Virus Genes (2009) 39:238–245
DOI 10.1007/s11262-009-0390-x

Oral immunization of mice with recombinant *Lactococcus lactis* expressing porcine transmissible gastroenteritis virus spike glycoprotein

Lijie Tang · Yijing Li

Received: 10 March 2009 / Accepted: 9 July 2009 / Published online: 24 July 2009

Abstract *Lactococcus lactis* NZ9000 was selected as an antigen delivery vehicle for mucosal immunization against porcine transmissible gastroenteritis virus (TGEV) infection. An approximately 70 kDa fragment of the N-terminal globular domain of the spike (S) protein (S_N protein) from the coronavirus TGEV was used as the transmissible gastroenteritis virus antigen model. Recombinant *L. lactis*, expressing the S_N protein, was constructed with the pNZ8112 plasmid. Expression and localization of the transcribed S_N protein from the recombinant L_{NZ}9000-rTGEV-S_N were detected via SDS-PAGE, Western blot, and immunofluorescence. BALB/c mice, orally immunized with L_{NZ}9000-rTGEV-S_N, produced local mucosal immune responses against TGEV. The induced antibodies demonstrated neutralizing effects on TGEV infection. These data indicated that the recombinant *L. lactis* could be a valuable tool in the development of future vaccines against TGEV.

Keywords TGEV S glycoprotein · *Lactococcus lactis* · Oral immunization

Introduction

Research carried out in recent years has led to the conviction that certain strains of lactic acid bacteria (LAB), in particular strains from the genera *Lactobacillus* and *Bifidobactrium*, may promote health in man and animals. LAB possesses a number of properties that make them attractive candidates for oral vaccination purposes. These bacteria are considered to be 'safe' organisms with a GRAS (Generally Recognized As Safe) status. In addition, *Lactobacilli* are able to survive passage through the upper gastrointestinal tract (GIT), and certain strains are capable of colonizing the intestinal tract [1–3]. These properties are particularly relevant when vaccines are to be applied in less industrialized countries. Furthermore, many bacterial, viral, and parasitic pathogens enter the body via the mucosal surfaces of the body. The first line of defense for humans and animals is found, not surprisingly, in the intestines, the largest immunological organ of the human body. More than 60% of antibodies produced daily are secreted into the GIT. Oral immunization frequently evokes both local and systemic immune responses, resulting in an effective elimination of foreign invaders [4–7]. These characteristics make them attractive candidates for delivering pharmaceutical compounds of interest to the mucosa, particularly vaccines and immunomodulators.

Transmissible gastroenteritis virus (TGEV) is a coronavirus that causes a highly contagious enteric disease in pigs, resulting in neonatal mortality and severe economical loses to the affected farms. The viral genome consists of a single-stranded, positive-sense 28.5 kb RNA and is constructed of three major structural proteins: S, M, and N, and a fourth structural protein, the small membrane (sM) protein [8, 9]. Protein S is the major inducer of TGEV neutralizing antibodies. The S protein has been used mainly for the induction of protective immunity against TGEV, and relevant neutralization epitopes have been mapped to the N-terminal domain of this protein. Four major antigenic sites have been described in

L. Tang
Life Science Department, Northeast Agricultural University, Harbin 150030, People's Republic of China

Y. Li (✉)
Veterinary Department, Northeast Agricultural University, Harbin 150030, People's Republic of China
e-mail: yijingli@yahoo.com

glycoprotein S, of which site A is immunodominant [10–12]. The protection of suckling piglets against TGEV infection is based on the uptake of specific lactogenic antibodies, mainly of the IgA class, from the milk of the TGEV-immune sows [13, 14].

In the present article, we used *Lactococcus lactis* NZ9000 as a vector to express the N-terminal globular domain of the spike (S) protein and to act as an antigenic carrier in oral vaccination. Our data indicated that intragastric inoculation with the recombinant bacteria L_{NZ}9000-rTGEV-S_N could induce specific immune responses against TGEV.

Materials and methods

Bacterial strain and growth conditions

Lactococcus lactis were grown at 30°C in M17 medium containing 0.5% glucose (GM17). When required, antibiotics (chloramphenicol) were added at 10 μg/ml for *L. lactis*, unless otherwise stated. Plating of bacteria was performed on GM17 medium with 1.5% agar. The antibiotic concentration used for the selection of *Lactococcus* transformants was 10 μg/ml of Chloromycetin (Cm, Sigma, St. Louis, MO).

