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Intragastric administration of *Lactobacillus casei* expressing transmissible gastroentritis coronavirus spike glycoprotein induced specific antibody production

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Abstract

Lactobacillus casei strain Shirota was selected as a bacterial carrier for the development of live mucosal vaccines against coronavirus. A 75 kDa fragment of transmissible gastroenteritis coronavirus (TGEV) spike glycoprotein S was used as the model coronavirus antigen. The S glycoprotein was cloned into a *Lactobacillus*/*E. coli* shuttle vector (pLP500) where expression and secretion of the glycoprotein S from the recombinant lactobacilli was detected via immunoblotting. Oral immunization of BALB/c mice with recombinant LcS that constitutively expresses the 75 kDa fragment of the glycoprotein S, induced both local mucosal and systemic immune responses against TGEV. Maximum titers of IgG (8.38 ± 0.19 ng/ml of serum) and IgA (64.82 ± 2.9 ng/ml of intestinal water) were attained 32 days post oral inturbation. The induced antibodies demonstrated neutralizing effects on TGEV infection.

Keywords: Coronavirus; Spike glycoprotein; Oral vaccination

1. Introduction

Although parenteral vaccination is usually highly effective in eliciting a protective immune response, the response obtained is not necessarily the one desired as many viral, bacterial and parasitic pathogens enter the body via the mucosal surfaces. Effective protection against mucosal infections requires the development of vaccines that are able to induce protective local immune responses in order to neutralize the pathogen at its infection point [1,2]. This can be achieved via oral vaccination where oral administration of antigens might stimulate the natural route of infection and be a more effective method of immunization [3]. The principle antibody type involved in mucosal immunity is secretory immunoglobulin A, the majority of which is released into the gastrointestinal fluid, saliva, tears, urine and other secretions [4,5]. Besides being more convenient and less expensive, mucosal immunization offers several advantages over parenterally administered vaccines whereby it not only enhances vaccine efficacy by simultaneously inducing mucosal and systemic immunity, but also minimize adverse vaccine effects by avoiding direct contact between potentially toxic vaccine components and the systemic circulation [6,7].

Lactobacillus strains have a number of properties that make them attractive candidates as delivery vehicles for the presentation to the mucosa of compounds with pharmaceutical interest, in particular vaccines and immunomodulators. Lactobacilli have been used in fermentation and preservation of food for decades, and are considered ‘generally regarded as safe’ (GRAS) microorganisms. In addition, lactobacilli are able to survive transit of the upper gastrointestinal tract and certain strains have been reported to be able to colonize the intestinal tract [8,9,31]. Findings indicating that certain *Lac-*

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tobacillus spp. can induce a non-specific immunoadjuvant effect [10] have provoked several studies aimed at determining the capability and feasibility of the application of these bacteria as safe oral vaccines [11–13,31].

Transmissible gastroenteritis coronavirus (TGEV), a member of the genus *Coronavirus*, family *Coronaviridae*, order *Nidovirales*, is an enteropathogenic coronavirus that causes a highly fatal acute diarrhea in newborn pigs [14]. It has been estimated to cost US pig producers about 100 million dollars annually due to death or poor growth of infected pigs [15]. The viral genome consists of a single-stranded, positive-sense 28.5 kb RNA and is constructed of three major structural proteins: a phosphorylated nucleoprotein (N protein) and two glycoproteins, the membrane (M) and the spike (S) protein [16]. The S protein has a membrane-anchoring domain and is highly glycosylated. It is also thought to be the viral attachment protein which interacts with the cell receptor, porcine aminopeptidase N (APN) [17,18]. As the major inducer of TGEV-neutralizing antibodies, the S protein has been used mainly for the induction of protective immunity to TGEV [19–21]. The protection of suckling piglets against TGEV infection is based on the uptake of specific lactogenic antibodies, mainly of the IgA class, in the milk of the TGEV-immune sows [22].

In this study, the potential of using *Lactobacillus casei* Shirota (LcS) to express heterologous coronaviral protein and to act as an antigen carrier for oral vaccination was analyzed. The viral antigen used is a 75 kDa fragment of TGEV glycoprotein S that encompasses all the four major antigenic domains critical for neutralization [23–25]. A constitutive *Lactobacillus* expression system that has been assembled into a plasmid vector series designated pLP500 [26] was used in this study. The immunogenicity of the recombinant LcS was analyzed post intragastric administration of live bacteria to the mice. To our knowledge, this is the first report on the cloning and expression of viral antigen in lactobacilli. Our data has also indicated that orogastric intubation of the recombinant LcS could induce a specific immune response against TGEV.

2. Material and methods

2.1. Bacterial strain and growth conditions

L. casei Shirota, isolated from Yakult cultured milk (Singapore), was grown in MRS broth (Difco Laboratories, Detroit, USA) at 37 °C with continuous shaking at 250 rpm.

