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Expression fusion immunogen by live attenuated *Escherichia coli* against enterotoxins infection in mice

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Summary

Previous epidemiological studies have shown that enterotoxins from enterotoxigenic *Escherichia coli* (ETEC) appear to be the most important causes of neonatal piglet and porcine post-weaning diarrhoea (PWD). Thus, it is necessary to develop an effective vaccine against ETEC infection. In the present study, the Kil cassette was inserted into the pseudogene *yaiT* by homologous recombination to create an attenuated *E. coli* double selection platform O142 (*yaiT*-Kil). After that, PRPL-Kil was replaced with a fusion gene (LTA1-STa$_{13}$-STb-LTA2-LTB-STa$_{13}$-STb) to establish oral vaccines O142(yaiT::LTA1-STa$_{13}$-STb-LTA2-LTB-STa$_{13}$-STb) (*ER-T*). Subsequently, BALB/c mice were orally immunized with *ER-T*. Results showed that serum IgG and faecal sIgA responded against all ETEC enterotoxins and induced F41 antibody in BALB/c mice by orogastrically inoculation with recombinant *E. coli ER-T*. Moreover, the determination of cellular immune response demonstrated that the stimulation index (SI) was significantly higher in immunized mice than in control mice, and a clear trend in the helper T-cell (Th) response was Th2-cell (IL-4) exceed Th1-cell (IFN-γ).Our results indicated that recombinant *E. coli ER-T* provides effective protection against ETEC infection.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is a bacterial pathogen responsible for severe diarrhoea diseases in animals and humans. Enterotoxigenic *Escherichia coli* leads to high morbidity and mortality both for neonatal and post-weaning pigs (Harvey *et al*., 2006). ETEC diarrhoea causes slow growth, weight loss and death, which result in considerable economic losses for hog producers worldwide (Trevisi *et al*., 2015). The major virulence factors of these bacteria are bacterial fimbriae/non-fimbrial adhesins and enterotoxins (Nataro and Kaper, 1998). Fimbriae/non-fimbrial adhesions mediate the attachment of bacteria to host intestinal villus and facilitate bacterial colonization. Then, signal peptide guides enterotoxins through the cell membrane, and causes epithelial cell chloride-ion secretion and preventing sodium chloride absorption, exacerbated secretory diarrhoea by simultaneous fluid movement into the lumen (Field *et al*., 1978). In the past decade, ETEC infection has been prevented using antibiotic agents (Smith *et al*., 2010). Recently, multi-resistance has been reported with increasing frequency in several countries worldwide, as hogs have frequently been treated as a group with excessive mass medication (Wang *et al*., 2010). Moreover, antibiotics could select resistant *Escherichia coli* to transfer their resistance plasmids to other bacteria that may include pathogens in the faecal flora (Nijsten *et al*., 1996). Thus, the objective of this study is the prevention of ETEC invasion by alternative methods (Bischoff *et al*., 2002). Immunization remains an effective approach for preventing infectious diseases. However, there is no broadly effective vaccine available for swine ETEC diarrhoea in China. An effective porcine ETEC vaccine should include all the enterotoxins antigens to lead to anti-heat-labile enterotoxin (anti-LT) and anti-heat-stable enterotoxins (anti-STa and anti-STb) immunity(Liu *et al*., 2014).

Recent studies showed that most commercial vaccines are administered by injection, stress response in newborn piglets is induced by repeated injection. Nevertheless, activation of secreted intestinal anti-ETEC responses is impossible to achieve by parenteral administration (Lasaro *et al*., 2005). Therefore, stimulating a protective immune response by colonization in the intestinal mucosa without causing inflammations is important for an ETEC vaccine. The gastrointestinal tract (GIT) is the animal's largest immunological organ, with a daily production of more than 60% of antibodies (Tang and Li, 2009). Mucosal immune activity plays a major role in neutralizing ETEC upon entry into the

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body (Kotton and Hohmann, 2004). Oral vaccination avoids the use of syringes, which evokes both local and systemic immune responses, and production of secreted immunoglobulin A (IgA) blocks bacterial attachment to the intestinal epithelial cells (Jertborn *et al.*, 1998). Furthermore, attenuated strains express more heterologous antigens simultaneously, and safely deliver multiple expressed antigens at mucosal sites (Charles and Dougan, 1990). In addition, oral bacterial vaccine vectors are stable in storage, simple to administer and inexpensive to manufacture (Ascon *et al.*, 1998).