Labeling of *Lactococcus* with cFDA-SE

Lactococcus lactis NZ9000 was labeled with 5′-(and 6′)-carboxyfluorescein diacetate succinimidyl ester, (cFDA-SE; Molecular Probes-Invitrogen, Carlsbad, CA; Sigma), a non-fluorescent, membrane-permeable ester, which is converted to a fluorescent derivative by a non-specific prokaryotic/eukaryotic intracellular esterase. The derivative is then covalently linked to an intracellular protein via the probe's succinimidyl group [15]. Briefly, a 100 mM stock solution of cFDA-SE was dissolved in dimethyl sulfoxide and then diluted in ethanol (1 ml, reagent grade). This solution was filter-sterilized before being aliquoted and stored at −20°C. *L. lactis* NZ9000 was grown overnight at 30°C in M17 medium containing 0.5% glucose. The bacterial cultures were centrifuged at 4,000×*g* for 10 min, and the pellets were washed twice in sterile PBS and adjusted to a concentration of 10^{10} CFU/ml before being labeled with cFDA-SE at 37°C for 20 min. Fluorescent labeling was terminated by pelleting the bacteria. The labeled bacteria were washed twice in sterile PBS to remove excess cFDA-SE and resuspended in sterile PBS. The flow cytometry profile (FACS Calibur TM: BD Biosciences, Cell Quest^{TM} Pro) showed that about 99.5% of cells were labeled.

Animal adhesion study

Seven-week-old BALB/c mice were obtained from the Laboratory Animal Center, Harbin Medical University of China. A group of 15 mice were dosed with approximately 10^9 cFDA-SE-labeled bacteria by oral administration. Another group of 15 mice was orally fed with sterile PBS as the control. Groups of three mice each were killed on days 1, 2, 4, 6, and 7, and then the duodenum, jejunum, ileum, and colon were extracted from each mouse. Individual sections were cut longitudinally, and residual food particles or fecal material was removed from the intestine before being examined for the presence of adhering cFDA-SE-labeled bacteria. This was performed by adding 150 μl of PBS to every 1.0 cm of tissue and dislodging microbes from the mucosal surface of the tissues. Cell extracts were fixed with formaldehyde (0.75%, v/v), prior to flow cytometry analysis [3].

Construction of the TGEV S_N gene expression plasmid

TGEV strain TH-98 was isolated and stored in our laboratory [16]. All DNA manipulations were performed according to standard procedures [17]. The pNZ8112 plasmid used in the present study is a cell-surface expression vector containing the ssUSP45 secretion signal peptide and anchor domain structure, a gift from NIZO, The Netherlands. A 2,037-bp fragment of the gene encoding TGEV antigen S that encompasses the four major antigenic neutralization epitopes was obtained by RT-PCR from TGEV genome. The oligonucleotides were: 5′-CTTGCATGCATGAAAAAACTGTTCGTTGTTCTGGTTGTAAT-3′ (forward) containing an *Sph*I site and 5′-CGTCTAGACACACCACTATTATCAGACGGTACACCCACTATGTTG-3′ (reverse) containing an *Xba*I site. PCR amplification conditions were as follows: 95°C for 5 min; 30 cycles of 94°C for 1 min, 57.9°C for 1.0 min and 72°C for 1.5 min; and 72°C for 10 min of final extension. The PCR product was digested by *Sph*I and *Xba*I, and inserted into the corresponding sites of pNZ8112.

Generation of recombinant *L. lactis* NZ9000 with the TGEV S_N protein

Electroporation was carried out according to the method described by Josson et al. [18] and Holo and Nes [19], with some modifications. Briefly, 10 μl of recombinant plasmid DNA was added to 150 μl of *L. lactis* NZ9000, gently mixed and placed at 4°C for 5 min. The mixer was subjected to a single electric pulse (25 μF of 2500 V/cm), resuspended in GM17 medium without antibiotics and incubated under anaerobic conditions at 30°C for 2 h. Recombinant strains were selected on GM17-agar medium

containing l0 μg/ml of Cm. The presence and integrity of the constructions were checked by extraction of recombinant plasmid DNA [20], restriction enzyme analysis, and sequencing.

Segregation and structural stability of pNZ8112-S_N

To assess the segregation stability of NZ8112-S_N in *L. lactis* NZ9000, the recombinant strain was grown in GM17 medium at 30°C without Cm. Serial subcultures were performed by diluting the culture in fresh broth (1:100) without antibiotics and growing until the mid-exponential phase was reached (OD600 at 1.0). After 80 generations, the percentage of Cm-resistant (Cm^r) colonies was determined by plating 100 μl of culture dilutions onto GM17 plates without Cm or plates supplemented with 10 μg/ml Cm. To determine the structural stability of the recombinant plasmid after 80 generations, several colonies were randomly picked from the GM17 plate and analyzed by colony PCR, using forward and reverse primers of the S gene.

Protein expression and localization analysis

To analyze the expression and localization of the S_N fusion protein, overnight cultures of strains L_{NZ}9000-rTGEV-S_N, NZ9000 (with plasmid pNZ8112), and NZ9000 were introduced into fresh medium at a ratio of 1:250. For the induction of the nisA promoter, strains were grown to an optical density of 0.5 at 600 nm, and nisin (Sigma) was added to a final concentration of 10 ng/ml. Growth was continued for 3 h. For NZ9000, growth was continued without addition of nisin until an optical density of 1.0 at 600 nm was reached. To prepare extracts, cells were pelleted by centrifugation at 5,000×*g* for 15 min at 4°C. The bacterial pellets were washed twice with sterile, 50 mM Tris-Cl (pH 8.0) and lysed in a Bead-Beater (Biospec, Bartlesville, USA) by vigorous shaking. The lysates were centrifuged at 15,000×*g* for l0 min, and the supernatants were stored at −20°C until further analysis. The bacterial protein extracts and supernatant were examined using 10% SDS-PAGE. Proteins were electrotransferred onto a nitrocellulose membrane, and the immunoblots were developed using mouse anti-TGEV serum (the antiserum obtained from BALB/c mice immunized with of TGEV). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma, 1:2000) was used, and visualization of the immunolabeled bands was carried out using the Chemiluminescent Substrate reagent (Pierce) according to the manufacturer's instruction.