2.2. Labeling of bacteria with fluorescence probe

L. casei Shirota was labeled with a protein dye, five-(and 6-) carboxyfluorescein diacetate succinimidyl ester, cFDA-SE (Molecular Probes, USA, 2 mg), a non-fluorescent membrane permeative ester which non-specific prokaryotic and eukaryotic intracellular esterases convert to a fluorescent derivative that is in turn covalently linked to intracellular proteins via the probe's succinimidyl group. Log-phase culture of LcS was harvested, washed twice with sterile phosphate-buffered saline (PBS) and adjusted to a concentration of 10^{10} CFU ml^{-1} prior to labeling with 50 μM cFDA-SE at 37 °C for 20 min. A 100 μM stock solution of cFDA-SE was prepared by being first dissolved in dimethyl sulfoxide (20 μl) (Merck, Darmstadt, Germany) and then further diluted in ethanol (1 ml; reagent grade). This solution was then filter sterilized (0.2-μm-pore-size Acrodisc filter; Gelman) before being aliquoted and stored at −20 °C. Fluorescent labeling was terminated by pelleting the bacteria, washing twice with PBS to remove excess cFDA-SE, and resuspending the pellet in PBS.

2.3. Adhesion study on animal

Eight-week-old female BALB/c mice, obtained from the Laboratory Animals Centre, National University of Singapore, were maintained at the Animal Holding Unit of the Department of Microbiology, National University of Singapore and had free access to a standard mouse diet and water. A group of 12 mice were orally dosed with approximately 10^9 cFDA-SE-labeled lactobacilli by orogastric intubation while the control mice had been orally fed with sterile PBS. Groups of three mice each were sacrificed on days 1, 2, 4, 6 and 7 after dosing by CO_2 asphyxiation, after which the intestine was extracted from each mouse and cut longitudinally. Visible residual food particles or fecal material were first removed from the intestine before being examined for the presence of adhering cFDA-SE-labeled LcS. 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 with the aid of a plunger from a syringe (1.0 ml; Terumo, Tokyo, Japan). Cell extracts were fixed with formaldehyde (0.75%, v/v) prior to flow cytometry analysis.

2.4. Flow cytometry analysis

The amount of cFDA-SE-stained LcS from intestinal cell extracts was enumerated from the counts of the reference glass beads included in the sample using an Epics Elite flow sorter (Coulter, Miami, FL) at a 488-nm excitation wavelength with a 15-mW argon laser with a 75-mm sort sense flow cell at 82.7 kPa of pressure. Upon excitation at 488 nm in the flow cytometer, cFDA-SE gives a maximal emission signal in the green at 518 nm. Data were recorded in the FCS2.0 file format by using Coulter Epics Elite (version 4.01) software and were then analyzed and converted into plots by using WinMDI (J. Trotter, Scripps Research Institute, La Jolla, CA).

2.5. Cloning of rTGEV-S gene into Lactobacillus/E. coli shuttle vector

All DNA manipulations were performed according to standard procedures [27]. A 2.3 kbp cDNA fragment (nu-

cleotides 33–2286), encoding for the N-terminal glycoprotein S from TGEV Miller strain (rTGEV-S), was first synthesized via reverse transcription from viral RNA before being amplified by polymerase chain reaction and subcloned into TOPO-XL vector (Invitrogen, California, USA). Subsequently, the DNA fragment was cloned into a *Lactobacillus*/*E. coli* shuttle vector pLP500, which is a kind gift of Prof. Pouwels PH of the TNO-Nutrition and Food Research Institute, The Netherlands, under the control of the constitutive promoter of L-(+)-lactate dehydrogenase (L-ldh) gene, yielding pLP500-rTGEV-S. The construct was verified by nucleotide sequencing.

2.6. Generation of LcS transformants secreting subunit TGEV glycoprotein S

Electroporation was carried out as described by the method of Josson et al. [28]. In brief, plasmid DNA (0.5 μg) was added to 100 μl of LcS (between 10^{10} and 10^{11} CFU ml^{-1}) and this suspension was subjected to a single electric pulse (25 μF at 8500 V/cm). Recombinant LcS strains were selected on MRS medium containing 5 μg/ml of erythromycin. The presence and integrity of the constructions carried by the LcS transformants were checked by extraction of plasmid DNA, followed by restriction analysis and sequencing.

2.7. Gel electrophoresis and immunoblotting

Overnight cultures of recombinant LcS (LcS-rTGEV-S), LcS containing the empty pLP500 vector (LcS-pLP500) and wild type LcS were centrifuged at 5000 × *g* for 15 min at 4 °C where the supernatants were harvested. The supernatants obtained were concentrated 10 times using Ultrafree-CL PBCC Centrifugal Filter Unit (Millipore, Billerica, USA). Twenty microliters of concentrated supernatant from the respective cultures were boiled in sample loading buffer (60 mM Tris, 2% SDS, 10% glycerol, 100 mM DTT pH6.8, 300 μl saturated bromophenol blue) and subjected to SDS–PAGE in 10% polyacrylamide gel. Proteins were transferred electrophoretically onto a nitrocellulose membrane [27] where the immunoblots were developed using convalescent swine serum against TGEV at a dilution of 1:1000. The convalescence serum was obtained from pigs that were inoculated by the oral route with 10^5 TCID50 of TGEV Miller strain and challenged intramuscularly 45 days post-inoculation. For the detection of specific antibody binding, horseradish peroxidase (HRP)-conjugated anti-swine IgG (ZYMED Laboratories Inc., San Francisco, USA) diluted at 1:10,000 were used. Visualization of the immunolabeled bands was then carried out using SuperSignal West Pico Chemiluminescent Substrate chemiluminescence reagent (Pierce Biotechnology, Rockford, USA), accordingly to manufacturer's instruction.

