 3.81^{Aa}	89.75 ± 5.09^{Aab}	100.65 ± 4.98^{Bb}	72.54 ± 7.64^{Bb}
L. acidophilus-S_2	18.89 ± 4.57^{a}	36.37 ± 3.92^{ABa}	45.37 ± 3.92^{Aa}	67.37 ± 3.92^{Aa}	79.49 ± 6.09^{Ab}	97.37 ± 3.92^{Bb}	63.14 ± 8.25^{Bb}

Different lowercase letters denote differences among groups ($p < 0.05$) between the assays for the same immune time. Different uppercase letters denote significant differences among groups ($p < 0.01$) between the assays for the same immune time.

Table 2
Serum IL-4 levels of the immunized mice (pg/mL).

Group	Days post immunization						
	0	7	14	21	28	35	42
PBS	30.35 ± 4.62^{a}	29.48 ± 3.52^{a}	28.65 ± 4.32^{a}	30.64 ± 3.83^{Bb}	29.32 ± 5.58^{Bb}	27.07 ± 4.47^{Cc}	32.31 ± 5.27^{Bb}
Inactivated vaccine	29.12 ± 4.37^{a}	30.44 ± 3.11^{a}	31.65 ± 5.03^{a}	43.54 ± 3.89^{Aa}	45.49 ± 6.36^{Aa}	67.12 ± 4.05^{Aa}	56.72 ± 4.53^{Aa}
L. acidophilus-S_1	29.98 ± 3.56^{a}	29.00 ± 6.42^{a}	30.43 ± 4.71^{a}	37.54 ± 3.03^{ABab}	40.43 ± 5.79^{Aa}	47.56 ± 5.08^{Bb}	31.50 ± 2.43^{Bb}
L. acidophilus-S_2	28.99 ± 3.64^{a}	31.54 ± 3.19^{a}	30.65 ± 4.12^{a}	32.37 ± 3.92^{ABb}	38.43 ± 4.76^{Aab}	42.87 ± 3.12^{Bb}	31.25 ± 5.67^{Bb}

Different lowercase letters denote differences among groups ($p < 0.05$) between the assays for the same immune time. Different uppercase letters denote significant differences among groups ($p < 0.01$) between the assays for the same immune time.

inactivated vaccine group was obviously higher than those of the oral vaccine groups between 35 dpi to 42 dpi ($p < 0.01$), but the differences between the two oral vaccine groups and the inactivated vaccine group were not significant ($p > 0.05$) at 7 dpi to14 dpi.

3.5. Antibody responses levels detection of oral immunized pigs

To determine the immunogenicity of the recombinant *L. acidophilus*-S_1, 9 pregnant sows were used for the program and the humoral immune responses were examined by indirect ELISA. The *L. acidophilus*-S_1 and commercial inactivated vaccine groups both showed growth in specific serum IgG after the first and second vaccination (Fig. 3), whereas no increasing trend was noticed in the control sow ($p < 0.01$). Furthermore, in contrast to the *L. acidophilus*-S_1 group, the commercial inactivated vaccine group demonstrated obviously higher serum IgG levels at 35 dpi ($p < 0.05$).

Piglets that regularly suckle the immune mother receive colostrum/milk antibody, a process that transfers passive immunity to the piglets (Makadiya et al., 2016). Therefore, we tested colostrum samples collected from the sows on the day of farrowing for the presence of PEDV specific SIgA antibodies. The levels of SIgA antibodies were obviously higher in colostrum of the recombinant *L. acidophilus*-S1 group compared to the control and commercial inactivated vaccine groups ($p < 0.01$) (Fig. 4A). Moreover, serum of piglets collected on day 4 after birth had also high PEDV specific IgG in the recombinant *L. acidophilus*-S1 group, while no specific antibodies were found in the control and commercial inactivated vaccine groups ($p < 0.01$) (Fig. 4B).

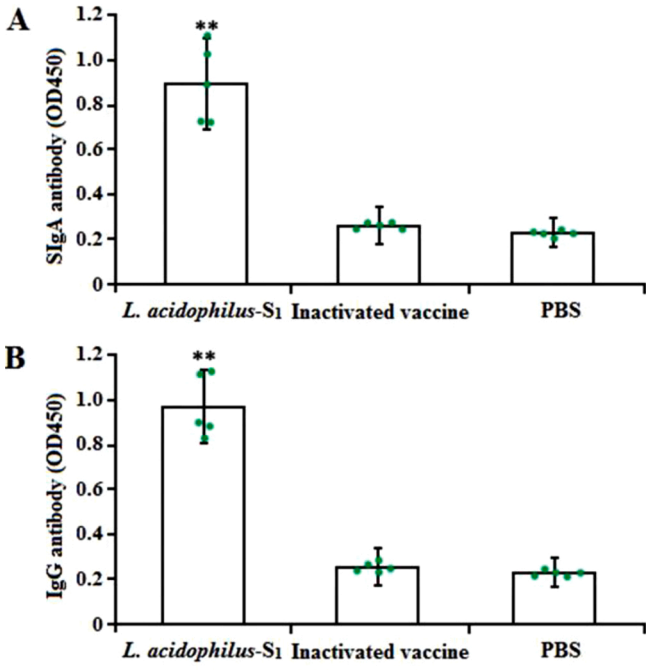


Fig. 4. SIgA levels of the immunized sows and IgG levels of piglets. (A) Anti-PEDV SIgA antibody levels of the sow groups immunized with PBS, commercial inactivated vaccine, and *L. acidophilus*-S_1; (B) Anti-PEDV IgG antibody levels of the piglets. ** mean highly significant difference compared with PBS mock and vaccine groups ($p < 0.01$). Statistical significance among different experimental groups was determined by t-test.