Immunofluorescence was also used to analyze the cell-surface display of S_N protein. Induced bacterial cultures of L_{NZ9000}-rTGEV-S_N (1.0 ml) were pelleted, washed twice with sterile PBS, resuspended in PBS containing mouse anti-S serum, and incubated at 37°C for 2 h. The cells were centrifuged and washed twice with sterile PBS to remove unbound antibodies. The pellets were incubated with FITC-conjugated goat anti-mouse IgG (Sigma) containing 1% Evans blue dye (Pierce) at 37°C for 2 h. The cells were washed twice with sterile PBS, resuspended in sterile PBS and laid on a glass slide. Confocal microscopy was used to visualize the fluorescence on the cell surface of L_{NZ9000}-rTGEV-S_N. The strain of *Lactococcus lactis* NZ9000 harboring the pNZ8112 plasmid and the wide-type *L. lactis* NZ9000 were used as controls.

Immunization

L_{NZ9000}-rTGEV-S_N was cultured, induced with nisin, and centrifuged. Cell pellets were washed once with sterile PBS and resuspended in PBS to a concentration of 10^{10} CFU/ml. The treatment group of mice received doses of 10^9 cells of L_{NZ9000}-rTGEV-S_N. The control mice were immunized with equivalent doses of *L. lactis* NZ9000 with pNZ8112. The sham control group received 0.1 ml doses of PBS. All the mice were immunized via oral administration. The immune protocol was administered on three consecutive days at days 0, 1, and 2. A booster immunization was given at days 14, 15, and 16, and a second booster was given at days 28, 29, and 30 [21].

ELISA analysis of serum and intestine mucus

Sera were prepared and stored at −20°C until required. The intestinal mucus of mice was extracted as described previously [22, 23]. Polystyrene microtiter plates were coated with the TGEV propagated on swine testicular (ST) cells overnight at 4°C, and the cultures of swine testicular (ST) cells were used as the negative control antigen. The ELISA plates were washed twice in PBS containing 1% Tween-20 and then saturated with PBS containing 5% skimmed milk at 37°C for 2 h. Serum or intestinal lavage was diluted tenfold in PBS containing 1% BSA and primary antibodies. After incubation at 37°C for 1 h, the plates were washed twice with PBS containing 1% Tween-20. Bound antibodies were detected using HRP-conjugated goat anti-mouse IgA or IgG (Sigma), followed by color development using *o*-phenylene diamine dihydrochloride (Sigma) as the substrate. The absorbance was measured at 490 nm, and the endpoint titers and concentrations of antibody (IgA/IgG) from the optical density (OD) taken from ELISA data were calculated according to Raghava et al. [24].

Neutralization ability of the induced antibodies

Intestinal fluids and serum samples obtained from the mice immunized with L_{NZ}9000-rTGEV-S_N were evaluated using

the plaque reduction assay to determine the neutralization ability. Lavages and serum obtained from the mice fed with the non-expressing strain or PBS were used as negative control, and 50 µl of samples in twofold serial dilutions (from 1:2 to 256) were prepared in 96-well plates. TGEV, adjusted to 100 TCID50 in 50 µl of virus diluent (10% concentrated Hank's balanced salt solution, 0.1% bovine serum albumin, pH 7.4), was added to the cell plate containing serially diluted intestinal lavage and serum. The antibody and virus mixture was mixed and incubated at 37°C for 1 h, and then 100 µl of ST cells (used for virus infection) were added to the antibody–virus mixture and incubated at 5% CO_2 and 37°C for 5 days. The overlay medium was then discarded, after which the wells were washed twice with sterile PBS (pH 7.4) and stained with 1% crystal violet solution. Differences in the number of plaques formed between treatments were examined, and the level of significance was determined by analysis of variance (ANOVA) followed by the Student's *t*-test.

Results

Adhesion of *L. lactis* NZ9000

The persistence of *L. lactis* NZ9000 in the intestinal tract was determined via oral administration of cFDA-SE-labeled *Lactococcus* to mice and sampling the different regions of the intestine, post-orogastric intubation. Flow cytometric analysis of cell extracts (Table 1) indicated that *Lactococcus* was able to survive and adhere to different regions of the intestinal tract. Adhesion was most prominent in the ileum, with $8.45 \pm 0.67 \times 10^4$ cells being detected on the first day. By the seventh day, the amounts of *L. lactis* NZ9000 that remained adhered to the intestinal mucosa were 22.17, 33.29, 36.33, and 35.76% of that observed on the first day in duodenum, jejunum, ileum, and colon, respectively.