2.8. Immunization of mice

Groups of three female BALB/c mice (8-week-old) were immunized orally either with recombinant LcS constitutively expressing rTGEV-S (harboring plasmid pLP500-rTGEV-S), a control non-expressor strain (harboring pLP500) or sterile phosphate buffer saline (PBS). Freshly cultured bacteria were harvested and washed twice with sterile PBS (pH 7.2). Oral doses of 2×10^9 cells (0.1 ml of the suspension) were 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. Sera were collected via tail-bleeding while collection of the intestinal fluid was performed as described previously [29]. In brief, four doses of 0.5 ml of the lavage solution [25 mM NaCl, 40 mM Na_2SO_4, 10 mM KCl, 20 mM $NaHCO_3$, and 48.5 mM polyethylene glycol (average MW = 3350)] were given intragastrically at 15 min intervals, after which the mice were given 0.1 mg of pilocarpine (Alcon-Couvreur, Puurs, Belgium) intraperitoneally 30 min after the last dose of lavage solution. A discharge of intestinal contents occurred regularly over the next 10–20 min. The collected intestinal discharges were brought up to 6 ml with PBS and vortexed vigorously before being centrifuged repetitively at 6000 × *g* for the clarification of the intestinal content.

2.9. ELISA

Polystyrene microtitre plates were coated overnight at 4 °C with 1 μg of purified TGEV glycoprotein S. The coating antigen was extracted from cell lysate of recombinant *E. coli* harboring and expressing a 1.4 kbp amino terminal fragment of S protein in the form of GST-fusion protein (TGEV-S-GST), and purified by passing the cell lysate through Glutathione Sepharose 4B affinity column (Amersham Biosciences Inc., Buckinghamshire, England). Serum or intestinal lavage samples were diluted two-fold and used as primary antibodies. Bound antibodies were detected using horseradish peroxidase conjugated goat anti-mouse IgA or IgG (ZYMED Laboratories Inc., San Francisco, USA), followed by colour development using *o*-phenylene diamine dihydrochloride (Sigma, St. Louis, USA) as substrate. Absorbance was then measured at 490 nm.

2.10. Plaque reduction assay

Intestinal fluids and serum samples from mice fed with rTGEV-S expressing LcS were evaluated using plaque reduction assay to determine the neutralization ability of the induced antibodies. Lavages and sera from non-expressor strain or PBS fed mice were taken as control. Fifty microlitres of samples in two-fold serial dilutions (from 1:2 to 1:4, 256) were prepared in microcentrifuge tubes. TGEV adjusted to 5×10^4 PFU in 50 μl of virus diluent (10% concentrated Hank's balanced salt solution, 0.1% bovine serum albumin; pH 7.2–7.4) was added to the tube containing seri-

ally diluted serum or intestinal lavage. The antibody and virus mixture was mixed, pulsed centrifuged and then incubated at 37 °C for an hour. A twenty-four well plate with confluent monolayer of swine testicular (ST) cells (ATCC, CRL-1746, USA) was used for virus infection. Before inoculation of the antibody-virus mixture, the cell monolayer was rinse once with virus diluent, after which 100 μl of the antibody-virus mixture was added to the appropriate wells. The plates were left at 37 °C for another hour, and rocked at 15 min intervals. After incubation, the inoculum was removed and the cell monolayer was rinsed once with virus diluent. Overlay medium (equal volume of concentrated cell culture medium and 2.5% carboxylmethyl–cellulose solution) was added to the wells and the plate was incubated in a 5% CO_2 incubator at 37 °C for 6 days. The overlay medium was then discarded, after which the wells were washed thrice with sterile PBS (pH 7.4) and stained with 1% crystal violet solution. Differences in the number of plaques formed between treatments were examined for the level of significance by Student's *t*-test after analysis of variance.

3. Results

3.1. Colonization ability of LcS

The ability of LcS to adhere and colonize the intestinal mucosal surface was determined by oral feeding of cFDA-SE labeled lactobacilli to mice and isolating the intestinal extracts from such mice various days post orogastric intubation. Flow cytometric analysis of cell extracts (Table 1) indicated that a portion of the orally fed LcS was able to survive and attach to different regions of murine intestinal tract. Adhesion was most prominent in ileum with 6.89×10^4 cells being detected in the ileum extract on day 1. By the seventh day, the amount of LcS that remained adhered to the intestinal mucosal were 10.60, 32.58, 30.48 and 33.66% of that on the first day in the duodenum, jejunum, ileum and colon, respectively.

Table 1
Average number of cFDA-SE labeled LcS 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
	Average cell no per intestinal part			
1	6.04E + 03	1.72E + 04	6.89E + 04	3.00E + 04
2	4.57E + 03	5.92E + 03	2.56E + 04	2.02E + 04
4	3.58E + 03	5.72E + 03	2.46E + 04	1.30E + 04
6	1.27E + 03	5.83E + 03	2.12E + 04	1.11E + 04
7	6.40E + 02	5.62E + 03	2.10E + 04	1.01E + 04
	Percentage of cells remaining as of day 1			
2	75.68	34.33	37.21	67.29
4	59.37	33.17	35.67	43.36
6	21.08	33.81	30.79	37.12
7	10.60	32.58	30.48	33.66

3.2. Expression of rTGEV-S by LcS

A 2.3 kbp fragment coding for the amino terminus of the TGEV S protein was amplified by polymerase chain reaction and cloned into the plasmid pLP500 [26], a plasmid with endogenous regulation elements from *Lactobacillus*, downstream of the secretion signal of *prt* P gene of *L. casei*, for secretable expression. The recombinant plasmid pLP500-rTGEV-S was transformed via electroporation into LcS. For control purposes, LcS was also transformed with the empty vector, pLP500.

