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Efficacy and immunogenicity of a live *L. acidophilus* expressing S_{AD} epitope of transmissible gastroenteritis virus as an oral vaccine

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Summary. – Transmissible gastroenteritis virus (TGEV) causes great economic loss to swine industry worldwide. Vaccination is an important method to control the TGEV infection. In this study, a TGEV oral vaccine was generated by transferring a eukaryotic expression recombinant plasmid carrying the S_{AD} (A and D antigenic sites of the S protein) epitope of TGEV into a swine-origin *Lactobacillus acidophilus* (*L. acidophilus*). In orally immunized BALB/c mice, the TGEV *L. acidophilus* oral vaccine induced significantly higher level of SIgA antibodies specific to TGEV compared with the mice immunized with a commercial inactivated TGEV vaccine and similar levels of IgG specific to TGEV as the inactivated vaccine. Furthermore, the TGEV *L. acidophilus* oral vaccine induced higher levels of IFN-γ, which suggested that the vaccine was able to induce immune response. In brief, this novel TGEV *L. acidophilus* oral vaccine could induce high levels of both mucosal and humoral immune responses, which has a potential to be used in the pig industries in the future.

Keywords: transmissible gastroenteritis virus (TGEV); live *L. acidophilus* oral vaccine; SIgA antibody; IgG antibody; IFN-γ; IL-4

Introduction

Transmissible gastroenteritis (TGE) is an acute, highly contagious, and rapidly spreading enteric disease leading to huge economic losses to the swine industry caused by transmissible gastroenteritis virus (TGEV), which is a member of the family *Coronaviridae* (Eleouet *et al*., 1995; Mendez *et al*., 1996). TGEV infection presents in swine of all ages, but its course is highly age-dependent, causing high mortality up to 100% in neonatal and young pigs under 2 weeks of age (Laude *et al*., 1993; Schwegmann-Wessels *et al*., 2003). TGEV is an enveloped virus containing a single-stranded, positive-sense polyadenylated RNA genome. The genome encodes four structural proteins: spike (S) protein, membrane (M) protein, nucleocapsid (N) protein, and the minor envelope (E) protein (Spaan *et al*., 1988; Laude *et al*., 1993; Schwegmann-Wessels *et al*., 2003). The S protein, carrying the major B-cell epitope, is a unique structural protein that induces neutralizing antibodies against TGEV (Jimenez *et al*., 1986; Gebauer *et al*., 1991). There are four antigenic sites (A, B, C, and D) on the N-terminus of the S protein (Correa *et al*., 1990; Delmas *et al*., 1990), among which the sites A and D are responsible for stimulating the production of neutralizing antibodies (Di-Qiu *et al*., 2011).

TGEV mainly causes gastrointestinal infection that could lead to local lesions, so the elicitation of an efficient immune response should be not only at systemic but also at mucosal level. Until now, vaccination appears to be the most effective

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Abbreviations: dpi = days post immunization; IFN-γ = interferon γ; IL-4 = interleukin 4; SIgA = secretory immunoglobulin A; TGEV = transmissible gastroenteritis virus

way to prevent TGEV infection, and mucosal immunization induces secretory immunoglobulin A (SIgA) that effectively neutralizes the TGEV. However, the commercial inactivated vaccine cannot induce mucosal immune response (Simkins *et al.*, 1992; Saif *et al.*, 1994; Tuboly and Nagy, 2001). *Lactobacillus*, a normal microbial flora in human and most animal intestines, has been widely used in food industry. It possesses the functions of balancing the gut flora, promoting digestion of food, and boosting immunity, which makes it one of the most attractive antigenic delivery vehicles for oral immunization being able to induce mucosal immune response (Pouwels *et al.*, 1998; Steidler *et al.*, 2000).

In this study, an eukaryotic recombinant expression plasmid pRc/CMV2-S_{AD}-Rep.8014 carrying the S_{AD} (A and D antigenic sites of the S protein) epitope of TGEV and replication gene Rep.8014 of *Lactobacillus acidophilus* (*L. acidophilus*) has been constructed and transformed into swine-origin *L. acidophilus*. Subsequently, the six-week-old BALB/c mice were orally immunized with the *L. acidophilus*. The results showed that the TGEV *L. acidophilus* oral vaccine stimulated significantly higher levels of specific SIgA against TGEV than commercial inactivated TGEV vaccine in mice, induced high levels of IgG antibodies, and upregulated the levels of IFN-γ, demonstrated that the vaccine was able to induce both humoral and mucosal immune responses.

