

# High-level mucosal and systemic immune responses induced by oral administration with *Lactobacillus*-expressed porcine epidemic diarrhea virus (PEDV) S1 region combined with *Lactobacillus*-expressed N protein

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**Abstract** To develop effective mucosal vaccine formulation against porcine epidemic diarrhea virus (PEDV) infection, the DNA fragments encoding spike protein immunodominant region S1 and nucleocapsid N of PEDV were inserted into pPG1 (surface-displayed) or pPG2 (secretory) plasmids followed by electrotransformation into *Lactobacillus casei* (Lc) to yield four recombinant strains: PG1-S1, PG2-S1, PG1-N, and PG2-N. After intragastric administration, it was observed that live Lc-expressing S1 protein combined with Lc-expressing N protein could elicit much more potent mucosal and systemic immune responses than the former alone ( $P < 0.001$ ), however slightly inferior to the latter alone ( $P > 0.05$ ). Furthermore, the surface-displayed mixture (PG1-S1+ PG1-N) revealed stronger immunogenicity than the secretory mixture (PG2-S1+ PG2-N) as well as PEDV-neutralizing potency in vitro ( $P < 0.001$ ). On 49th day after the last immunization, splenocytes were prepared from mice immunized with surface-displayed mixture, secretory mixture and negative control to be stimulated by purified N and S protein, respectively. The results of ELISA analysis showed that N protein was capable of inducing a higher level of IL-4 ( $P < 0.001$ ) and IFN- $\gamma$  ( $P < 0.001$ ) than S1 protein in the immunized mice. Taken together, 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 S1 region.

**Keywords** Combined intragastric immunization · Porcine epidemic diarrhea virus · *Lactobacillus casei* · N protein and S1 region

## Introduction

Porcine epidemic diarrhea virus (PEDV) causes lethal diarrhea in piglets, resulting in great economic losses all over the world, and is a member of *Coronaviridae*, causing severe entero-pathogenic diarrhea especially in piglets. PEDV belongs to group I coronavirus which consists of PEDV, transmissible gastroenteritis virus (TGEV), human coronavirus 229E (HCoV-229E) (Vijgen et al. 2004), feline coronavirus (FeCoV), canine coronavirus (CCV), and human coronavirus NL63 (NL or New Haven) (BaoXian et al. 2009; van der Hoek et al. 2004). PEDV and TGEV caused the diseases characterized by rather similar clinical symptoms which make it rather difficult to draw a clear dividing line between them. Both of them replicate in the differentiated enterocytes of the small intestine and cause severe diarrhea (Sanchez et al. 1992). Several viral proteins are important for inducing an immune response to PEDV: the spike protein (S, 180–220 kDa), the membrane protein (M, 27–32 kDa), and the nucleoprotein (N, 55–58 kDa) (Egberink et al. 1988). The S glycoprotein of PEDV possessed an immunodominant region S1 (Liyun et al. 2008) and plays a crucial role in the early steps of infection. It mediates binding of the virus to the cell surface and the subsequent fusion between the viral and cell membranes (Yeo et al. 2003; Duarte and Laude 1994). PEDV N protein forms a helical nucleocapsid with genomic RNA and is the predominant antigen produced in coronavirus-infected

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cells, thus making it a major viral target. Therefore, N protein as a sensitive diagnostic antigen was used to develop specific and sensitive enzyme-linked immunosorbent assay (ELISA) (Hou et al. 2007a; Rodak et al. 2005). However, N protein was not able to induce immunoprotection against PEDV infection owing to an envelope surrounding the virion shielding (Ge et al. 2009; Hou et al. 2007b).

Lactic acid bacteria (LAB) have long been used in industrial food fermentation and preservation and are known for beneficial effects on the health of humans and animals and considered a generally-regarded-as-safe microorganism (Pouwels et al. 1996; Claassen et al. 1995). Many strains of LAB are able to survive and colonize in the intestinal tract (Wei et al. 2010; Davidson et al. 2011; Mohamadzadeh et al. 2010; Moeini et al. 2011; Fredriksen et al. 2010; Yigang et al. 2007). LAB are used as a live carrier delivering model pathogenic antigen on mucosal surface to induce systemic and mucosal effective immune responses, which may be preferable in terms of safety control, minimization of side effects, and non-specific immunoadjuvant effect (Galdeano and Perdigon 2006; Ogawa et al. 2006); this has provoked serial studies aimed at determining the capability and feasibility of the application of LAB as safe oral vaccines (Claassen et al. 1995; Pouwels et al. 1996; Maassen et al. 1998).

Like many other pathogens, PEDV initiates its infectious cycle at the mucosal surfaces, especially the intestinal mucosal epithelial surface (Ben Salem et al. 2010; Duarte and Laude 1994). Efficient protection against mucosal infections requires the development of new vaccines that induce protective immune responses not only at the systemic level but also at the mucosal level in order to inhibit the entry of virus into the body through the mucosal approach (Mota et al. 2006). This can best be achieved by mucosal vaccination, a route which offers several advantages over the traditional parenteral vaccines. The potential development of lactic acid bacteria to deliver heterologous antigens to the mucosal immune system has been proposed; these have many properties that make them attractive candidates as antigen delivery carriers for the presentation to the mucosa of compounds with pharmaceutical interest, in particular vaccines (Perdign et al. 2001; Mercenier et al. 2000; Wells et al. 1996).