The expression of rTGEV-S by LcS was analyzed by immunoblot assay using recombinant LcS harboring either pLP500-rTGEV-S or the empty vector and wild type LcS as control. Western blot analysis of supernatant from overnight culture of LcS-rTGEV-S revealed a protein band (75 kDa) corresponding to the expected molecular weight of the rTGEV-S (Fig. 1, lane 3). The reactivity of the 75 kDa band with a polyclonal TGEV-specific convalescence swine serum gave an indication of the immunogenicity of the expressed protein. In contrast, no signal was detected in culture medium and culture supernatants of control strain harboring pLP500 or wild type LcS (Fig. 1, lanes 2, 4 and 5). Samples of purified recombinant TGEV S protein, TGEV-S-GST, was also included as positive control (Fig. 1, lane 1).

3.3. Immune responses induced by intragastric immunization

BALB/c mice receiving three doses of LcS expressing rTGEV-S on days 0–2 were primed for a secondary response to rTGEV-S following booster oral administrations on days 14–16 and 28–30. BALB/c mice was chosen as the model as it was utilized by Liu et al. to evaluate the immunogenicity of TGEV DNA vaccine [30].

Immunological responses of the oral fed mice to rTGEV-S were determined by probing blots of 10-fold concentrated

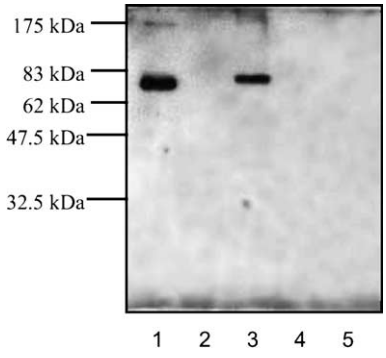

Fig. 1. Expression of rTGEV-S from LcS transformants. Supernatants from overnight cultures of LcS-rTGEV-S (lane 3), LcS-pLP500 (lane 4) or LcS (lane 5) were separated by SDS–PAGE 10% and transferred electrophoretically to nitrocellulose before being incubated with convalescent swine anti-TGEV serum at a dilution of 1:1000. Bound antibodies were detected with HRP-conjugated anti-swine IgG antibodies. Lane 1: purified recombinant TGEV S protein; lane 2: MRS broth.

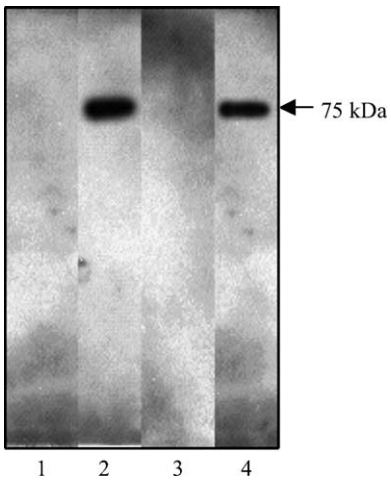

Fig. 2. Immunogenicity of induced intestinal IgA and serum IgG specific for rTGEV-S protein. Western blot was performed using intestinal lavages (lane 2) and sera (lane 4) from mice orally fed with LcS-rTGEV-S. Controls of preimmunized samples of intestinal lavage (lane 1) and serum (lane 3) were also included. Antibody binding was determined using goat anti-mouse IgA and IgG conjugated with HRP. Intestinal IgA in intestinal lavage and serum IgG bound to the 75 kDa S protein where as no band was observed on membranes probed with preimmune lavage and serum.

LcS-rTGEV-S supernatant with intestinal lavages and sera extracted from these mice. The detection of a 75 kDa rTGEV-S band on the respective membranes blotted with intestinal lavages and sera, indicated that mucosal and systemic immune responses against TGEV were elicited (Fig. 2). The local response was further studied by measuring the anti-rTGEV-S IgA response in intestinal lavages post intragastric immunization. The concentration of mucosal IgA antibodies against rTGEV-S in the intestinal lavages was determined via ELISA, using purified TGEV-S-GST protein as coating antigen. Results were expressed as titers that were determined by expression of the test samples to a standard curve generated by serial dilution of commercially purchased IgA (ZYMED Laboratories Inc., San Francisco, USA) of known titer. As shown in Fig. 3, there was no substantial difference in mucosal IgA levels between experimental group and control groups prior to intervention while oral immunization of rTGEV-S expressing strains elicited an antigen-specific mucosal IgA response. The antibody titer of the rTGEV-S specific response in BALB/c mice following immunization with recombinant LcS was detectable as rapidly as day 18, raising to titers of 3.98 ± 0.18 ng/ml of lavage and 3.63 ± 0.19 ng/ml of lavage by day 32 and 48, respectively. Since an approximate volume of 2 ml of intestinal lavage was extracted from each mouse, the maximum amount of IgA in the entire intestinal tract would be 7.96 ± 0.36 ng. From the estimation of a previous study [31], it was derived that the intestinal water covering the surface of the entire gastrointestinal tract of mice adds up to 122.8 ± 3.2 mg. As such, the maximum titer of IgA induced by oral administration of recombinant LcS amounted to 64.82 ± 2.9 ng/ml of intestinal water.