Material and Methods

Construction of the shuttle plasmid pRc/CMV2-S_{AD}-Rep.8014. To amplify the TGEV S_{AD} epitope gene (333 amino acids), one pair of PCR primers (targeting nucleotides 1005 to 2305 of the S gene; F: 5'-AGTAAGCTTATGATCAGGTTTAACCTTA AT-3' and an antisense R: 5'-TAAGCGGCCGCTTAATTATCAGACGGTACAC-3' with *Hind*III and *Not*I restriction sites) was designed based on sequence deposited in GenBank (Acc. No. AY587882). The S_{AD} gene was amplified by RT-PCR from TGEV cDNA (Shandong Provincial Center for Animal Disease Control and Prevention, China). The PCR product was identified and purified by gel electrophoresis. Both purified S_{AD} gene and plasmid pRc/CMV2 (SIGMA, Germany) were subjected to *Hind*III and *Not*I double digestion, respectively, and then ligated by T4 DNA ligase (TIANGEN, Beijing, China). The recombinant pRc/CMV2-S_{AD} was sequenced and analyzed. The plasmids pGEM-T-Rep.8014 (Shandong Provincial Center for Animal Disease Control and Prevention, China) carrying replication gene Rep.8014 of *L. acidophilus* and pRc/CMV2-S_{AD} were then each separately subjected to a *NotI* and *ApaI* double digestion and ligated by T4 DNA ligase**.** The resulted recombinant pRc/CMV2-S_{AD}-Rep.8014 was then sequenced and analyzed.

Immunofluorescence assay (IFA). To check the expression of S_{AD} epitope from pRc/CMV2-S_{AD}-Rep.8014, the PK15 cells were transfected with the recombinant plasmid pRc/CMV2-S_{AD}-Rep.8014 and the negative control plasmid pRc/CMV2 with Vigofect Transfection reagent (Vigorous Biotechnology, Beijing, China), respectively. After incubation for 48 hours, the IFA was conducted with the swine TGEV-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. The fluorescence was observed under fluorescence microscope (Olympus, Japan).

Generation of recombinant L. acidophilus carrying recombinant plasmid pRc/CMV2-S_{AD}-Rep.8014. 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 (Landete *et al.*, 2014). *L. acidophilus* SW1 colony was inoculated into 3 ml of MRS broth (Hopebiol, Qingdao, China) for static cultivation at 37°C for 16 h. All of the bacterial cells were added into 150 ml MRS broth to further culture for 2–3 h until the OD_{600} of 0.1–0.2 was reached. Penicillin then was added to a final concentration of 5 μg/ml. In the early-log phase (OD_{600} 0.2–0.3, incubation for 1–1.5 h), the cells were harvested, chilled on ice for 10 min and washed twice with ice-cold EPWB (0.6 mM NaH_2PO_4, 0.1 mM $MgCl_2$, pH 7.4). Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA) was used for electroporation. Total of 1 μg pRc/CMV2-S_{AD}-Rep.8014 plasmid DNA was blended with 100 μl of the ice-cold cell suspension in a 0.2 cm cuvette and then put on the ice for 10 min. After that, the mixture was exposed to a high voltage pulse (peak field strength of 13 kV/cm, capacitance of 25 μF and resistance of 400 Ω). After electroporation, the bacterial cells were plated on selective MRS agar containing 5 μg/ml of ampicillin to inoculate for three days at 37°C. PCR was conducted with the primers (100 pmol each) targeting the partial S_{AD} gene (498 bp) to confirm the carrying of the gene, P1: 5'-AGG TGA CGT ATT GTA ATA GTT A-3' and an antisense P2: 5'-TTC TTG TAC GGG CAG CTA CAT C-3' in an EmeraldAmp® PCR Master Mix (TaKaRa, Dalian, China) in the final volume of 20 μl. Following denaturation at 94 °C for 7 min, 32 cycles of amplification (30 s at 94 C, 30 s at 53°C and 30 s at 72°C) were performed with the final extension at 72°C for 10 min. Meanwhile, the non-electroporated bacterial cells were set as a negative control. The products were analyzed on 0.8% agarose gel and verified by nucleotide sequencing.

Protein expression and purification of S_A epitope. The S_A epitope was amplified from the recombinant plasmid pRc/CMV2-S_{AD}-Rep.8014 by PCR and cloned into the pET-32a (QIAGEN, Germany) vector. Positive recombinant plasmids were transformed into *Escherichia coli* BL21 (TIANGEN, Beijing, China) for protein expression. Briefly, the positive BL21 colony containing recombinant expression vector pET-30a-S_A 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 of the desired protein at 37°C for 6 h.

The bacteria were pelleted at 8,000 rpm for 20 min, then resuspended in PBS and sonicated on ice (160 W, ultrasound for 2 s at 2 s intervals on ice, for 3 min totally). After sonication, the lysate

was centrifuged at 8,000 rpm for 10 min. The supernatant and precipitate were collected and subjected to SDS-PAGE and Western blot. The expressed protein was purified according to the procedure of His·Bind® Purification Kit (Novagen, USA). The concentration of the S_A fusion protein was determined according to the instructions of the BCA Protein Assay Kit manufacturer of (Beyotime, Shanghai, China). The Western blot was conducted with swine TGEV-positive serum as the first antibody and HRP-conjugated goat anti-swine IgG antibody (BioDee, Beijing, China) as the second antibody. The luminescence was visualized using a DAB color development kit (TIANGEN, Beijing, China).