In this study, we have established a recombinant *E. coli* strain in pseudogene positions *yaiT* expressing LTA1-STa$_{13}$-STb-LTA2-LTB-STa$_{13}$-STb fusion immunogen. Using an oral vaccine, we have gained detailed insight into the immune responses in mouse models. The statistics show that oral immunization with *ER-T* can elicit more potent systemic and mucosal immune response in mice.

Results

Establish double selection platform and assess its performance

PCR was used to identify O142 (*yaiT*-Kil) using primers T1 and T4. The products showed that the *E. coli* O142: ΔSTa (*yaiT* gene, 800 bp), O142:△STa/pKil-donor (*yaiT* gene, 800 bp; pKil-donor, 3800 bp) and O142 (*yaiT*-Kil) (*yaiT* with Kil cassette flanked, 3800 bp) were of expected size (Fig. 1, panel B). The double selection platforms were plated on MacConkey agar 18 h later, the platform strains grew normally at 30°C. Platform-expressed Kil gene caused cells to die at 43°C (Fig. 1, panel C).

Expression of the fusion protein by recombinant E. coli

ER-T was verified by PCR using the primers T1 with P2. The product of *ER-T* was of expected size (3000 bp) for LTA1-STa$_{13}$-STb-LTA2-LTB-STa$_{13}$-STb fusion gene, meanwhile, there were no bands in O142:△STa, O142