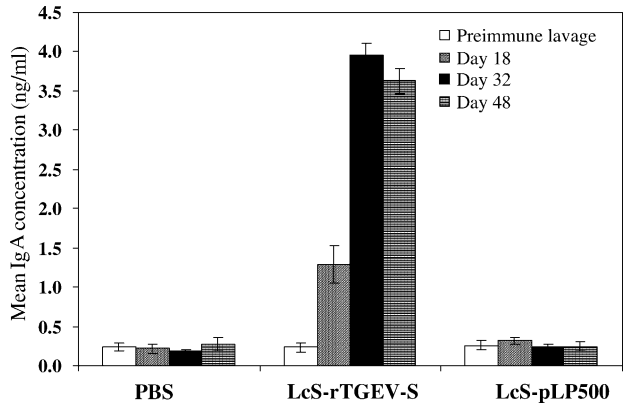

Fig. 3. rTGEV-S specific local IgA response in murine intestinal lavage after intragastric immunization. Groups of three mice received three consecutive doses of 10^9 LcS-rTGEV-S, three times at 2-week intervals. Control mice received 10^9 CFU of LcS containing pLP500 while a naïve group received buffer dose. Intestinal lavages that were collected 2 days after the first boost, 2 and 16 days after the second boost, were analyzed via ELISA, using TGEV-S-GST as the coating antigen. Bars represent the mean IgA titer ± S.E.M. in each group.

Likewise, serum concentration of rTGEV-S specific IgG from immunized mice were also determined. All animals that were orally fed with rTGEV-S expressing LcS sero-converted after the second dose (Fig. 4). Elicitation of TGEV specific serum IgG was found to be prompter and stronger on comparison to the induction of mucosal IgA in the intestine. A titer of 6.03 ± 0.32 ng/ml of serum of rTGEV-S specific IgG has been attained after the first boost which continued to increase to a level of 8.38 ± 0.18 ng/ml after the second boost. No significant induction of anti-rTGEV-S antibodies was observed in the control groups of mice that received PBS or LcS harboring the empty vector (Figs. 3 and 4). Taken together these results, the recombinant LcS generated in this study was able

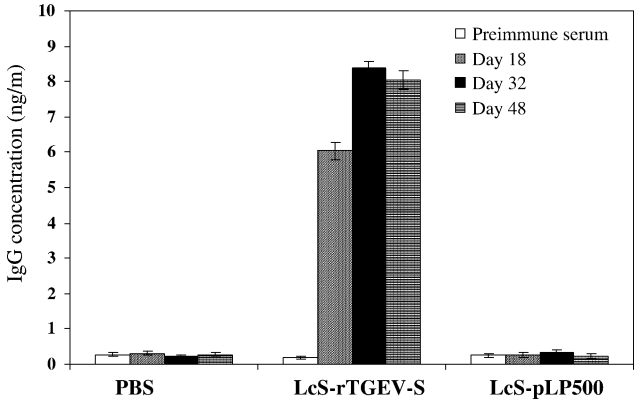

Fig. 4. Anti-rTGEV-S serum IgG titers induced after intragastric immunization with recombinant LcS. Sera from groups of three mice immunized orally with 10^9 LcS (TGEV-S expressor or control non-expressor strains) were tested for the presence of rTGEV-S specific IgG by ELISA, using TGEV-S-GST as the coating antigen. Negative control sera from mice fed with PBS were also assayed. Bars represent the mean IgG titer ± S.E.M. in each group.