Therefore, developing novel and efficient vaccine for intragastric administration based on *Lactobacillus casei* (Lc) was a better formulation for immunoprophylaxis against PEDV infection. In this study, the well-characterized PEDV genes S1 and N with an outstanding potential to function as antigens were cloned into shuttle vector pPG1 and pPG2 based on xylose promoter followed by transformation into Lc to yield the recombinant strains PG1-S1 and PG2-N. In addition, PG2-S1 and PG1-N were constructed previously in our laboratory (JunWei et al. 2009; Ge et al. 2009). The

results appear in the trend of PG1-N>PG2-N>PG1-N + PG1-S1>PG2-N + PG2-S1 and PG1-N + PG1-S1>PG2-N + PG2-S1>PG1-S1>PG2-S1 in the titers of serum IgG and secretory IgA against N and S1 after oral administration in mice with the recombinant Lc. Furthermore, N protein can induce splenocytes of immunized mice to yield a higher level of IL-4 and IFN- $\gamma$  than S1 protein.

## Materials and methods

### Bacterial strains and growth conditions

*L. casei* ATCC 393 was a plasmid-free strain grown in MRS medium (Sigma) at 37 °C, without shaking. Chloramphenicol (Sigma, Shanghai, China) was utilized at a concentration of 10  $\mu\text{g/ml}$ . For the cloning of plasmids, *Escherichia coli* JM109 was used in this study and grown in LB medium containing 100  $\mu\text{g/ml}$  of ampicillin. The plasmids pPG1 and pPG2 were kindly gifted by Prof. Jos Seegers (NIZO Institute, Netherlands).

### Plasmid procedure and eletrotransformation

DNA fragments encoding PEDV spike protein immunodominant region S1 (2,310 bp) and nucleocapsid (1,320 bp) were obtained from the plasmid pMD18-T Simple-S1 and pMD18-T Simple-N by *Bam*HI and *Xho*I digestion. They were inserted in the corresponding site in plasmids pPG1 and pPG2 to generate new plasmids pPG1-S1 and pPG2-N. There is an anchoring matrix-encoding *pgsA* derived from *Bacillus subtilis* behind the target gene in the pPG1 as a type of surface-displayed expression. pPG1 and pPG2 has the ssUSP secretion signal before the target gene to ensure the target protein outside the Lc.

Preparation and electrotransformation of competent *L. casei* were performed as described previously (Lee et al. 2006). Briefly, a stationary phase (16–18 h) culture of recipient *L. casei* was inoculated (1/50 inoculum) (*v/v*) into 100 ml MRS broth and incubated at 37 °C without shaking. The cells were harvested at OD<sub>600</sub> 0.3–0.4 by centrifugation at 3,000 $\times$ *g* for 10 min at 4 °C and washed twice with an equal culture volume of ice-cold sucrose–magnesium chloride electroporation buffer (SMEB) (250 mM sucrose, 1 mM MgCl<sub>2</sub>, 5 mM sodium phosphate, pH 7.4). The cells were concentrated 100-fold of the original culture volume in ice-cold SMEB buffer.

Plasmid DNA was mixed with 100  $\mu\text{l}$  of ice-cold bacterial suspension. The suspension was transferred into a pre-cool Gene Pulser™ disposable cuvette (inter-electrode distance 0.2 cm). A single electric pulse (1.5 V, 200  $\Omega$ , 25  $\mu\text{F}$ ) was delivered by a Gene Pulser™ (Bio-Rad, USA). Immediately following discharge, the suspension

was diluted with 900  $\mu\text{l}$  of recovery medium (MRS broth with 0.3 M sucrose). The bacteria were incubated for 3.5 h at 37 °C to allow recovery and expression of antibiotic resistance marker and then plated on MRS agar supplemented with 10  $\mu\text{g}/\text{ml}$  chloramphenicol.

Taken together, two novel recombinant *L. casei* have been constructed and designated PG1-S1 and PG2-N.

#### SDS-PAGE, immunoblotting, and immunofluorescence

For analysis of the expression of S1 and N protein in PG1-S1 and PG2-N, transformed bacteria were grown overnight in basal MRS medium containing chloramphenicol (Cm, 10  $\mu\text{g}/\text{ml}$ ) supplemented with xylose at 37 °C. Bacterial cells were collected by centrifugation at 3,000g for 15 min. The pellets were washed twice with sterile phosphate-buffered saline (PBS, pH 7.4) and lysed in Bead-Beater (Biospec, Bartlesville, OK, USA) by vigorous shaking. The cell debris was centrifuged at 3,000g for 10 min and the supernatant was analyzed via 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting was carried out as follows: protein extractions were electrotransferred on a nitrocellulose membrane and the blots were developed using mouse anti-pPEDV (purified PEDV) serum (previously prepared in our laboratory) at a dilution of 1:500 with PBS (pH 7.4). Horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) was utilized as a secondary antibody at a dilution of 1:8,000, which was visualized with the Chemiluminescent Substrate reagent (Pierce, Rockford, IL, USA) following the manufacturer's instruction. The recombinant *L. casei* harbored empty plasmids pPG1 and pPG2 which acted as negative controls, named PG1 and PG2, respectively.

For detection of the surface-displayed expression of PG1-S1, immunofluorescence was used as described previously (Cortes-Perez et al. 2003). In short, PG1-S1 cells were cultured and induced in MRS overnight at 37 °C. The cell pellets were sequentially incubated with mouse anti-pPEDV serum (1:200), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Invitrogen) secondary antibodies (1:5,000) and analyzed with confocal microscopy.

The secreted N protein expression by PG2-N was examined in the supernatant. Overnight cultures of PG2-N in MRS supplemented with xylose were obtained by centrifugation at 12,000 $\times$ g for 5 min. The supernatants were concentrated 20 times using a Centrifugal Filter Unit (Millipore, Billerica, MA, USA) and then examined using 12% SDS-PAGE and immunoblotting as described above.