Establishment of S_A-based indirect ELISA for detection of antibodies against S_{AD} epitope. To detect the IgG and SIgA antibodies specific to TGEV S_A epitope, indirect ELISA methods were developed referring to published protocols with minor modifications (Liu *et al.*, 2009). The optimal concentrations of serum and coating antigen was determined by a checkerboard titration, 96-well microtiter plates coated with two-fold diluted S_A protein antigen (from 12 μg/well to 0.325 μg/well, 100 μl/well) were incubated at 37°C for 1 h and then at 4°C overnight in bicarbonate buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, pH 9.6). Each well was washed four times with 200 μl PBS-0.1% Tween 20 (PBST), then incubated with 100 μl blocking buffer at 37°C for 2 h. After washing for four times with PBST, the wells were incubated with two-fold diluted mouse TEGV positive serum and negative serum (from 1:25 to 1:400, 100 μl/well) at 37°C for 1 h. 100 μl HRP-conjugated goat anti-mouse IgG antibody or HRP-conjugated goat anti-mouse IgA antibodies (1:6,000 dilutions, 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 mol/l 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 checkerboard test.

Immunization of mice and sample collection. A total of 24 six-week-old female BALB/c mice (Experimental Animal Center of Shandong University, China) were randomly separated into 3 groups. A group of mice were orally immunized with 10^9 CFU (colony-forming units) of recombinant *L. acidophilus* (100 μl of the suspension). Another group was subcutaneously immunized with 100 μl TGE-PED-Rotavirus inactivated vaccine (Lanzhou Pharmaceutical Factory of Biology, China). The last group was orally administered with 100 μl sterile phosphate buffer saline (PBS). All mice were boosted at 2 weeks post first immunization by the same strategy. Sera were collected via tail-bleeding at 0, 14, 28 and 42 days post immunization (dpi) for the detection of TGEV-specific antibody, interferon γ (IFN-γ) and interleukin 4 (IL-4).

IFN-γ and IL-4 levels detection in mouse sera. Serum IFN-γ levels were detected by an IFN-γ detection kit according to the manufacturer's instructions (R&D Systems, Shanghai, China). A standard curve was generated by using mouse IFN-γ standard by two-fold serial dilution in Calibrator Diluent RD5Y from 600 pg/ml to 9.4 pg/ml. Subsequently, the mouse IFN-γ standard dilutions and mouse IFN-γ control were coated onto 96-well microtiter plates for 2 h at room temperature. Meanwhile, serum samples were coated onto ELISA wells as primary antibodies. Mouse IFN-γ Conjugate used as secondary antibody was added to each well. The OD_{450} values were read and then the concentrations (pg/ml) of IFN-γ of mice were determined according to the standard curve.

Serum IL-4 levels were detected similarly by an IL-4 detection kit (R&D Systems, Shanghai, China). Mouse IL-4 Standard was diluted in Calibrator Diluent RD5Y by two-fold serial dilution between 500 pg/ml and 7.8 pg/ml, then coated onto 96-well microtiter plates for 2 h at room temperature. Serum samples were three-fold diluted in Calibrator Diluent RD5Y before being coated. The ELISA was performed as above and the concentration of IL-4 was determined based on the IL-4 standard curve.

Statistical analysis. All of the data were analyzed using SPSS 19.0 software and the values were presented as mean ± standard deviation (SD), with $P < 0.05$ and $P < 0.01$ considered as statistically significant and highly significant, respectively.

Ethics statement. All animal studies in this study were conducted in accordance to the guidelines of the Animal Care and Use Committee of Shandong Agriculture University, and all animal studies protocols are approved by Shandong Agriculture University.

Results

Construction and identification of recombinant plasmid pRc/CMV2-S_{AD}-Rep.8014

The pRc/CMV2-S_{AD} plasmid was obtained by amplifying and sub-cloning the S_{AD} fragment into pRc/CMV2 vector, then the replication gene Rep.8014 of *L. acidophilus* from pGEM-T-Rep.8014 was subcloned into pRc/CMV2-S_{AD}, resulting into pRc/CMV2-S_{AD}-Rep.8014. To verify the expression of S_{AD}, indirect immunofluorescence was conducted in PK15 cells transfected with pRc/CMV2-S_{AD}-Rep.8014. The results showed that positive fluorescence signal was observed in the cytoplasm under a fluorescence microscope (Olympus, Japan), while there was no fluorescence in the empty vector-transfected cells, indicated that the S_{AD} epitope was expressed successfully in PK15 cells (Fig. 1a,b).