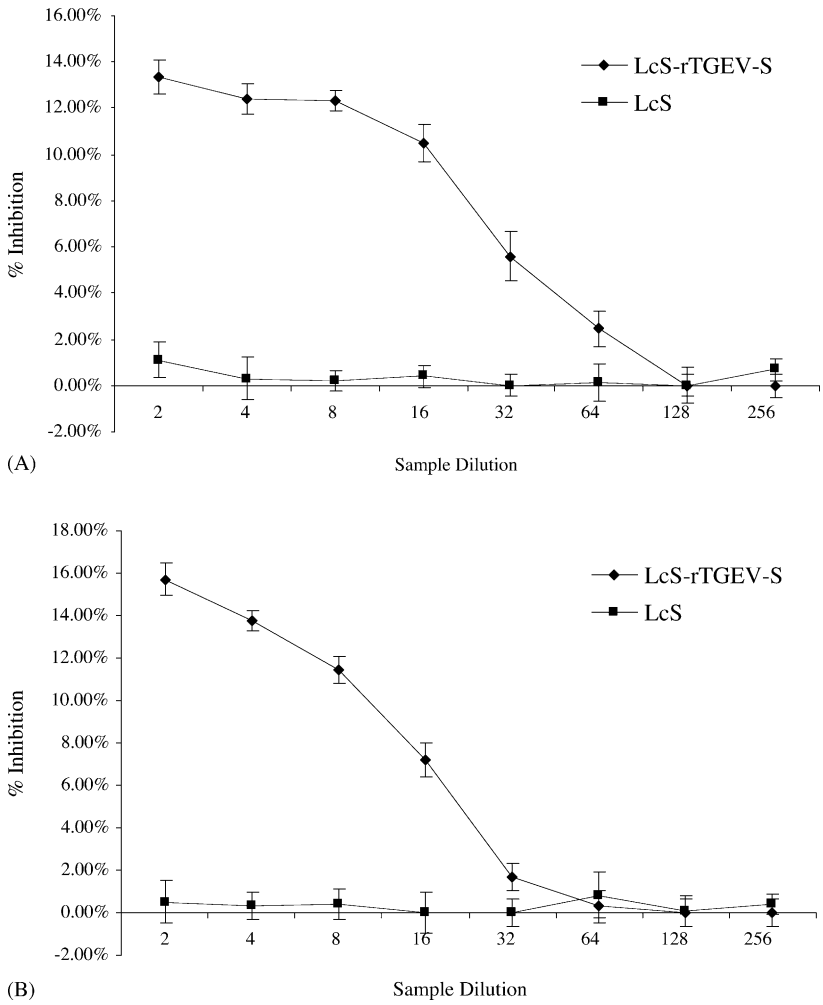

Fig. 5. Inhibition of viral plaque formation by (A) intestine lavages and (B) sera prepared from mice fed recombinant LcS. Maximum reduction in number of plaques, expressed as a percentage of plaques obtained for the negative control samples, using (A) intestinal lavages or (B) sera collected from mice fed with rTGEV-S-expressing LcS was $13.35 \pm 0.77\%$ and $15.70 \pm 0.77\%$, respectively. Results are mean values and standard errors of triplicates.

to elicit both TGEV specific systemic and mucosal antibody responses upon oral administration.

Plaque reduction assays were performed to further determine whether the antibody responses were specific against TGEV glycoprotein S. Results demonstrated that the presence of anti-rTGEV-S IgA or IgG in the culture medium conferred statistically significant neutralizing effects ($p < 0.05$) on TGEV infection (Fig. 5). A near $15 \pm 0.77\%$ reduction in the number of plaques was consistently observed when plaque reduction assays were carried out using two- to eight-folds diluted intestinal lavages or sera from LcS-rTGEV-S fed mice. The inhibitory effect decreased gradually on further dilutions and reached a level similar to that of the buffer control or the control non-expressor strain at dilutions 1:128 and 1:64 of intestinal lavage and sera, respectively.

4. Discussion

For many pathogens, the initial infection occurs at the mucosa of the lungs and intestines. It is therefore important to develop vaccines that induce protective immune responses where initial infection and replication of the pathogen is prevented at the mucosa via the mucosal immunity, mediated predominantly by secretory IgA [32]. Furthermore, since most viral infections that have gained entrance through the mucosal surfaces will become systemic, the vaccines should also elicit specific immunity in the systemic lymphoid tissues. With the widespread threat of many emerging pathogenic viruses (such as SARS Coronavirus, Hantan and influenza viruses) to human life, challenges have been spawned to develop safe anti-viral vaccine for preventive use. As such, a great deal of research is currently focused on the development of adequate mucosal vaccines, with various vaccine delivery systems being explored for oral application.

To date, only a few systems have been described using *Lactobacillus* spp. as a carrier for expressing heterologous bacterial antigens in a form that can be presented to and processed by the immune system of the mammalian host [11–13]. In this study, we engineered for the first time, a *Lactobacillus* spp. to express a coronaviral protein, TGEV S protein, into the exter-

nal milieu. A 75 kDa band corresponded to the expected size of rTGEV-S, was detected in the supernatant of overnight recombinant LcS culture when probed with convalescent swine serum. Hence, indicating that the recombinant LcS was able to express and secrete S protein fragment without compromising its antigenic properties. IgA is the predominant antibody at the mucosal surface as it is locally produced at a level that exceeds that of all of other immunoglobulins. Therefore, it is likely that to be effective, an oral TGEV vaccine will have to induce a specific intestinal IgA response. The recombinant LcS was shown to be able to elicit both mucosal and systemic humoral responses after oral immunization in mice, which is in agreement with a study conducted by Enjuanes and coworkers [33]. Likewise, Enjuanes and coworkers developed an attenuated strain of *Salmonella typhimurium* expressing the amino terminus of TGEV S protein. However, the protein fragment encoded by the constructed *S. typhimurium* lacks the antigenic domain A of S protein. Nevertheless, specific immune responses against TGEV were induced by the recombinant *S. typhimurium*, though the protective effects of the elicited antibodies were not assessed. On comparison of the two delivery systems illustrated above, the probiotic effects and the harmless nature of *Lactobacillus* spp. would render it more appropriate an oral vaccine carrier as to a natively pathogenic bacteria.

The oral immunization regime used, which consisted of three sets of three successive daily doses of the experimental vaccine, was adapted from the procedure of Challacombe [34], who found that this pattern of immunization 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 systemic antibody response to rTGEV-S can be elicited in all mice receiving recombinant LcS intragastrically.