Cell-surface expression of the TGEV S_N antigen

The gene encoding the S_N protein was amplified and cloned as an *Sph*I–*Xba*I fragment into the corresponding sites of the expression plasmid, generating pNZ8112-S_N (Fig. 1). The recombinant plasmid was electroporated into *L. lactis* NZ9000, producing the recombinant strain L_{NZ}9000-rTGEV-S_N.

L_{NZ}9000-rTGEV-S_N colonies were grown overnight in basal GM17 medium and then induced with nisin. The cell

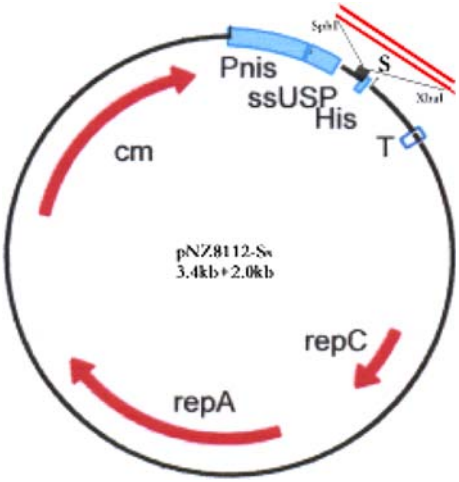

Fig. 1 Map of pNZ8112-S_N. Cm^r resistance determinant; repA and repC replication elements; *nis*A-promoter and multiple cloning sites with terminator sequence behind the mcs are shown. The *usp*45 signal sequence ssUSP is translationally fused to the *nis*A-promoter. Possibility for C-terminal His tag is present. This is a broad-range host vector with the *cat*-gene and an *Nco*I-site for translational fusions. The S_N gene was obtained from TGEV genome RNA by RT-PCR

Table 1 Average number of cFDA-SE labeled *L. lactis* isolated from different sections of murine intestinal tract and the percentage of the initial population (day 1) that remained attached various days post-oral intubation

Days post-oral feeding	Duodenum	Jejunum	Ileum	Colon
1	8.93 ± 0.64E + 03	1.55 ± 0.86E + 04	8.45 ± 0.72E + 04	6.74 ± 0.69E + 04
2	6.82 ± 0.38E + 03	6.36 ± 0.57E + 03	6.03 ± 0.54E + 04	4.33 ± 0.36E + 04
4	3.89 ± 0.52E + 03	5.23 ± 0.48E + 03	4.35 ± 0.61E + 04	2.42 ± 0.28E + 04
6	2.93 ± 0.68E + 03	5.99 ± 0.81E + 03	3.35 ± 0.27E + 04	2.63 ± 0.61E + 04
7	1.98 ± 0.71E + 03	5.16 ± 0.29E + 03	3.07 ± 0.38E + 04	2.41 ± 0.56E + 04
	Percentage of cells remaining as of day 1(%)			
2	76.37	41.03	71.36	64.24
4	43.56	33.74	51.48	35.90
6	32.81	38.65	39.64	39.02
7	22.17	33.29	36.33	35.76

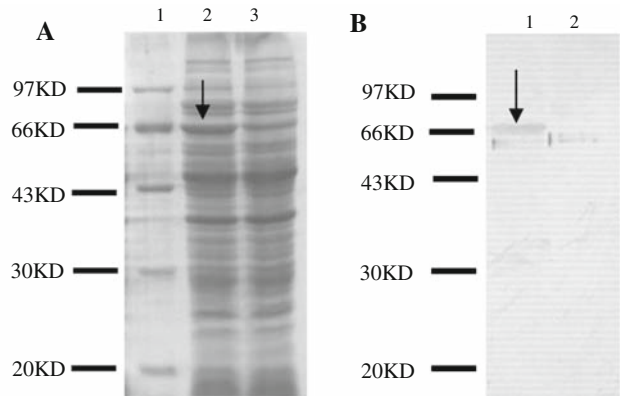

Fig. 2 The SDS-PAGE (**a**) and Western blot (**b**) analyses of the cell lysates from the expressed product. Lane a1: MW, molecular mass marker; Lane a2: proteins from L_{NZ}9000-rTGEV-S_N induced by nisin; Lane a3: proteins from *Lactococcus lactis* NZ9000 harboring pNZ8112, induced by nisin; Laneb1: western blot analysis of the product of L_{NZ}9000-rTGEV-S_N; Lane b2: western blot analysis of the product of *Lactococcus lactis* NZ9000 harboring pNZ8112. *Arrow* indicates the expressed protein