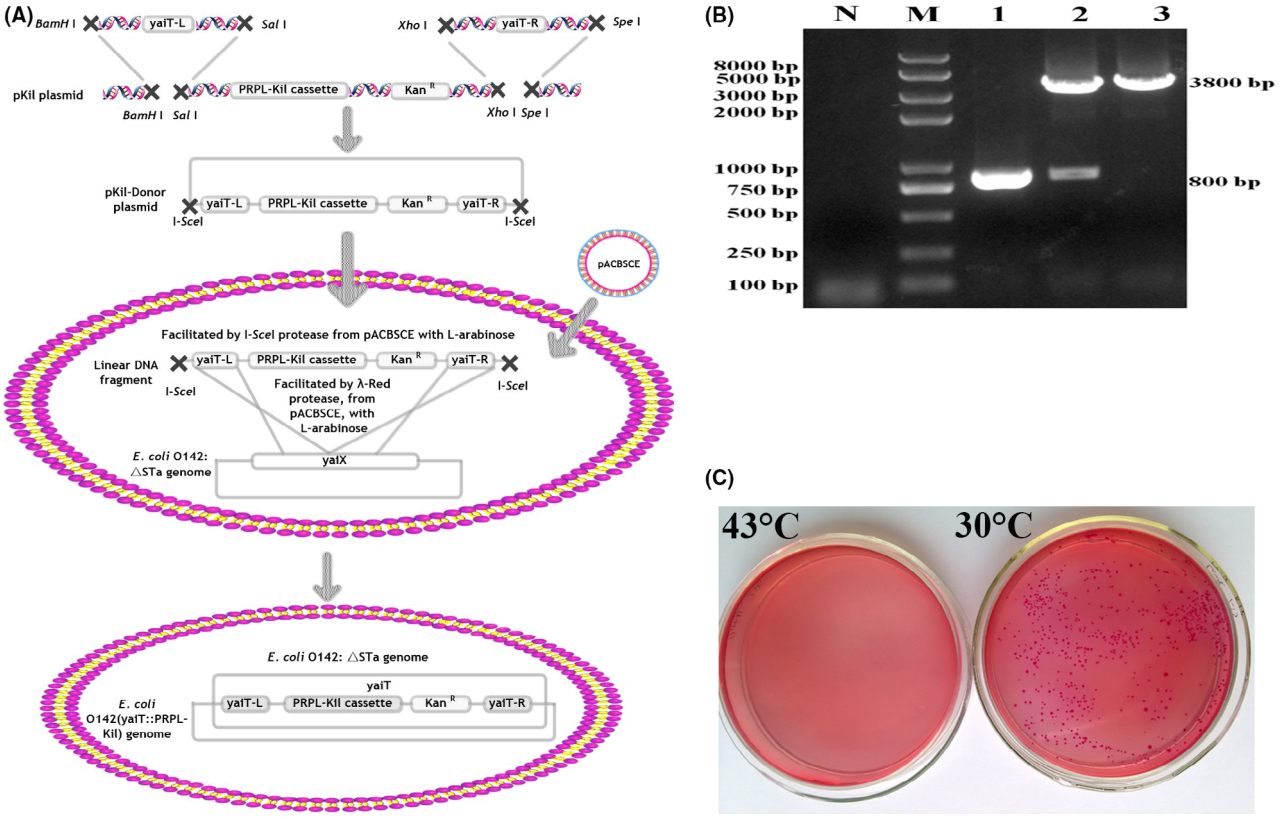


Fig. 1. The schematic outline of the recombinant strategy for constructing the double selection platform.
Panel A. Construction of the double selection platform O142(yaiT::PRPL-Kil) by homologous recombination. The pKil-donor plasmid and the pACBSCE plasmid are co-transformed into the Attenuated *E. coli* O142:△STa. L-arabinose induction promotes expression of the I-SceI recombinase system and the λ-Red endonuclease. I-SceI generates a linear DNA fragment from the pKil-donor plasmid that is a substrate for recombination with the pseudogene mediated by the λ-Red system.
Panel B. PCR analysis of chromosomal DNA from double selection platform O142(yaiT::PRPL-Kil) by using the primers T1 and T4. N: PCR negative control; M: molecular size marker; 1: PCR product of *E. coli* O142: △STa; 2: PCR product of O142: △STa/pKil-donor; 3: PCR product of O142(yaiT::PRPL-Kil).
Panel C. Inversion screen test of the double selection platform. a: O142(yaiT::PRPL-Kil) incubated at 43°C, cannot grow on MacConkey agar plates; b: O142(yaiT::PRPL-Kil) incubated at 30°C, grown normally.

(yaiT-Kil) (Fig. 2, panel C). After sequencing, we found that fusion genes (LTA1-STa$_{13}$-STb-LTA2-LTB-STa$_{13}$-STb cassette) were correct insertions of the *E. coli* O142:△STa (data not shown). In addition, Western blot analysis of LTA1-STa$_{13}$-STb-LTA2-LTB-STa$_{13}$-STb fusion protein, and the expected sizes (17 kDa and 35 kDa) were observed (Fig. 2, panel D).

Feasibility analysis

Mice were orally inoculated with *ER-T*, after 4 h gut/carcass mass ratios (G/C = 0.065 ± 0.006) remained normal, *E. coli* O142 and *E. coli* 344-C induced a significant increase of intestinal fluid accumulation in mice model (G/C = 0.125 ± 0.005, G/C = 0.107 ± 0.003), indicating that