Generation of recombinant L. acidophilus carrying recombinant plasmid pRc/CMV2-S_{AD}-Rep.8014

The pRc/CMV2-S_{AD}-Rep.8014 was transformed into Swine-origin *L. acidophilus* SW1 by electroporation assay. To determine whether the bacterial strain is carrying the recombinant plasmid pRc/CMV2-S_{AD}-Rep.8014 after the electroporation assay, specific primers of TGEV S_A gene were used for colony identification. In order to test if the pRc/CMV2-S_{AD}-Rep.8014 could be carried in recombinant *L. acidophilus* steadily, the positive *L. acidophilus* was cul-

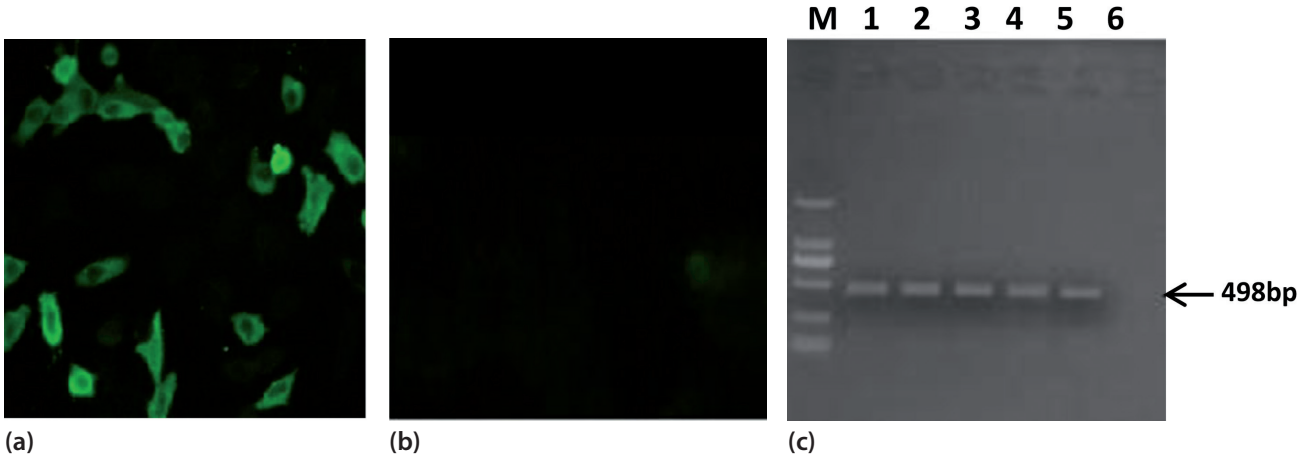

Fig. 1

Expression of the TGEV-S_{AD} protein in PK15 cells

(a) PK15 was transfected with the eukaryotic plasmid pRc/CMV2-S_{AD}-Rep.8014. **(b)** PK15 was transfected with the empty plasmid pRc/CMV2). **(c)** Identification of pRc/CMV2-S_{AD}-Rep.8014 in recombinant *L. acidophilus* after 5 passages by PCR (M, DL2000 DNA Marker. Lane 1–5, S_A gene fragments amplified by PCR from the 5 passages. Lane 6, Negative control).

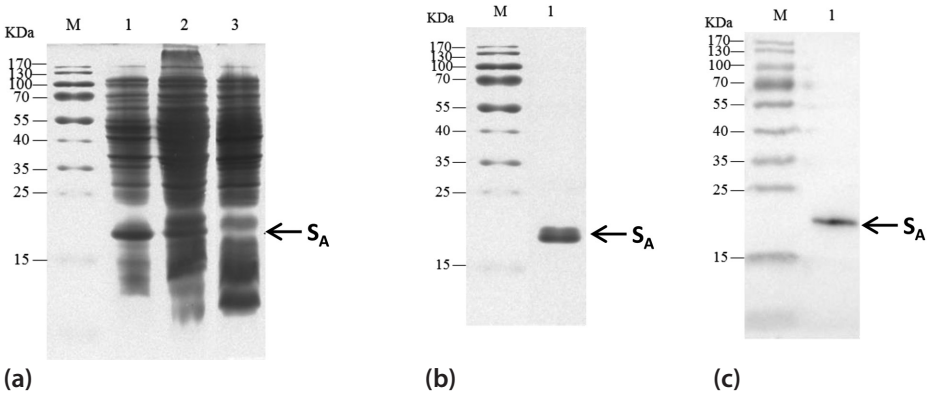

Fig. 2

Expression, purification and identification of recombinant SA fusion protein in BL21 *E. coli*

(a) SDS-PAGE analysis of S_A fusion protein. (M, Protein Marker. Lane 1, Precipitate of BL21 *E. coli* containing pET30a-S_A induced by IPTG. Lane 2, Supernatant of BL21 *E. coli* containing pET30a-S_A induced by IPTG. Lane 3, Control: BL21 *E. coli*). **(b)** SDS-PAGE analysis of the purified S_A fusion protein. (M, Protein Marker. Lane 1, Purified S_A fusion protein). **(c)** Western Blotting of S_A fusion protein using TGEV-positive swine serum as first antibody. (M, Protein Marker. Lane 1, Purified S_A fusion protein).

tured for 5 generations and analyzed through PCR, and the gene was detected as expected (Fig. 1c). Amplification products were analyzed by agarose gel electrophoresis and the results showed an about 500 bp band was amplified from all 5 passages that was consistent with the expected objective band size (498 bp).