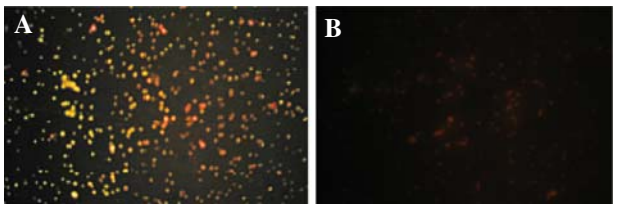

Fig. 3 Detection of the expressed product by indirect immunofluorescence (40×). **a** The recombinant strain of L_{NZ}9000-rTGEV-S_N. The bacteria exhibit strong *yellow* and *green* fluorescence; **b** The *Lactococcus lactis* NZ9000 strain with pNZ8112. There is no fluorescence. The cells were stained *red*. (Color figure online)

lysates were analyzed by SDS-PAGE and Western blot using mouse anti-TGEV serum. Coomassie blue gel-staining showed an approximately 70 kDa fusion protein band in L_{NZ}9000-rTGEV-S_N (Fig. 2a lane 2), but not in *L. lactis* NZ9000 with pNZ8112 (Fig. 2a lane 3) or wild-type *L. lactis* NZ9000 (data not shown). Similarly, an immune-reactive band was detected (Fig. 2b lane 1) via Western blot in a similar position, as observed in the SDS-PAGE in L_{NZ}9000-rTGEV-S_N (Fig. 2a lane 2). As the negative control, *L. lactis* NZ9000 with pNZ8112 did not display the corresponding immunoreactive band (Fig. 2b lane 2). These results show that the nisin promoter from pNZ8112 could efficiently induce the expression of the TGEV heterologous protein.

The immunofluorescence assay was developed with the mouse anti-TGEV serum and FITC fluorescence-conjugated goat anti-mouse IgG to confirm the cell-surface display of the S_N fusion protein. The results indicated that there was green–yellow fluorescence on the cell surface of

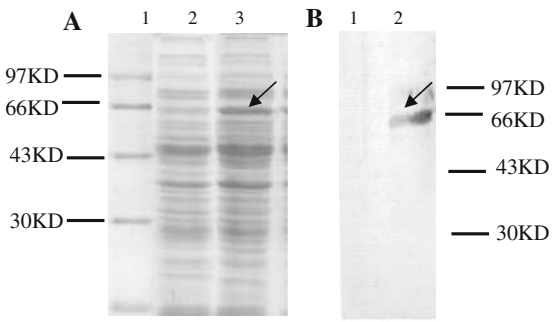

Fig. 4 Expression and identification of the S_N protein in L_{NZ}9000-rTGEV-S_N subcultured for 80 generations without selection for Cm^r. Lane a1: MW, molecular mass marker; lanes a2 and a3: Coomassie blue gel staining shows an approximately 70 kDa fusion protein expressed from L_{NZ}9000-rTGEV-S_N, induced by nisin or uninduced (lane a2); lanes b1 and b2: immunoreactive band of protein expressed by L_{NZ}9000-rTGEV-S_N. No immunoreactive bands were observed in the cell lysates from the recombinant cells without induction by nisin (lane B1). *Arrow* indicates the expressed protein

L_{NZ}9000-rTGEV-S_N (Fig. 3a), and the control bacteria were red, as determined with the Evans blue dye (Fig. 3b). The localization analysis results suggested that the S_N protein could be exposed externally on the cell wall. The S protein could not be detected in the supernatant from induced cultures of L_{NZ}9000-rTGEV-S_N, even after concentrating the supernatant by 50-fold in a Millipore Ultrafree-15 column (data not shown).

Next, the segregation and structural stability of pNZ8112-S_N in *L. lactis* NZ9000 were analyzed after 80 generations of subcultures in GM17 broth without Cm. The results indicated that the recombinant plasmid was stable and maintained its Cm^r phenotype and structural arrangement. Thus, the recombinant *Lactococcus* could still express the protein of interest (Fig. 4).

Immune responses of the recombinant *L. lactis*

The mucosal immune response was investigated by measuring the anti-TGEV-S_N IgA level in intestinal lavages following intragastric immunization. The concentration of mucosal IgA against TGEV-S_N was determined by ELISA, using the TGEV cultures as the coating antigen. The results showed that there was no substantial difference in mucosal IgA levels between experimental and control groups, prior to intragastric immunization. However, oral immunization with recombinant L_{NZ}9000-rTGEV-S_N elicited an antigen-specific, mucosal IgA response. After the second booster, a high level of anti-TGEV-S_N IgA was observed in intestinal lavages of mice immunized with L_{NZ}9000-rTGEV-S_N (Fig. 5), whereas no anti-S_N-specific IgA was detected in controls. When the ST cell culture was used as the negative control antigen, no anti-S_N-specific IgA was detected (data