#### Immunization and sample collection

Groups of ten female SPF Balb/C mice (8 weeks old) were immunized with PG1, PG2, PG1-S1, PG2-S1, PG1-N,

PG2-N, PG1-S1+ PG1-N (mixture), PG2-S1+ PG2-N (mixture), and PBS that were grown overnight in basal MRS medium containing Cm (10  $\mu\text{g}/\text{ml}$ ) supplemented with xylose at 37 °C, respectively. For the oral route,  $2 \times 10^9$  cells in 0.1 ml PBS (pH 7.4) suspension was administered on three consecutive days at days 0–2, 14–16 (first booster), and 28–30 (second booster). The mice were handled and maintained under strict ethical conditions according to the international recommendations for animal welfare.

On days 0 (pre-immune), 7, 21, 35, 49, 63, and 77, sera were prepared from blood samples collected from the tail vein; feces of 200 mg was directly obtained from the anus of the mice and subsequently suspended and lysed in 400  $\mu\text{l}$  of PBS containing 0.01 M EDTA–Na<sub>2</sub> overnight at 4 °C, which was followed by centrifuge 12,000 $\times$ g for 30 min and the supernatant were stored at –20 °C; vaginal, ophthalmic, and nasal fluids were obtained by washing the respective organs three times with 50  $\mu\text{l}$  sterile PBS. The cleared extracts of all samples were collected by centrifugation and stored at –20 °C for the analysis afterward; in addition, it was supplemented with protease inhibitors. On days 0 and 77, the intestinal mucus of mice was extracted as described previously (Robinson et al. 1997; Elson et al. 1984).

#### Enzyme-linked immunosorbent assay

ELISA plates were coated overnight at 4 °C with the purified S1 or N protein. After the wells were blocked for 2 h at 37 °C with PBS containing 5% skimmed milk, mucosal or serum samples were serially diluted in PBS-1% BSA and added in triplicates and incubated for 1 h at 37 °C. After the plates were washed three times with 0.05% PBS-Tween 20, horseradish peroxidase-conjugated goat anti-mouse IgG or IgA antibody (Invitrogen) was added into each well (1:5,000) and incubated for an additional 1 h at 37 °C. After another round of washing, color development was carried out using *o*-phenylenediamine dihydrochloride as substrate, and then absorbance was measured at 490 nm. Sera and mucosal samples from Balb/C mouse received recombinant Lc or PBS as blank control (background). Endpoint titers were defined as the highest dilution that gave an absorbance two times higher than the background.

#### Neutralization ability of the induced antibodies

Intestinal fluids and serum samples from mice immunized with six kinds of recombinant strains expressing S1 and N were evaluated using a plaque reduction assay to determine the neutralization ability of the induced antibodies according to the method described by Hou et al. (2007a). In brief, lavages and sera from non-expressor fed mice were used as

negative control. Fifty microliters of samples in twofold serial dilutions was prepared in microcentrifuge tubes. PEDV adjusted to  $1 \times 10^{10}$  pfu/ml in 50- $\mu$ l virus diluent was added to the tube containing serially diluted serum or intestinal lavage. The antibody and virus mixture was mixed, pulsed, centrifuged, and then incubated at 37 °C for 1 h. A 96-cell plate with confluent monolayer of *Vero* cell (used for virus infection) inoculated with antibody–virus mixture at 37 °C for another 1 h rocked at 20-min intervals. Then, the inoculum was removed and overlaid with medium (equal volume of concentrated cell culture medium containing 2.5% carboxymethyl celluloses) and the plate was incubated in a 5% CO<sub>2</sub> incubator at 37 °C for 3 days. The IgA or IgG against PEDV as positive control was kindly provided by Prof. Liyun Yu (China, IgA or IgG neutralization titer  $\geq$  1:256). The inhibitory rate was calculated as described previously by Hou et al. (2007b). The overlay medium was then discarded, after which the wells were washed three times with sterile PBS, pH 7.4 and stained with 1% crystal violet solution. The same experiment was performed twice and the statistical comparison was made by Student's *t*-test.

#### Cytokine ELISA

At 11 weeks post-immunization, spleen cells were cultured in 10% FCS/RPMI-1640 upon stimulation with 5  $\mu$ g/ml Concanavalin A (Con A) or 10  $\mu$ g/ml soluble purified S1 or N protein at 37 °C in 5% CO<sub>2</sub>. Supernatant fluids were collected at 72 h, and IL-4 assays and IFN- $\gamma$  assays were performed using ELISA kits according to the manufacturer's instructions (Biosource International, Camarillo, CA, USA). The cytokine concentrations were calculated according to the standard curve obtained for each ELISA plate.

#### Statistical analysis

Data are the geometric means from  $n=10$  mice per group and representative of duplicate experiments. The error bars represent standard deviations. Results were expressed as mean  $\pm$  standard deviation (SD). *P* value which was less than 0.05 as determined by Student's *t*-test was considered significant.

## Results

#### S1 protein surface-displayed expression by *L. casei*

Overnight cultures of PG1-S1 and PG1 induced in basal MRS supplemented with xylose were pelleted and cell lysates were analyzed by SDS-PAGE and immunoblotting, which showed that there was ca. 84-kDa fusion protein in

the cell lysates of PG1-S1 induced by xylose (Fig. 1, lane 1), but not in that of PG1 (Fig. 1, lane 2).

The immunofluorescence reaction of PG1-S1 and PG1 induced by xylose developed with the mouse anti-S1 serum and FITC-conjugated goat anti-mouse IgG also showed that there was only green fluorescence on the cell surface of PG1-S1 (Fig. 2a), but not on that of PG1 (Fig. 2b).