Establishment of S_A-based indirect ELISA for the detection of antibody against S_{AD} epitope

To establish S_A-based indirect ELISA, the S_A epitope was expressed and purified from *E. coli* BL21. As shown in Fig. 2, the expression of the S_A epitope protein was assessed via SDS-PAGE, a band with the expected molecular mass of 17 kDa was observed upon staining with Coomassie brilliant blue (Fig. 2a). The S_A epitope protein was purified successfully with His·Bind Purification Kit (Fig. 2b), and the concentration of the purified protein is (1.448 mg/ml) was determined referring to BCA Protein Assay calibration curve. The purified S_A epitope protein was further identified by Western blot using TGEV-positive swine serum (Fig. 2c). A checkerboard titration was used to determine the optimal dilutions of antigen and serum. The optimal antigen concentration and serum sample dilution were set at 4 μg/ml and 1:100, respectively.

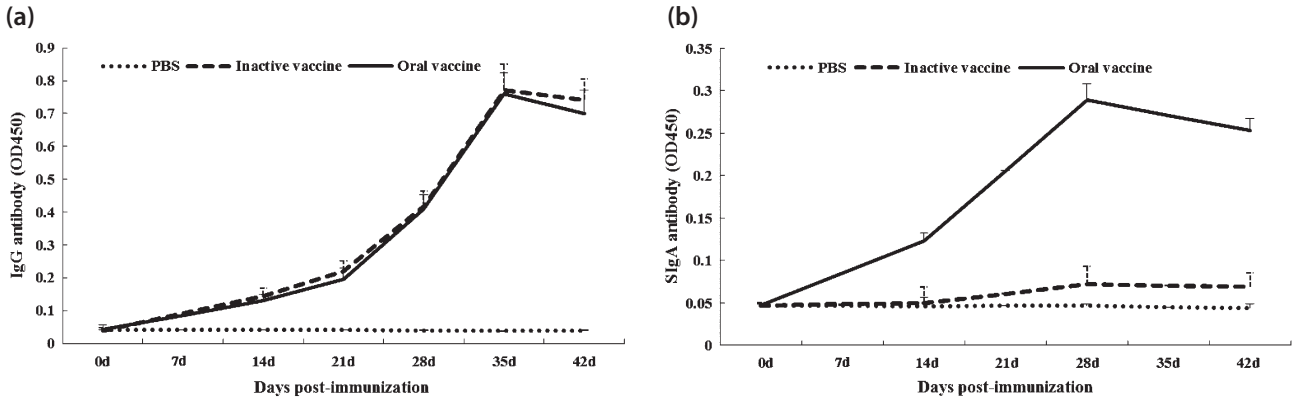

Fig. 3

The IgG and SIgA antibody levels in the immunized mice

(a) The anti-TGEV IgG antibody levels in the mouse groups immunized with PBS, inactivated vaccine, oral vaccine. The IgG levels were monitored at 0, 14, 21, 28, 35 and 42 days post immunization. **(b)** The anti-TGEV SIgA antibody levels of the mouse groups immunized with PBS, inactivated vaccine, oral vaccine. The antibodies were detected by indirect ELISA at different days post immunization, using TGEV S protein as the coating antigen. The SIgA levels were monitored at 0, 14, 28, and 42 days post immunization. * means significant difference compared with the PBS mock and inactivated vaccine group (P <0.05), ** means highly significant difference compared with the PBS mock and inactivated vaccine group (P <0.01).

Antibody detection of immunized mice

An indirect ELISA was used to detect serum and mucosal antibody levels against TGEV of mice immunized with *L. acidophilus* carrying the recombinant plasmid pRc/CMV2-S_{AD}-Rep.8014. The levels of IgG antibodies of orally immunized mice and commercial inactivated vaccine immunized mice began to increase at 14 dpi and reached the peak at 35 dpi (Fig. 3a). The TGEV *L. acidophilus* vaccine induced similar TGEV-specific IgG level as the commercial inactivated vaccine. While the IgG antibody levels of the oral vaccine group were slightly lower than those of inactivated vaccine group between 14 dpi to 42 dpi, but the differences between the two immunized groups were not significant (P >0.05) (Fig. 3a). Notably, the TGEV *L. acidophilus* vaccine was able to induce significantly higher levels of TGEV-specific SIgA antibody in mice than commercial inactivated vaccine (Fig. 3b). The SIgA antibody was detectable at two weeks after first immunization, however, limited level of SIgA was detected in inactivated vaccine group and SIgA antibody was undetectable in the PBS mock group. All the data indicated that the oral recombinant TGEV *L. acidophilus* induced both humoral and mucosal immunity.

Table 1. Serum IFN-γ levels in the immunized mice (pg/ml)

Group	Days post immunization					
	0	14	21	28	35	42
PBS	19.95±4.50	20.94±3.43	29.69±3.47	32.06±4.99	27.47±3.00	24.73±4.00
Inactivated vaccine	18.70±4.20	33.83±3.59*	73.66±4.24**	125.00±4.59**	106.53±9.62**	94.16±4.72**
Oral vaccine	19.19±4.07	31.14±2.98*	71.86±3.32**	118.20±7.59**	100.12±7.56**	92.03±7.10**

In the same column, *means significant difference compared with the PBS mock group (P <0.05), **means highly significant difference compared with PBS mock group (P <0.01).