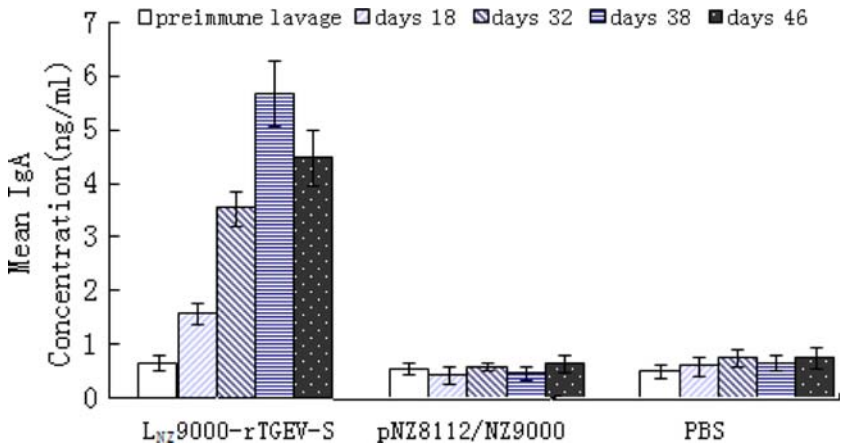

Fig. 5 Anti-TGEV-S_N-specific IgA level in intestinal lavage after intragastric immunization. Mice received three consecutive doses of 109 CFU/ml L_{NZ}9000-rTGEV-S_N, 3 times within a 2-week period. Control mice received 109 CFU/ml pNZ8112/NZ9000, while the negative group received PBS. Intestinal lavages were collected on days 18, 32, 38, and 46 after the first immunization, and were analyzed via ELISA using TGEV as the coating antigen. *Bars* represent the IgA titer ± S.E.M in each group

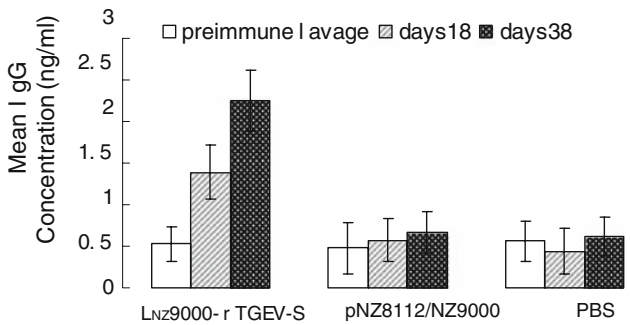

Fig. 6 Anti-TGEV-S_N serum IgG level after intragastric immunization. Sera from groups of mice immunized orally with 109 CFU/ml L_{NZ}9000-rTGEV-S_N and equivalent doses of pNZ8112/NZ9000 and PBS were analyzed for the presence of anti-TGEV-S_N-specific IgG by ELISA, using TGEV as the coating antigen. *Bars* represent the IgG titer ± S.E.M in each group

not shown). Likewise, a similar result was observed with the detection of anti-TGEV-S_N-specific IgG (Fig. 6).

Plaque reduction assay

Plaque reduction assays were performed to detect whether the antibody responses were specific to the TGEV antigen. Results demonstrated that the presence of anti-TGEV-S_N IgA or IgG in the culture medium conferred statistically significant neutralizing effects ($P < 0.05$) on TGEV infection (Fig. 7). A consistent reduction of 22.3 ± 3.28% in the number of plaques was observed when plaque reduction assays were carried out using two- to eightfold dilutions of sera, and a reduction of 35.6 ± 3.79% was seen with two- to eightfold dilutions of intestinal lavages, which were obtained from the mice immunized with L_{NZ}9000-rTGEV-S_N.

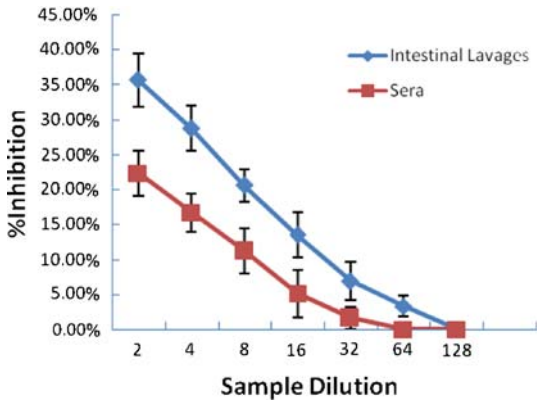

Fig. 7 Inhibition of viral plaque formation in intestinal lavages and sera after intragastric immunization. Maximum reduction in the number of plaques is expressed as a percentage of plaques obtained for the negative control samples. Intestinal lavages or sera values collected from mice fed with L_{NZ}9000-rTGEV-S_N were 35.6 ± 3.79 and 22.3 ± 3.28%, respectively. Results are mean values and standard errors of triplicates

The inhibitory effect decreased gradually with further dilution and reached a level similar to that of the buffer control or the non-expressing control strain at dilutions 1:64 and 1:128 of intestinal lavage and sera, respectively.

Discussion

In this report, we found that the globular part (N-terminal domain) of the TGEV spike protein (glycoprotein S) expressed in *L. lactis* NZ9000 retained its antigenic properties and elicited neutralizing antibodies, when used to immunize animals. For many pathogens, the initial infection mainly occurs at the mucosa tissues of intestines.

Therefore, it is important to develop vaccines that elicit protective immune responses against infection and replication of the pathogens at the mucosal level. Vaccines administered by parenteral routes generally fail to stimulate mucosal immune responses. Mucosal immunization is a proven, effective approach against colonization of pathogens and their further spread to the systemic circulation [4]. Therefore, it is necessary to develop efficient and safe antigen vectors that could trigger mucosal and systemic immune responses. One promising approach relies on the use of live bacterial vehicles. The potential of recombinant LAB to deliver heterologous antigens to the mucosal immune system has been under investigation [25–27]. This approach offers a number of advantages over the traditional parenteral vaccination, such as non-invasiveness and the possibility to elicit both mucosal and systemic immune responses. A great deal of research, currently being explored for oral application, focused on the development of adequate mucosal vaccines with various vaccine delivery systems. Among available approaches that efficiently stimulate mucosal responses, the use of *Lactococcus* carriers to deliver vaccine antigens probably constitutes one of the most successful strategies.