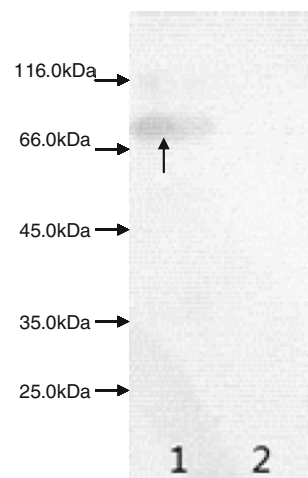
#### N protein secretory expression by *L. casei*

Cell lysates and supernatant of overnight cultures of PG2-N and PG2 in basal MRS medium with additional xylose were obtained by centrifugation, which were subjected to SDS-PAGE and immunoblotting to examine the target protein expression. An immunoreactive band of ca. 48 kDa was detected in the cell lysates (Fig. 3, lane 1) and supernatant (Fig. 3, lane 3), whereas there was no band in the negative control lane (Fig. 3, lanes 2 and 4). The results demonstrated that PEDV nucleocapsid was able to be induced by xylose in *L. casei* ATCC 393.

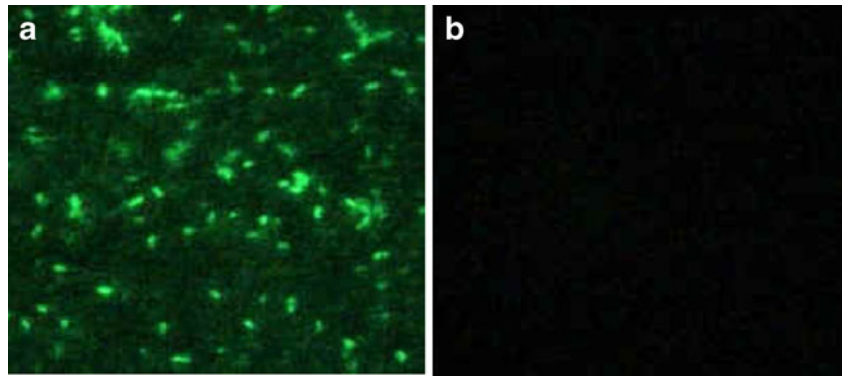
#### Immune responses against recombinant Lc by intragastric immunization

Balb/c mice were immunized orally with recombinant Lc. As negative and blank controls, mice were immunized with PG1, PG2, and PBS (data not shown), respectively. The mucosal response was further investigated by measuring anti-S1 or anti-N sIgA level in the feces, vaginal, nasal, and ophthalmic lavage fluids and intestinal mucus post-intragastric immunization via ELISA. The level of specific IgG anti-S1 or anti-N in the sera of experimental mice indicated that systemic immune response was induced by the recombinant Lc. The concentration of sIgA or IgG antibody was expressed as titers that were determined by the expression of the test samples to a standard curve generated by serial dilution of commercially purchased IgA or IgG (Sigma) of known concentration.

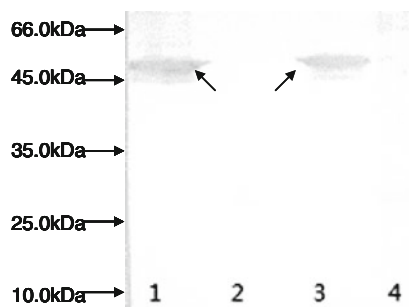
**Fig. 1** PEDV S protein immunodominant region S1 expressed in PG1-S1. Western blot analysis showed an immunoreactive band of 75-kDa fusion protein in lysates of PG1-S1 (lane 1), but not in that of PG1 (lane 2)



**Fig. 2** The immunofluorescence analysis of S1 surface-displayed expression by *L. casei*. **a** There was green fluorescence on the surface of PG1-S1, **b** there was no immunofluorescence reaction on the PG1 cell surface



Since 7 days after the first immunization, the higher levels of IgA specific for S1 that could be observed in the animal group that received a mixture of PG1-S1+ PG1-N or PG2-S1 + PG2-N in four kinds of samples including nasal, feces, ophthalmic, and vaginal lavage fluids (Fig. 4a–d) are significantly different from the groups that received PG1-S1 ( $P<0.001$ ,  $P<0.001$ ,  $P<0.001$ ,  $P<0.001$ , and  $P<0.001$ ) and PG2-S1 ( $P<0.001$ ,  $P<0.001$ ,  $P<0.001$ ,  $P<0.001$ , and  $P<0.001$ ), respectively. Analysis of the sera obtained from the same animals revealed that the animals that displayed the higher levels of IgA also displayed a higher level of IgG (Fig. 4e), showing correlation in the production of these two kinds of antibody. On the 77th day, there was an obvious trend of PG1-S1+ PG1-N > PG2-S1 + PG2-N > PG1-S1 > PG2-S1 in the titers of IgA in the intestinal mucus (Fig. 4f). However, higher levels of IgA or IgG specific for N were induced by PG1-N and PG2-N but not PG1-S1+ PG1-N and PG2-S1 + PG2-N (Fig. 5a–e). The trend of PG1-N>PG2-N>PG1-S1+ PG1-N>PG2-S1 + PG2-N was observed in the intestinal mucus IgA titer on the 77th day (Fig. 5f). The levels of IgG or IgA in all animal groups did not show significant difference from each other before the first immunization (pre-immune). By contrast, as might be expected, no significant elicitation of antibodies was observed in negative or blank control (data not shown).