In order to confirm the efficacy of the induced antibodies in inhibiting the virus, we tested whether intestinal lavages and sera can inhibit the infection of ST cells in plaque reduction neutralization assay. Serum and intestinal samples collected from mice fed with rTGEV-S expressing LcS demonstrated statistically significant inhibition (14–16% reduction in plaque formation) of plaque formation by TGEV. Interestingly, despite the lack of posttranslational modifications in prokaryotic expression system, the 75 kDa fragment of TGEV S protein expressed by LcS was recognized by convalescent swine serum, and able to elicit significant immune protection, albeit low level against TGEV. In a study conducted by Hu et al., inoculation of animals with recombinant *E. coli* expressing high levels of glycoprotein S did not induce neutralizing antibodies or confer protection in vivo [35]. On the other hand, Escribano and coworkers have developed arabidopsis transgenic plants expressing N-terminal domain of TGEV S protein that elicited neutralizing antibodies when used to immunize mice [36]. The use of plant as an oral vaccine delivery vehicle however, would only result in short-term induction of the immune system against the expressed antigen and hence, requires consistent and frequent feeding of mice with the transgenic plant if long-term effect is desired. In contrast, the development of a delivery system like *Lactobacillus* spp. that is able to adhere and colonize the intestinal tract, permits continuous expression of antigen in the intestine, resulting in extended protection of the vaccinated subject from TGEV infection. It was shown in this study that 6.04×10^3 to 6.89×10^4 of the orally fed LcS was able to adhere to the intestinal tract of mice, where the percentage of LcS that remained in the intestinal tract after the initial attachment as of day 1 varies from 10.60 to 33.60%. In particular, the amount of LcS that remain attached were maintained at a fairly constant percentage of 32.58–34.33% and 30.48–37.21% in the jejunum and ileum respectively, indicating that at least one-third of the LcS which had adhered to the jejunum and ileum were retained in the intestinal tract for a week. The recombinant LcS generated in this study also demonstrates similar adhesion and colonization capability as its native counterpart (results not shown). As such, oral immunization regime could be developed based on the adherence ability of the vaccine carrier. In our case, single dose of the lactobacilli vaccine is required instead of three consecutive doses at each immunization, and booster doses administered at intervals of 7 days instead of 14 days would be sufficient for consistent expression of TGEV spike protein in the intestine.

In the present study, LcS has been demonstrated to be able to survive the transit of the upper gastrointestinal tract and was able to express and secrete heterologous coronaviral protein that induced specific immune responses against the antigen within the murine intestine milieu when orally administered. The mechanism of how the secreted antigen was also able to transude into the intravascular compartment to induce systemic immune responses will be further investigated. Nevertheless, LcS would serve as a potential delivery vehicle for oral vaccine.

References

[1] Mesteck J. The common mucosal immune system and current strategies for induction of immune responses in external secretions. J Clin Immunol 1987;7:265–76.
[2] McGhee JR, Mestecky J, Dertzbaugh MT, Eldridge JK, Hirasawa M, Kiyono H. The mucosal immune system: from fundamental concepts to vaccine development. Vaccine 1992;10:75–88.
[3] Staats HF, Jackson RJ, Marinaro M, Takahashi I, Kiyono H, McGhee JR. Mucosal immunity to infection with implications for vaccine development. Curr Opin Immunol 1994;6:572–83.
[4] Underdown BJ, Mestecky J. Mucosal immunoglobulin. In: Ogra P, Mesteck J, Lamm ME, Strober W, McGhee JR, Bienestock J, editors. Handbook of mucosal immunology. Boston: Academic Press; 1994. p. 79–98.
[5] Brandtzaeg P. Distribution and characteristics of mucosal immunoglobuilin-producing cells. In: Handbook of immunology. Boston: Academic Press; 1994. p. 251–79.
[6] Russell-Jones GJ. Oral vaccine delivery. J Control Release 2000;65:49–54.
[7] Chen H. Recent advances in mucosal vaccine development. J Control Release 2000;67:117–28.