Table 2. Serum IL-4 levels in the immunized mice (pg/ml)

Group	Days post immunization					
	0	14	21	28	35	42
PBS	29.86±1.68	30.00±2.29	30.43±3.50	30.77±1.56	31.05±2.64	31.41±2.23
Inactivated vaccine	29.31±2.58	30.50±2.34	30.44±2.23	47.16±2.66*	34.86±5.10	33.66±4.01
Oral vaccine	29.43±2.06	30.39±2.20	29.36±2.06	42.98±3.88*	34.17±5.12	33.33±3.74

In the same column, *means significant difference compared with the mock group (P <0.05)

Detection of serum IFN-γ and IL-4 of immunized mice

The serum IFN-γ levels of the two immunized groups began to increase after immunization and reached the peak at 28 dpi. The levels then decreased slowly from 28 dpi to 42 dpi, and they were significantly (P <0.01) higher than those of PBS group from 14 dpi to 42 dpi (Table 1). However, there was no significant difference between the two immunized groups from 0 dpi to 42 dpi (Table 1). From immunization to 21 dpi, there was no significant change of the IL-4 levels in the serum of all groups (Table 2). At 28 dpi, the IL-4 levels of the both groups were significantly (P <0.05) higher than those of PBS group (Table 2). At 28 dpi, the IL-4 levels in sera of the immunized mice reached the peak and decreased thereafter.

Discussion

The use of probiotics as a supplement has been well studied recently, focusing on their ability to provide natural additives in feeds. Studies have shown that *L. acidophilus* and its by-products support immune function in pigs. They have been selectively used as vaccine vector, which delivers the antigen for mucosal immunization. The oral vaccine with recombinant *L. acidophilus* expressing foreign antigen has been studied and induced local mucosal immune responses (Tang and Li, 2009; Wang *et al.*, 2012; Chamcha *et al.*, 2015; Jee *et al.*, 2017). For most of enteric viruses, the mucosal immunity plays an important role when the pathogens initiate the infection at the mucosa of intestines, such as porcine epidemic diarrhea virus (PEDV) and TGEV. The maternal vaccination routes generally fail to provide enough protection to local intestinal infections in the offsprings, while the mucosal SIgA has proven effect to limit the colonization by the pathogen, therefore against further spread of the disease (Jiang *et al.*, 2016). Thus, many studies employed *L. acidophilus* as a vector expressing the antigen in order to induce SIgA in the intestines (Ho *et al.*, 2005; Jiang *et al.*, 2016; Yu *et al.*, 2017). Studies have shown that *L. acidophilus* suppresses intestinal inflammation and its fermentation products can attenuate the acute phase response in weaned pigs (Burdick Sanchez *et al.*, 2018; Kim *et al.*, 2018). Furthermore, the adjuvant effect of *L. acidophilus* on immune response to DNA vaccine such as foot and mouth disease has been studied (Su *et al.*, 2014). In the present study, *L. acidophilus* is used as a vector, which is originally isolated from healthy pig intestine in our previous study, thus it has a potential to colonize in the pig intestine.

A previous study has found that live bacterial vectors could induce immune reactions, including humoral, cellular, and more importantly, mucosal immunity against pathogen infection (Mercenier *et al.*, 2000). In this study, we have developed an oral vaccine encoding S_{AD} antigenic sites of TGEV glycoprotein S1 protein, delivered by live *L. acidophilus*. The results suggested that the live oral TGEV *L. acidophilus* vaccine was able to induce TGEV-specific humoral antibody. Moreover, the TGEV specific SIgA levels of oral vaccine group were significantly (P <0.01) higher than those of inactivated vaccine group after 14 dpi, suggested the TGEV *L. acidophilus* vaccine efficiently induced mucosal immunity in mice (Fig. 3).

IFN-γ and IL-4 are important indicators for mouse cellular immunologic response. According to the functional difference, activated $CD4^+$ T cells are classified into at least two subgroups: Th1 and Th2. The Th1-like phenotype is distinctly associated with levels of interleukin-2 (IL-2) and IFN-γ (Robinson *et al.*, 1993; Ulmer *et al.*, 1993, 1997, 1998; Wang *et al.*, 1993). The Th2-like phenotype is predominantly characterized by the increasing levels of IL-4, interleukin 5 (IL-5) and interleukin 6 (IL-6) (Lekutis *et al.*, 1997). In this study, the serum levels of IFN-γ of the mice immunized with oral vaccine were significantly (P <0.01) higher than those of the PBS group from 14 dpi to 42 dpi (Table 2), whereas the serum levels of IL-4 of the oral vaccine group were significantly higher than those of the PBS group only at 28 dpi. The results indicated that the TGEV oral vaccine might mainly enhance a Th1-type immune response.