**Fig. 3** Secretory expression of PEDV nucleocapsid (N) in PG2-N. An immunoreactive band of 55-kDa fusion protein in lysates and supernatant (lanes 1 and 3), but not in that of PG2, was shown by Western blot analysis

### Plaque reduction assay

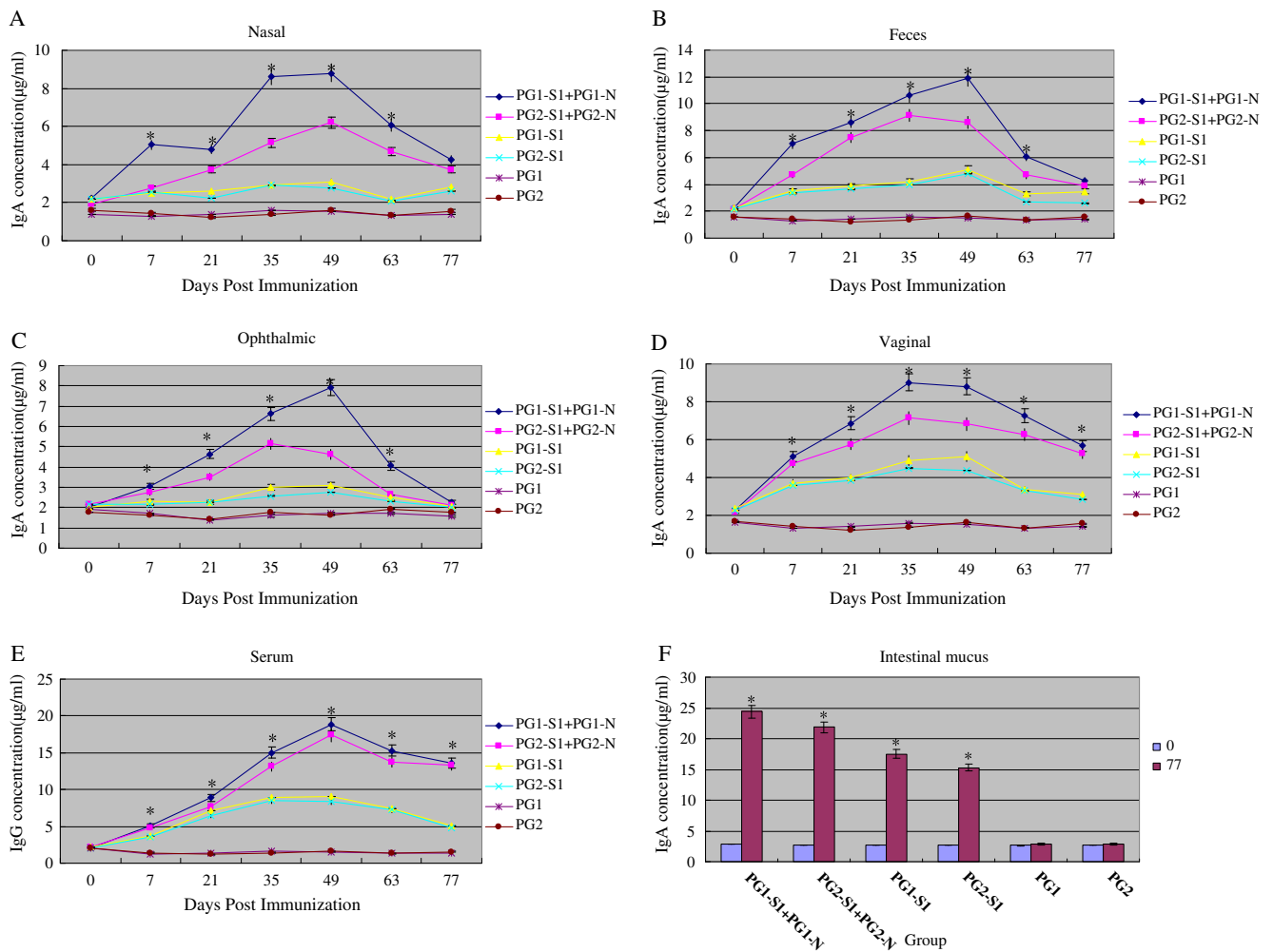
Plaque reduction assays were performed to further detect whether the antibody responses were against PEDV. Results demonstrated that the presence of anti-S1 IgA or IgG in the culture medium conferred statistically significant neutralizing effects ( $P<0.05$ ) on PEDV infection. It was consistently observed that antibody response of mice orally immunized with a mixture displayed stronger PEDV-neutralizing activity than Lc-expressing S1 from fourfold to 128-fold dilution of sera (Fig. 6a) or intestinal lavage fluids (Fig. 6b), which was exhibited by reduction in the number of plaque. Not surprisingly, no neutralizing activity was observed in the sera of mice that received PG1 and PG2.

### Cytokine responses

ELISA results obtained with culture supernatants harvested at 72 h showed that, in response to S1 or N, spleen cells from the PG1-S1+ PG1-N group produced higher levels of Th1-associated cytokine IFN- $\gamma$  (Fig. 7a) and Th2-associated cytokine IL-4 (Fig. 7b) than PG2-S1+ PG2-N ( $P<0.05$ ), PG1 ( $P<0.001$ ), PG2 ( $P<0.001$ ), and PBS ( $P<0.001$ ) (data not shown). In addition, the results showed that N protein was capable of inducing a higher level of IL-4 ( $P<0.001$ ) and IFN- $\gamma$  ( $P<0.001$ ) than S1 protein.