The use of LAB as a live vehicle for the delivery of antigens or other therapeutic molecules for mucosal immunization has been proposed. However, only a few of systems have been described using *Lactobacillus casei* strains as a carrier of heterologous viral antigens in a form that can be presented to and processed by the mammalian host [28]. The TGEV S protein was expressed by *Lactobacillus casei* [29]. *Lactobacillus* is the resident strain present in the intestinal tract of mammals. It may produce immunological tolerance, and a practical application for its use should be identified. IgA is the predominant antibody at the mucosal surface, as its local production is greater than other immunoglobulins. Therefore, an efficient TGEV oral vaccine should be able to induce a specific mucosal IgA response. We evaluated the immunogenicity of L_{NZ}9000-rTGEV-S_N using BALB/c mice as the animal model. The L_{NZ}9000-rTGEV-S_N produced both mucosal and systemic immune responses after intragastric administration. The oral administration regimen consisted of three sets of three successive daily doses of L_{NZ}9000-rTGEV-S_N by vaccination. This protocol was adapted from Challacombe's procedure [21], which was consistently effective when particulate oral vaccines were used to immunize mice. Three successive daily doses of recombinant bacteria were required in order to ensure that a systemic antibody response to TGEV could be elicited in all mice that received the recombinant strain, intragastrically.

In order to confirm the efficiency of the induced antibodies to inhibit the virus, we tested whether intestinal lavages and sera could inhibit the infection of ST cells in a plaque reduction assay. Serum and intestinal samples collected from mice immunized with L_{NZ}9000-rTGEV-S_N demonstrated statistically significant inhibition. An important feature of a delivery system is the ability to adhere to and persist in the intestinal tract. *Lactobacilli* could survive passage through the upper GIT and colonize the intestinal tracts [30, 31]. In addition, *Lactobacilli* have intrinsic adjuvant activity [32, 33]. In the present study, we investigated the potential adhesion and persistence of NZ9000 in the mouse intestine by using cFDA-SE. It was shown that 8.93×10^3 CFU/ml to 8.45×10^4 CFU/ml of *L. lactis* NZ9000, orally fed to mice, adhered to the different sections of intestinal tract. The percentage of *L. lactis* NZ9000 that remained in the different sections of the intestinal tract after initial attachment on the first day varied from 41.03 to 76.37%.

In the present study, we demonstrated that *L. lactis* NZ9000 was able to survive in the upper GIT and express and secrete the heterologous TGEV S_N protein that induced specific immune responses after oral administration. Currently, we are in the process of confirming the immune protection of the recombinant *Lactococcus* expressing TGEV S_N protein in pigs. We are also investigating the mechanisms by which the antigen enters intravascular compartments and induces systemic immune responses. The probiotic effects and the harmless nature of *Lactococcus lactis* NZ9000 would make it an ideal candidate for delivering heterologous antigens in an oral vaccine.

Conclusions

The present study describes a novel approach for preventing the spread of transmissible gastroenteritis in pigs. We described the capability of *L. lactis* NZ9000 to adhere to the GIT. We believe that our data could be valuable to intensive host-pathogen interaction studies and preventing TGEV outbreaks. The recombinant *Lactococcus* expressing S_N protein induced both local mucosal and systematical immune responses against TGEV. The induced antibodies demonstrated neutralizing effects on TGEV infection. These data indicated that the recombinant *Lactococcus* could be a useful tool in the development of future vaccines against TGEV.

Acknowledgments This work was supported by Grant 706019 from the National Natural Sciences Funds of China and Grant CX2008 from the supported program for innovative research team of Northeast Agricultural University (NEAU). The authors wish to thank NIZO Food Research for providing the pNZ8112 plasmid and the bacterial strain, *Lactococcus lactis* NZ9000.