[8] Alander M, Satokari R, Korpela R, Saxelin M, Vilpponen-Salmela T, Mattila-Sandholm T, et al. Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG, after oral consumption. Appl Environ Microbiol 1999;65:351–4.
[9] Cesena C, Morelli L, Alander M, Siljander T, Tuomola E, Salminen S, et al. *Lactobacillus crispatus* and its non-aggregating mutant in human colonization trials. J Dairy Sci 2001;84:1001–10.
[10] Link-Amster H, Rochat F, Saudan KY, Mignot O, Aeschlimann JM. Modulation of a specific humoral immune response and changes in the intestinal flora mediated through fermented milk intake. FEMS Immunol Med Microbiol 1994;10:55–63.
[11] Shaw DM, Gaerthe B, Leer RJ, Van Der Stap JG, Smittenaar C, Heijne Den Bak-Glashouwer M, et al. Engineering the microflora to vaccinate the mucosa: serum immunoglobulin G responses and activated draining cervical lymph nodes following mucosal application of tetanus toxin fragment C-expressing lactobacilli. Immunology 2000;100:510–8.
[12] Zegers ND, Kluter E, van Der Stap H, van Dura E, van Dalen P, Shaw M, et al. Expression of the protective antigen of *Bacillus anthracis* by *Lactobacillus casei*: towards the development of an oral vaccine against anthrax. J Appl Microbiol 1999;87:309–14.
[13] Reveneau N, Geoffroy MC, Locht C, Chagnaud P, Mercenier A. Comparison of the immune responses induced by local immunizations with recombinant *Lactobacillus plantarum* producing tetanus toxin fragment C in different cellular locations. Vaccine 2002;20:1769–77.
[14] Cavanagh D. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch Virol 1997;142:629–33.
[15] Wesley RD, Woods RD. Partial passive protection with 2 monoclonal antibodies and frequency of feeding of hyperimmune anti-tgev serum for protection of 3 day old piglets from a tgev challenge infection. Tektran Agricultural Research Service 2000. http://www.nal.usda.gov/ttic/tektran/data/000011/57/0000115783.html.
[16] Saif LJ, Wedley R. Transmissible gastroenteritis virus. In: Straw BE, Allaire S, Mengeling WL, Taylor DJ, editors. Disease of swine. 8th ed. Ames: Iowa State University Press; 1982. p. 295–325.
[17] Krempl C, Schultze B, Launde H, Herrler G. Point mutations in the S protein connects the sialic acid binding activity with the enteropathogenicity of transmissible gastroenteritis coronavirus. J Virol 1997;71:3285–7.
[18] Schultze B, Krempl C, Ballesteros ML, Shaw L, Schauer R, Enjuanes L, et al. Transmissible gastroenteritis coronavirus, but not the related porcine respiratory coronavirus, has a salic acid (*N*-glycolylneuraminic acid) binding activity. J Virol 1996;70:5634–7.
[19] Delmas B, Gelfi J, Laude H. Antigenic structure of transmissible gastroenteritis virus. II. Domains in the peplomer glycoprotein. J Gen Virol 1986;67:1405–18.
[20] Jiménez G, Correa I, Melgosa MP, Bullido MJ, Enjuanes L. Critical epiptopes in transmissible gastroenteritis virus neutralization. J Virol 1986;60:131–9.
[21] Laude H, Gelfi J, Lavenant L, Charley B. Single amino acid changes in the viral glycoprotein M affect induction of alpha interferon by the coronavirus transmissible gastroenteritis virus. J Virol 1992;66:743–9.
[22] Bohl EH. Transmissible gastroenteritis. In: Leman AD, Glock RD, Mengeling WL, Penny RHC, School E, Straw B, editors. Disease of swine. Ames: Iowa State University Press; 1981. p. 198–208.
[23] Delmas B, Rasschaert D, Godet M, Gelfi J, Laude H. Four major antigenic sites of the coronavirus transmissible gastroenteritis virus are located on the amino-terminal half of spike glycoprotein S. J Gen Virol 1990;71:1313–23.
[24] Gebauer F, Posthumus WP, Correa I, Sune C, Smerdou C, Sanchez CM, et al. Residues involved in the antigenic sites of transmissible gastroenteritis coronavirus S glycoprotein. Virology 1991;183:225–38.
[25] Enjuanes L, Sune C, Gebauer F, Smerdou C, Camacho A, Anton IM, et al. Antigen selection and presentation to protect against transmissible gastroenteritis coronavirus. Vet Microbiol 1992;33:249–62.
[26] Pouwels PH, Vriesema A, Martinez B, Tielen FJ, Seegers JF, Leer RJ, et al. Lactobacilli as vehicle for targeting antigens to mucosal tissues by surface exposition of foreign antigens. Methods Enzymol 2001;336:369–89.
[27] Sambrook J, Fritisch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory; 1989.
[28] Josson K, Scheirlinck T, Michiels F, Platteeuw C, Stanssens P, Joos H, et al. Characterization of a Gram-positive broad-host-range plasmid isolated from *Lactobacillus hilgardii*. Plasmid 1989;21:9–20.
[29] Elson CO, Ealding W, Lefkowitz J. A lavage technique allowing repeated measurement of IgA antibody in mouse intestinal secretions. J Immunol Methods 1984;67:101–8.
[30] Liu C, Kokuho T, Kubota T, Watanabe S, Inumaru S, Yokomizo Y, et al. DNA mediated immunization with encoding the nucleoprotein gene of porcine transmissible gastroenteritis virus. Virus Res 2001;80:75–82.
[31] Lee YK, Ho PS, Low CS, Arvilommi H, Salminen S. Permanent colonization by *Lactobacillus casei* is hindered by the low rate of cell division in mouse gut. Appl Environ Microbiol 2004;70:670–4.
[32] McGhee JR, Mestecky J. In defense of mucosal surfaces. Development of novel vaccines for IgA responses protective at the portals of entry of microbial pathogens. Infect Dis Clin N Am 1990;4:315–41.
[33] Smerdou C, Urniza A, Curtis III R, Enjuanes L. Characterization of transmissible gastroenteritis coronavirus S protein expression products in avirulent *S. typhimurium* delta cya delta crp: persistence, stability and immune response in swine. Vet Microbiol 1996;48:87–100.
[34] Challacombe SJ. Salivary antibodies and systemic tolerance in mice after oral immunization with bacterial antigens. Ann NY Acad Sci 1983;409:177–93.
[35] Hu S, Bruszewski J, Smalling R, Browne JK. Studies of TGEV spike protein gp195 expressed in *E. coli* and by a TGEV-vaccinia virus recombinant. Adv Exp Med Biol 1985;185:63–82.
[36] Gomez N, Carrillo C, Salinas J, Parra F, Borca MV, Escribano JM. Expression of immunogenic glycoprotein S polypeptides from transmissible gastroenteritis coronavirus in transgenic plants. Virology 1998;249:352–8.