### Discussion

PEDV causes acute enteritis in pigs. The death rate of infected piglets is over 95%, leading to significant economic losses in swine husbandry (de Arriba et al. 2002; Yeo et al. 2003). Hence, it is important to develop an effective vaccination to protect against PEDV infection. Because PEDV replicate mainly in the villi of the small intestines (Ben Salem et al. 2010), the recombinant live mucosal vaccines expressing pathogen-derived antigens can be an alternative method for providing protection (JunWei et al. 2009; Jun-wei et al. 2010). Mucosal immunization offers a number of advantages



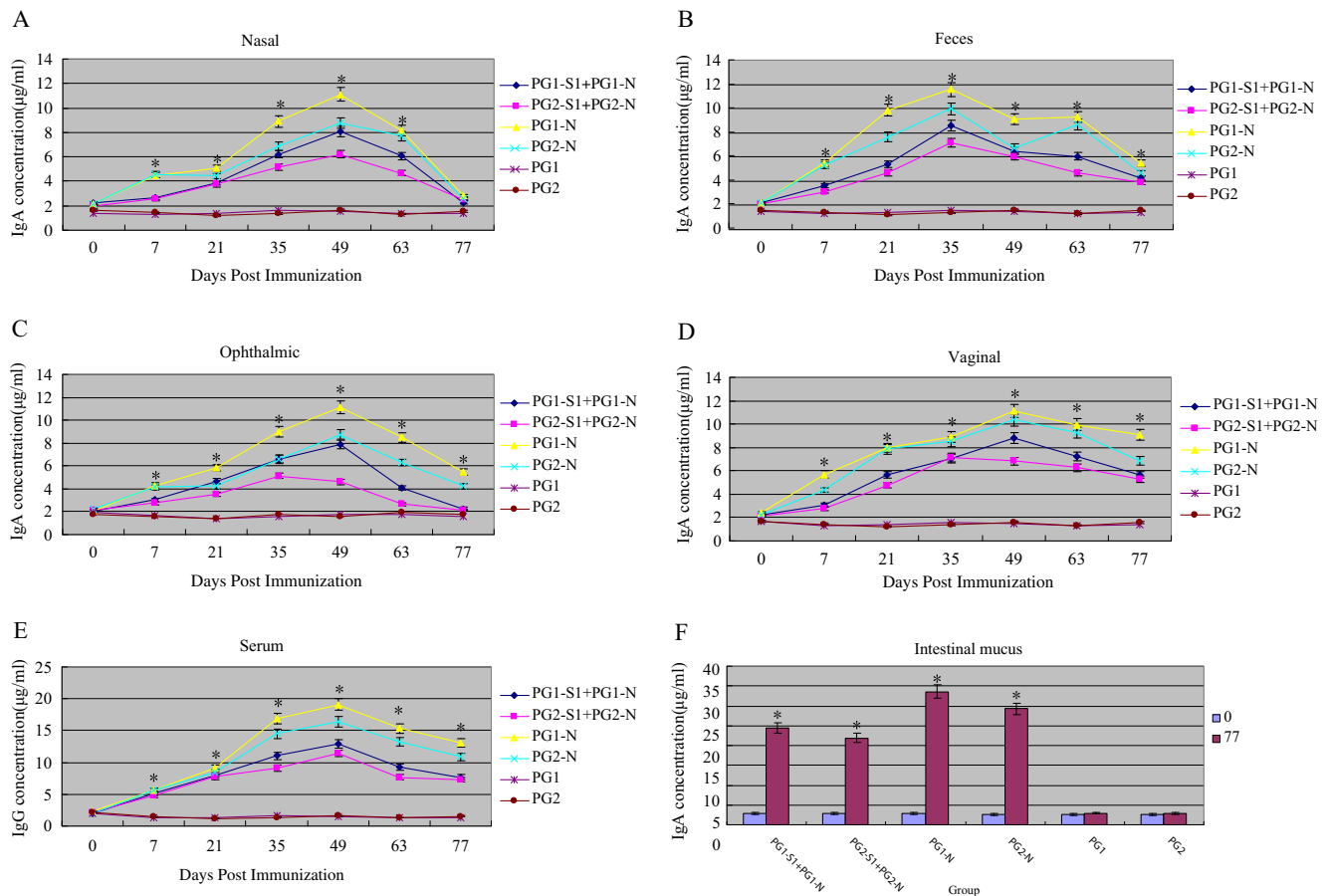
**Fig. 4** IgA and IgG levels of mucosal and serum samples (specific for S1). Samples of nasal (a), feces (b), ophthalmic (c), vaginal lavage fluids (d), intestinal mucus (e), and serum (f) from mice immunized

with PG1-S1+ PG1-N, PG2-S1+ PG2-N, PG1-S1, PG2-S1, PG1, and PG2. Values are means ( $\pm$ standard deviation) of three replicates per treatment in one experiment, which was repeated twice. \* $P < 0.001$

over other routes of antigen delivery, including convenience, cost-effectiveness, and induction of both mucosal and systemic immune responses (Shaw et al. 2000; Seegers 2002; Xin et al. 2003; Mannam et al. 2004; Lee et al. 2006). The goal is to provide the first line of defense by effectively eliminating pathogens at the mucosal surface. There were several studies focused on surface-displayed expression system based on bacteria (Ge et al. 2009; Hou et al. 2007a; Lee et al. 2006). However, surface display of antigens on the bacterial surface has been problematic because large antigens perturb membrane topology. True surface exposure of antigens requires a transmembrane anchor that is long enough to cross the cell wall (Lee et al. 2006). At least 100 amino acids are needed to properly cross the cell wall (Leenhouts et al. 1999). In this report, we have developed a surface display system using the poly- $\gamma$ -glutamate synthetase A protein (pgsA) as the transmembrane anchor to present heterologous proteins on Lc. We adapted pgsA derived from

the pgsBCA enzyme complex of *B. subtilis* isolated from chung-kook-jang (a traditional Korean soybean paste used as a food additive).