References

1. M. Alander, R. Satokari, R. Korpela, M. Saxelin, T. Vilpponen-Salmela, T. Mattila-Sandholm, A. von Wright, Appl. Environ. Microbiol. **65**, 351 (1999)
2. C. Cesena, L. Morelli, M. Alander, T. Siljander, E. Tuomola, S. Salminen, T. Mattila-Sandholm, A. von Wright, J. Dairy Sci. **84**, 1001 (2001)
3. Y.K. Lee, P.S. Ho, C.S. Low, H. Arvilommi, S. Salminen, Appl. Environ. Microbiol. **70**, 670 (2004). doi:10.1128/AEM.70.2.670-674.2004
4. L.G. Bermúdez-Humarán, P. Langella, N.G. Cortes-Perez, A. Gruss, R.S. Tamez-Guerra, S.C. Oliveira, O. Saucedo-Cardenas, R.M. de Oca-Luna, Y.L. Loir, Infect. Immun. **71**, 1887 (2003). doi:10.1128/IAI.71.4.1887-1896.2003
5. B.J. Underdown, J. Mestecky, Mucosal Immunoglobulin, in *Handbook of Mucosal Immunology*, ed. by P. Ogra, J. Mesteck, M.E. Lamm, W. Strober, J.R. McGhee, J. Bienestock, et al. (Academic Press, Boston, 1994), pp. 79–98
6. H.F. Staats, R.J. Jackson, M. Marinaro, I. Takahashi, H. Kiyono, J.R. McGhee, Curr. Opin. Immunol. **6**, 572 (1994). doi:10.1016/0952-7915(94)90144-9
7. P.H. Pouwels, R.J. Leer, W.J.A. Boersma, The potential of *Lactobacillus* as a carrier for oral immunization. J. Biotechnol. **44**, 183 (1996). doi:10.1016/0168-1656(95)00140-9
8. L.J. Saif, R. Wedley, in *Disease of Swine*, 8th edn., ed. by B.E. Straw, S. Allaire, W.L. Mengeling, D.J. Tayler (Iowa State University Press, Ames, 1982), p. 295
9. S. Paul, Adv. Virus Res. **66**, 193 (2006). doi:10.1016/S0065-3527(06)66005-3
10. D. Cavanagh, Arch. Virol. **142**, 629 (1997)
11. B. Delmas, J. Gelfi, H. Laude, J. Gen. Virol. **67**, 1405 (1986)
12. G. Jimenez, I. Correa, M.P. Melgosa, M.T. Bullido, L. Enjuanes, J. Virol. **60**, 131 (1986)
13. N. Gomez, C. Carrillo, J. Salinas, F. Parra, M.V. Borca, J.M. Escribano, Virology **249**, 352 (1998). doi:10.1006/viro.1998.9315
14. S. Hu, J. Bruszewski, R. Smalling, J.K. Browne, Adv. Exp. Med. Biol. **185**, 63 (1985)
15. S.A. Weston, C.R. Parish, J. Immunol. Methods **133**, 87 (1990)
16. R.E.N. Xiao-feng, L.I. Yi-jing, L.I. Guang-xing, L.I.U. Bao-quan, Chin. J. Vet. Med. **7**, 3 (2002). doi:0529-6005(2002)07-0003-04
17. J. Sambrook, W. Russel, *Molecular Cloning: A Laboratory Manual*, 3rd edn. (Cold Spring Harbor Laboratory Press, New York, 2002), pp. 597–628
18. K. Josson, T. Scheirlinck, F. Michiels, C. Platteeuw, P. Stanssens, H. Joos, Plasmid **21**, 9 (1989)
19. H. Holo, I.F. Nes, Appl. Environ. Microbiol. **55**, 3119 (1989)
20. D.G. Anderson, L.L. McKay, Appl. Environ. Microbiol. **46**, 549 (1983)
21. S.J. Challacombe, Ann. NY Acad. Sci. **409**, 177 (1983). doi:10.1111/j.1749-6632.1983.tb26868.x
22. J.M. Chatel, P. Langella, K. Adel-Patient, J.M. Wal, G. Corthier, Clin. Diagn. Lab. Immunol. **8**, 545 (2001). doi:10.1128/CDLI.8.3.545-551.2001
23. B. Corthésy, S. Boris, P. Isler, C. Grangette, A. Mercenier, J. Infect. Dis. **192**, 1441 (2005). doi:10.1086/444425
24. G.P.S. Raghava, A.K. Joshi, J.N. Agrewala, J. Immunol. Methods **153**, 263 (1992)
25. H. Chen, J. Control Release **67**, 117 (2000). doi:10.1016/S0168-3659(00)00199-1
26. C. Grangette, H. Muller-Alouf, D. Goudercourt, M.C. Geoffroy, M. Turneer, A. Mercenier, Infect. Immun. **69**, 1547 (2001). doi:10.1128/IAI.69.3.1547-1553.2001
27. M.L. Oliveira, A.P. Areas, I.B. Campos, V. Monedero, G. Perez-Martinez, E.N. Miyaji, L.C.C. Leite, K.A. Aires, P. Lee Ho, Microbes Infect. **8**, 1016 (2006). doi:10.1016/j.micinf.2005.10.020
28. G. M.Oddone, Q.Q. Lan, H. Rawsthorne, D.A. Mills, D.E. Block, Biotechnol. Bioeng. **96**, 1127 (2006). doi:10.1002/bit.21192
29. P.S. Ho, J. Kwang, Y.K. Lee, Vaccine **23**, 1335 (2005). doi:10.1016/j.vaccine.2004.09.015
30. L. Scheppler, M. Vogel, A.W. Zuercher, M. Zuercher, J.E. Germond, S.M. Miescher, M. Stadler, Vaccine **26**, 2913 (2002). doi:10.1016/S0264-410X(02)00229-3
31. G.J. Russell-Jones, J. Control Release **65**, 49 (2000). doi:10.1016/S0168-3659(99)00231-X
32. J.H. Kim, D.A. Mills, Plasmid **58**, 275 (2007). doi:10.1016/j.plasmid.2007.05.004
33. G. Perdigon, R. Fuller, R. Raya, Curr. Issues Intest. Microbiol. **2**, 27 (2001)