In conclusion, the TGEV *L. acidophilus* oral vaccine administered to mice could provoke high levels of humoral, cellular immune responses, and especially the production of specific mucosal SIgA antibody, which plays a critical role in anti-TGEV immune response. However, whether this TGEV *L. acidophilus* oral vaccine could provide sufficient protection in swine model needs further test. Taken together, we have demonstrated that live *L. acidophilus* expressing S_{AD} epitope of TGEV as an oral vaccine is immunogenic and has potential to be used in the industry.

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References

Burdick Sanchez NC, Carroll JA, Broadway PR, Bass BE, Frank JW (2018): Supplementation of a Lactobacillus acidophilus fermentation product can attenuate the acute phase response following a lipopolysaccharide challenge in weaned pigs. Animal 1–9. https://doi.org/10.1017/S1751731118001222

Chamcha V, Jones A, Quigley BR, Scott JR, Amara RR (2015): Oral Immunization with a Recombinant Lactococcus lactis-Expressing HIV-1 Antigen on Group A Streptococcus Pilus Induces Strong Mucosal Immunity in the Gut. J. Immunol. 195, 5025–5034. https://doi.org/10.4049/jimmunol.1501243

Correa I, Gebauer F, Bullido MJ, Sune C, Baay MF, Zwaagstra KA, Posthumus WP, Lenstra JA, Enjuanes, L (1990): Localization of antigenic sites of the E2 glycoprotein of transmissible gastroenteritis coronavirus. J. Gen. Virol. 71 (Pt 2), 271–279. https://doi.org/10.1099/0022-1317-71-2-271

Delmas B, Rasschaert D, Godet M, Gelfi J, Laude H (1990): Four major antigenic sites of the coronavirus transmissible gastroenteritis virus are located on the amino-terminal half of spike glycoprotein S. J. Gen. Virol. 71 (Pt 6), 1313–1323. https://doi.org/10.1099/0022-1317-71-6-1313

Di-Qiu L, Xin-Yuan Q, Jun-Wei G, Li-Jie T, Yan-Ping J, Yi-Jing L (2011): Construction and characterization of Lactobacillus pentosus expressing the D antigenic site of the spike protein of Transmissible gastroenteritis virus. Can. J. Microbiol. 57, 392–397. https://doi.org/10.1139/w11-027

Eleouet JF, Rasschaert D, Lambert P, Levy L, Vende P, Laude H (1995): Complete sequence (20 kilobases) of the polyprotein-encoding gene 1 of transmissible gastroenteritis virus. Virology 206, 817–822. https://doi.org/10.1006/viro.1995.1004

Gebauer F, Posthumus WP, Correa I, Sune C, Smerdou C, Sanchez CM, Lenstra JA, Meloen RH, Enjuanes L (1991): Residues involved in the antigenic sites of transmissible gastroenteritis coronavirus S glycoprotein. Virology 183, 225–238. https://doi.org/10.1016/0042-6822(91)90135-X

Ho PS, Kwang J, Lee YK (2005): Intragastric administration of Lactobacillus casei expressing transmissible gastroentritis coronavirus spike glycoprotein induced specific antibody production. Vaccine 23, 1335–1342. https://doi.org/10.1016/j.vaccine.2004.09.015

Jee PF, Tiong V, Shu MH, Khoo JJ, Wong WF, Abdul Rahim R, AbuBakar S, Chang LY (2017): Oral immunization of a non-recombinant Lactococcus lactis surface displaying influenza hemagglutinin 1 (HA1) induces mucosal immunity in mice. PLoS One. 12, e0187718. https://doi.org/10.1371/journal.pone.0187718

Jiang X, Hou X, Tang L, Jiang Y, Ma G, Li Y (2016): A phase trial of the oral Lactobacillus casei vaccine polarizes Th2 cell immunity against transmissible gastroenteritis coronavirus infection. Appl. Microbiol. Biotechnol. 100, 7457–7469. https://doi.org/10.1007/s00253-016-7424-9

Jimenez G, Correa I, Melgosa MP, Bullido MJ, Enjuanes L (1986): Critical epitopes in transmissible gastroenteritis virus neutralization. J. Virol. 60, 131–139.

Kim DH, Kim S, Lee JH, Kim JH, Che X, Ma HW, Seo DH, Kim TI, Kim WH, Kim SW, Cheon JH (2018): Lactobacillus acidophilus suppresses intestinal inflammation by inhibiting endoplasmic reticulum stress. J. Gastroenterol. Hepatol. 34, 178–185. https://doi.org/10.1111/jgh.14362

Landete JM, Arques JL, Peiroten A, Langa S, Medina M (2014): An improved method for the electrotransformation of lactic acid bacteria: A comparative survey. J. Microbiol. Methods. 105, 130–3. https://doi.org/10.1016/j.mimet.2014.07.022

Laude H, Van Reeth K, Pensaert M (1993): Porcine respiratory coronavirus: molecular features and virus-host interactions. Vet. Res. 24, 125–150.

Lekutis C, Shiver JW, Liu MA, Letvin NL (1997): HIV-1 env DNA vaccine administered to rhesus monkeys elicits MHC class II-restricted CD4+ T helper cells that secrete IFN-gamma and TNF-alpha. J. Immunol. 158, 4471–4477.