Mucosal delivery of vaccines induces mucosal immunity more efficiently than parenteral immunization (Mutwiri et al. 1999). The surfaces of the respiratory, gastrointestinal, and urogenital tracts, which are not covered by skin, are referred to as mucosa (Butler 1999). In total, the mucosa covers an area that is about 200 times larger than the skin. This large area is not as resistant to penetration as the skin because it is often limited by a thin epithelial layer without keratinization. A number of pathogens initiate infection through the mucosal surfaces. The mucosal surfaces, particularly the respiratory and digestive tracts, are exposed daily to a wide variety of foreign organisms and antigens. In addition, immunoglobulin A (IgA) is the primary immunoglobulin isotype induced at the mucosal surface. Secretory IgA (sIgA) in mucosal secretions provides



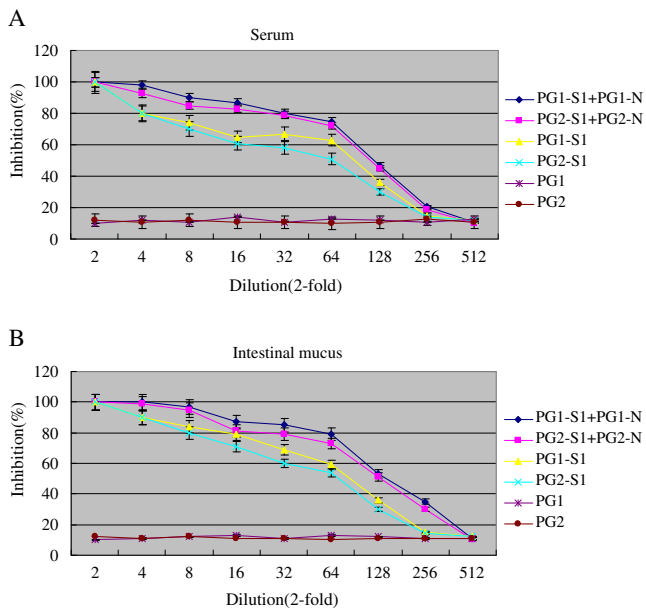
**Fig. 5** IgA and IgG levels of mucosal and serum samples (specific for N). Samples of nasal (a), feces (b), ophthalmic (c), vaginal lavage fluids (d), intestinal mucus (e), and serum (f) from mice immunized

with PG1-S1+ PG1-N, PG2-S1+ PG2-N, PG1-S1, PG2-S1, PG1, and PG2. Values are means ( $\pm$ standard deviation) of three replicates per treatment in one experiment, which was repeated twice. \* $P < 0.001$

protection against bacterial and viral pathogens and neutralization microbial toxins (Zhang et al. 2002; Yigang and Yijing 2008). Thus, they must be very effective in dealing with the large amounts of foreign antigens. The mucosa and associated lymphoid tissue is an attractive target for vaccine strategies because they harbor the early stages of infection (Ogra et al. 2001). Our observation supports the concept of a mucosal immune system in which mucosally situated IgA and IgG plasma cell progenitors are stimulated selectively by mucosal immunization and predicts that mucosal immunization can provoke both mucosal and circulating antibody responses better than parenteral immunization (Heritage et al. 1996).

In this report, we have developed recombinant *L. casei* (Lc) anchoring PEDV spike protein immunodominant region S1 (PG1-S1) and secreting nucleocapsid protein (PG2-N), respectively. The corresponding recombinant strains PG2-S1 (JunWei et al. 2009) and PG1-N (Ge et al. 2009) have been constructed in our previous work. Lc-expressing S1, Lc-expressing N, and a mixture of equal parts of Lc-expressing S1 and Lc-expressing N were used to vaccinate mice via oral route, respectively. Although the

amount of Lc-expressing S1 in the mixture was just half as much as that of Lc-expressing S1 alone for oral administration, a much higher level of IgG (serum) or IgA (mucus) specific for S protein was elicited by the mixture, which was consistent with PEDV neutralizing activity reflected by plaque reduction assay. However, antibody response specific for N protein induced by the mixture was inferior to that by Lc-expressing N. In view of the above-mentioned facts, we could speculate that N protein seems to play a crucial role in the production of higher-level antibody by the mixture, but S protein does not. Subsequently, we observed that the secretory level of IFN- $\gamma$  and IL-4 of splenocytes isolated from mice immunized with the mixture stimulated by N protein was obviously higher than S protein. Theoretically, IL-4 could effectively advance the proliferative differentiation of B cells to plasma cells secreting a large amount of antibody. IFN- $\gamma$  as an important member of cytokines has a vital role to play in directing immune responses and regulating immune network, especially the aspect of antiviral effect. As described above, it was undoubtedly logical that the mixture induced higher



**Fig. 6** Antibody neutralizing activity against PEDV in vitro. **a** Serum and **b** intestinal mucus prepared from mice immunized with PG1-S1+PG1-N, PG2-S1+PG2-N, PG1, and PG2. Results are mean values and standard errors of triplicates