4. Discussion

To date, the most effective measure to control viral infectious diseases still depends on vaccination in China. PED is a highly infectious disease with intestinal tissue tropism. Immunization with non-oral vaccines mainly stimulates the body to produce IgG. IgG in serum cannot neutralize the free PEDV particles in the intestine and cannot prevent the invasion of PEDV in the intestine (Song and Park, 2012). Oral vaccines cannot only stimulate the body to produce IgG, but also stimulate the intestinal mucosa to produce SIgA, protecting pigs from infection (Liu et al., 2012). However, if a live-attenuated PEDV vaccine is directly orally immunized, it will be damaged after entering the gastrointestinal tract. Therefore, there are broad prospects for development of a live vector oral vaccine which can effectively stimulate mucosal immunity by simulating the natural infection route of PEDV.

Lactobacillus is a normal intestinal bacterium and has adjuvant properties. It can combine immune adjuvant and target antigen to produce antigens and regulate the body's immune system for a long time to continuously stimulate the body's mucosal surface lymphocytes to produce specific antibodies (Yu et al., 2013). As is well known, the most important features of *Lactobacillus* are its non-toxic side effects and safety in clinical use, and the entire surface of most *Lactobacillus* is covered with a single crystal protein material. This structure can secrete proteins, so can be used as a secretory expression vector. *Lactobacillus*-based vaccines will receive increasing attention. The mechanism by which the *Lactobacillus* vaccine induces an immune response is unknown. In short, based on the outstanding advantages of *Lactobacillus* itself, it will become an attractive engineering bacterium and represents a safe, emerging, and promising oral vaccine carrier (Song et al., 2014; Shi et al., 2014; Yu et al., 2017; Song et al., 2019).

In previous studies, a genetically engineered *Lactobacillus casei* (*L. casei*) oral vaccine (pPG-COE-DCpep/L393) expressing a dendritic cell (DC)-targeting peptide fused with PEDV COE antigen induced significant levels of anti-PEDV specific IgG and IgA antibody responses in mice and piglets, and the protective efficacy against PEDV infection in piglets reached 60% (Wang et al., 2017; Hou et al., 2018). Moreover, recombinant *L. casei* could also inhibit inflammation in mesenteric lymph nodes (Makadiya et al., 2016). *L. casei* 393 could colonize the intestine transiently and was applied as a delivery vector and potent adjuvant. Scientists demonstrated that targeting ligands could favorably increase the bioavailability of the vaccine and help to elicit mucosal and systemic immune responses (Ma et al., 2018). Different from expressing PEDV antigen protein in *L. casei* ATCC 393 in previous res (Liu et al., 2012; Hou et al., 2018), the S protein expressed in swine-origin *L. acidophilus* named SW1 was isolated from healthy pigs in this study, which could facilitate the recombinant bacteria persisting in the gastrointestinal tract and expression of the antigen protein.

In China, outbreaks of PED have caused great economic losses to the

swine industry in recent years (Li et al., 2012; Zhang et al., 2017). It is speculated that genetic mutations lead to less protective effect of the traditional vaccine. G3 group of PEDV is the main predominant in China, and SS6 and COE domains in S protein displayed 2-aa or 3-aa mutations in most field strains of G3 group compared with traditional strain CV777 (Zhang et al., 2017). In this study, S_1 and S_2 genes were amplified from PEDV CH-SDBZ-1-2015 strain, which was located in G3 group. We developed two oral vaccines encoding S_1 and S_2 epitope domains of PEDV spike protein, delivered by live *L. acidophilus*. The mice immunization results suggested that oral *L. acidophilus* vaccines were able to induce PEDV specific humoral antibodies. Moreover, PEDV specific SIgA levels of oral *L. acidophilus* vaccine groups were significantly higher than the commercial inactivated vaccine group after 14 dpi, and the *L. acidophilus*-S_1 vaccine efficiently induced mucosal immunity in mice compared to the *L. acidophilus*-S_2 group (Fig. 2). The serum levels of IFN-γ and IL-4 indicated that our two oral *L. acidophilus* vaccines might mainly enhance a Th1-type immune response to stimulate the cellular immune responses. Although mice are not susceptible to PEDV infection, good immune responses of the oral *L. acidophilus* vaccine in pigs were also observed in this study. Sows vaccinated two times with *L. acidophilus*-S_1 had higher IgG antibody levels in the serum as compared to the control sows (Fig. 3). Also, obviously higher levels of SIgA antibodies were found in the colostrum of the orally vaccinated sows. Furthermore, maternal transferring of antibody was demonstrated, as only the serum of suckling piglets had higher levels of PEDV specific IgG. All data indicated that the oral recombinant *L. acidophilus*-S_1 delivering the PEDV specific antigens could act as a novel mucosal vaccine formulation and provide a useful strategy to induce efficient immune responses against PEDV infection.

In this study, the oral vaccines were designed as only S_1 or S_2 protein was expressed in recombinant *L. acidophilus*. In previous reseach, Lc-expressed N protein as molecular adjuvant or immunoenhancer was able to effectively facilitate the induction of mucosal and systemic immune responses by Lc-expressing S_1 region (Liu et al., 2012). An oral vaccine using *L. acidophilus* to co-express the S and N proteins of PEDV should be developed to yield better immune efficacy.

In summary, an oral vaccine strategy using *L. acidophilus* to deliver S_1 and S_2 epitopes of PEDV spike protein was explored to develop an anti-PEDV vaccine for oral administration in this study. We demonstrated that the genetically engineered pRc/CMV2-S_1-Rep.8014 could efficiently induce mucosal, humoral, and cellular immune responses against PEDV, suggesting a promising vaccine strategy.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2020.108827.

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