Liu B, Li G, Sui X, Yin J, Wang H, Ren X (2009): Expression and functional analysis of porcine aminopeptidase N produced in prokaryotic expression system. J. Biotechnol. 141, 91–96. https://doi.org/10.1016/j.jbiotec.2009.02.005

Mendez A, Smerdou C, Izeta A, Gebauer F, Enjuanes L (1996): Molecular characterization of transmissible gastroenteritis coronavirus defective interfering genomes: packaging and heterogeneity. Virology 217, 495–507. https://doi.org/10.1006/viro.1996.0144

Mercenier A, Muller-Alouf H, Grangette C (2000): Lactic acid bacteria as live vaccines. Curr. Issues. Mol. Biol. 2, 17–25.

Pouwels PH, Leer RJ, Shaw M, Heijne den Bak-Glashouwer MJ, Tielen FD, Smit E, Martinez B, Jore J, Conway PL (1998): Lactic acid bacteria as antigen delivery vehicles for oral immunization purposes. Int. J. Food. Microbiol. 41, 155–167. https://doi.org/10.1016/S0168-1605(98)00048-8

Robinson HL, Hunt LA, Webster RG (1993): Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. Vaccine. 11, 957–960. https://doi.org/10.1016/0264-410X(93)90385-B

Saif LJ, van Cott JL, Brim TA (1994): Immunity to transmissible gastroenteritis virus and porcine respiratory coronavirus infections in swine. Vet. Immunol. Immunopathol. 43, 89–97. https://doi.org/10.1016/0165-2427(94)90124-4

Schwegmann-Wessels C, Zimmer G, Schroder B, Breves G, Herrler G (2003): Binding of transmissible gastroenteritis coronavirus to brush border membrane sialoglycoproteins. J. Virol. 77, 11846–11848. https://doi.org/10.1128/JVI.77.21.11846-11848.2003

Simkins RA, Weilnau PA, Bias J, Saif, LJ (1992): Antigenic variation among transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus strains detected with monoclonal antibodies to the S protein of TGEV. Am. J. Vet. Res. 53, 1253–1258.

Spaan W, Cavanagh D, Horzinek MC (1988): Coronaviruses: structure and genome expression. J. Gen. Virol. 69 (Pt 12), 2939–52. https://doi.org/10.1099/0022-1317-69-12-2939

Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E (2000): Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. Science 289, 1352–1355. https://doi.org/10.1126/science.289.5483.1352

Su J, Li J, Zheng H, You Y, Luo X, Li Y, Li X, Ma X, Li J, Dou Y, Cai X (2014): Adjuvant effects of L. acidophilus LW1 on immune responses to the foot-and-mouth disease virus DNA vaccine in mice. PLoS One 9, e104446. https://doi.org/10.1371/journal.pone.0104446

Tang L, Li Y (2009): Oral immunization of mice with recombinant Lactococcus lactis expressing porcine transmissible

gastroenteritis virus spike glycoprotein. Virus Genes 39, 238–245. https://doi.org/10.1007/s11262-009-0390-x

Tuboly T, Nagy E (2001): Construction and characterization of recombinant porcine adenovirus serotype 5 expressing the transmissible gastroenteritis virus spike gene. J. Gen. Virol. 82, 183–190. https://doi.org/10.1099/0022-1317-82-1-183

Ulmer JB, Deck RR, DeWitt CM, Donnelly JJ, Friedman A, Montgomery DL, Yawman AM, Orme IM, Denis O, Content J, Huygen K, Liu MA (1997): Induction of immunity by DNA vaccination: application to influenza and tuberculosis. Behring. Inst. Mitt. 79–86.

Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ, Gromkowski SH, Deck RR, DeWitt CM, Friedman A, et al. (1993): Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259, 1745–1759. https://doi.org/10.1126/science.8456302

Ulmer JB, Fu TM, Deck RR, Friedman A, Guan L, DeWitt C, Liu X, Wang S, Liu MA, Donnelly JJ, Caulfield MJ (1998): Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA. J. Virol. 72, 5648–5653.

Wang B, Ugen KE, Srikantan V, Agadjanyan MG, Dang K, Refaeli Y, Sato AI, Boyer J, Williams WV, Weiner DB (1993): Gene inoculation generates immune responses against human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA 90, 4156–4160. https://doi.org/10.1073/pnas.90.9.4156

Wang Z, Gao J, Yu Q, Yang, Q (2012): Oral immunization with recombinant Lactococcus lactis expressing the hemagglutinin of the avian influenza virus induces mucosal and systemic immune responses. Future. Microbiol. 7, 1003–1010. https://doi.org/10.2217/fmb.12.69

Yu M, Wang L, Ma S, Wang X, Wang Y, Xiao Y, Jiang Y, Qiao X, Tang L, Xu Y, Li Y (2017): Immunogenicity of eGFP-Marked Recombinant Lactobacillus casei against Transmissible Gastroenteritis Virus and Porcine Epidemic Diarrhea Virus. Viruses 9, 274. https://doi.org/10.3390/v9100274