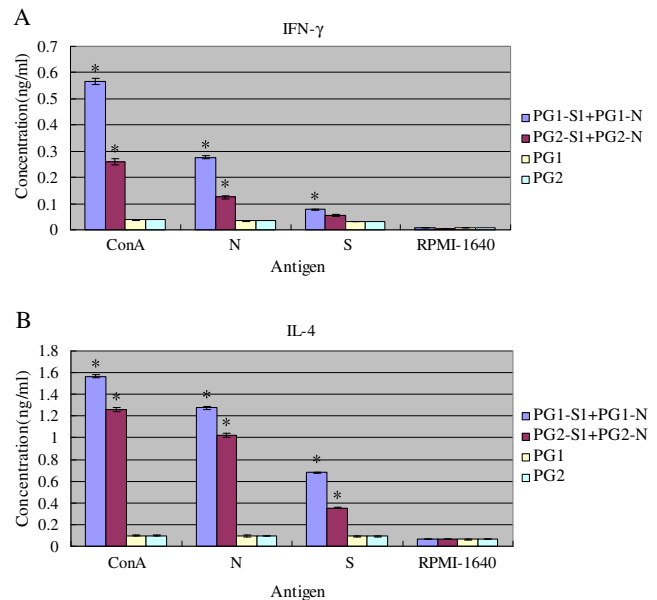
antibody responses against S protein. In theories, IL-4 could inhibit IL-12 pathway so that the secretion of IFN- $\gamma$  is down-regulated. However, the result showed that IL-4 and IFN- $\gamma$  were together induced by N or S1 to make Th1/Th2 relatively balanced with the greater amount of IL-4 than IFN- $\gamma$ , which demonstrated that Th2 response was a leading one.

The target protein expression completely depended on the xylose induction in this study owing to the *L. casei* expression systems based on xylose operon regulation. According to the above statement, the induced recombinant strains were able to stimulate the mucosal immune system of the gastrointestinal tract for several days but not a lifetime after oral administration in mice, which did not result in immunotolerance against target antigen. When the induced recombinant bacteria colonized on the mucosal surface, although xylose was not present, the target protein has been still keeping a secretory or trans-membrane state in the host cell and was recognized, processed, and presented by relevant lymphocytes in the mucosal immune system, which could induce local immune responses.

We performed a comparison of immunogenicity between surface-displayed (PG1) and secretory (PG2) recombinant strains after inoculation to mice via intragastric route. Statistically significant differences were observed since the seventh day after the first immunization. As far as the same antigen was concerned, recombinant Lc PG1 was greater than Lc PG2 in the induction of antibody immune responses, which was a conspicuous event in this study. On

the premise of the same bacterial amounts, the quantity of antigen anchored on the surface of Lc PG1 was far larger than Lc PG2 (data not shown) when recombinant Lc colonized the mucosal surface of the gastrointestinal tract because Lc PG2 secreted antigen into the external milieu. In addition, both Lc PG1 and Lc PG2 are inducible expression systems based on xylose operon and could not keep a sustainable expression of the target protein. In summary, recombinant Lc PG1 possessed stronger immunogenicity. Moreover, we have tried S1-N fusion protein expressed in Lc but failed using various promoters, which indicated that not every gene can be expressed efficiently in a host organism.

In conclusion, we have demonstrated that the nucleocapsid protein of PEDV displayed on the surface of *L. casei* was able to reinforce surface-displayed S1 to elicit a higher level of both systemic and mucosal immune responses, although its antibodies cannot neutralize PEDV. Taking the difficult expression of the whole S protein of PEDV in *L. casei* into consideration, an oral vaccine that induces neutralization of an antibody must include S protein immunodominant region S1 (Ho et al. 2005; JunWei et al. 2009; Liyun et al. 2008). For a successful immune response against an antigen, it is necessary that the antigen is present in sufficient quantities and in a form that can be recognized by antigen-presenting cells of the immune system. Consequently, *L. casei* suitably acts as mucosal vaccine carrier against gastrointestinal tropism pathogens. Although Balb/C mouse is not a genuine and susceptible animal



**Fig. 7** Cytokine responses of spleen cells from mice immunized with PG1-S1+PG1-N, PG2-S1+PG2-N, PG1, and PG2. Bars represent the means ( $\pm$ standard deviation) of three replicates per treatment in one experiment, which was repeated twice. \* $P < 0.001$



model for PEDV, the results of our study at least indicate that *L. casei* surface-displayed S1 combined with *L. casei* surface-displayed N could act as a novel mucosal vaccine formulation that may present significant opportunities for PEDV vaccine development.

In recent several years, the development of an oral vaccine based on lactic acid bacteria as delivery vehicles has made great strides. By combining knowledge from the different disciplines, virology, molecular biology, immunology, bioinformatics, ecology, and epidemiology, even greater progress is expected to be made towards understanding of the importance of live bacterial carriers and the underlying principles of mucosal vaccination therein (Collins and Schodel 1997). The elucidation and characterization of the physical structure, chemical composition, and bioinformation of bacterial cell wall and surface protein have provided new and challenging opportunities which allow us to better understand the interaction between intestinal *Lactobacilli* and mucosal tissues. The recent development of molecular biological techniques could permit the construction of lactic acid bacteria-presenting foreign antigens exposed to the different immune organizations and for the first time make it opportunistic to further explore the potential of these safe food bacteria as vaccine vehicles (Gentshev et al. 2001).

In spite of the rapid and concurrent development of related knowledge and techniques, a few problems must be solved before the first commercial mucosal vaccine based on lactic acid bacteria comes out. Although it is possible to determine the relative accurate level of expressed antigen in the test tube, we cannot precisely monitor gene expression in the gastrointestinal tract. Therefore, it is necessary to develop novel methods to analyze expressed genes encoding antigens in situ so as to control the efficiency and persistence of expression. In addition, what is the optimal proportion of Lc-expressing S1 and Lc-expressing N in a mixture yielding better immune efficacy and how long could recombinant bacteria persist in the gastrointestinal tract remain in need of further research.

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