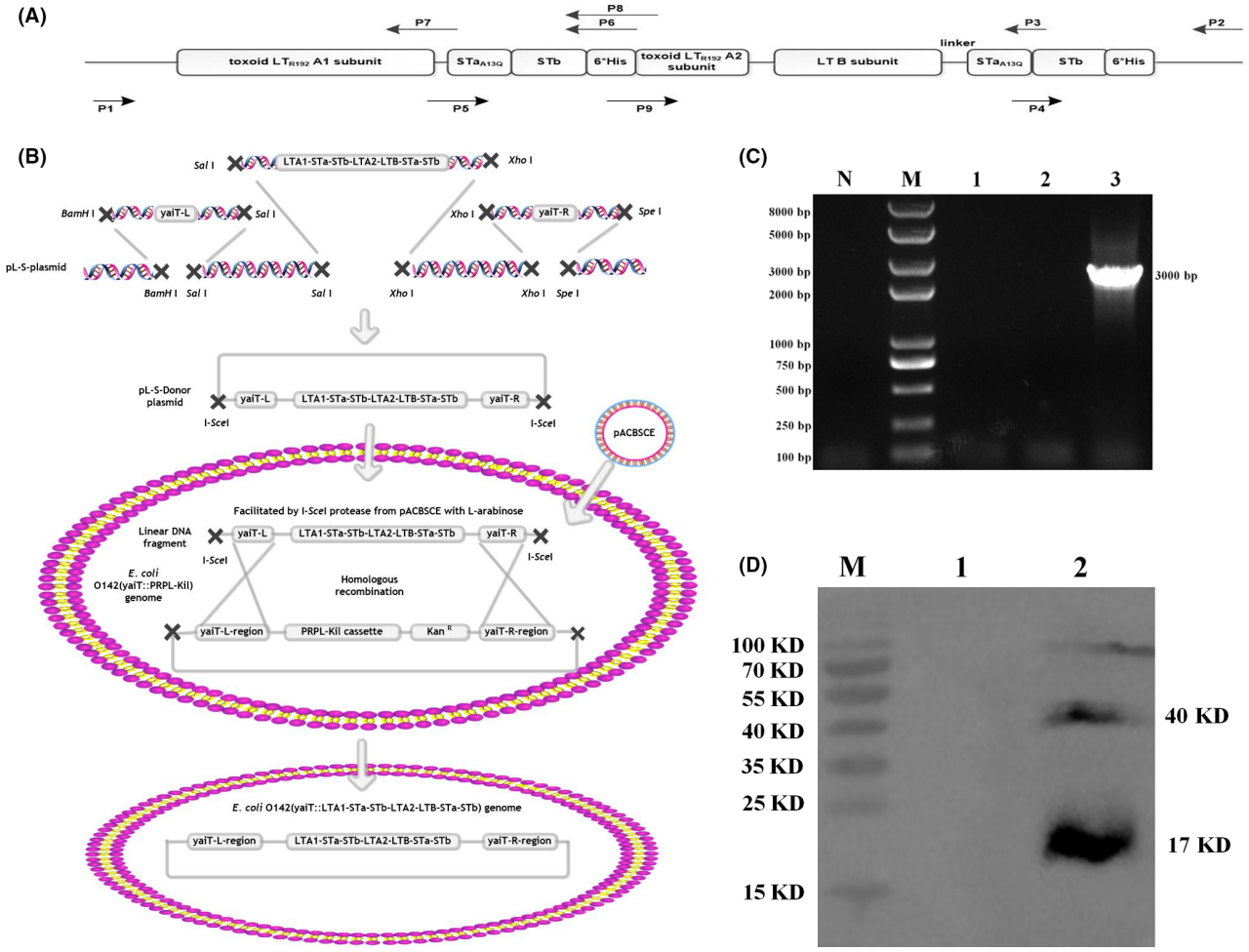


Fig. 2. The schematic outline of the recombinant strategy for constructing the recombinant *E. coli* O142(yaiT::LTA1-STa13-STb-LTA2-LTB-STa13-STb) for oral vaccine candidate.
Panel A. The full-length porcine LT192 operon was used conjugate with LT192, STa13, STb for generating LTA1-STa13-STb-LTA2-LTB-STa13-STb fusion antigen and the native LT promoter was retained which expressed without induction. PCR primers P1 and P2 amplified the entire LT cassette including the native LT promoter and terminator. Primers P2 paired with P4 amplified the STa13-6 × His-terminator chimeric gene. Primers A1 and A7 mutated the LT gene for LT192.
Panel B. Construction of the recombinant *E. coli* O142(yaiT::LTA1-STa13-STb-LTA2-LTB-STa13-STb) according to Gene Doctoring method. The pL-S-donor plasmid and the recombineering plasmid pACBSCE are co-transformed into the double selection platform. Arabinose induction promotes expression of the λ-Red gene products and I-SceI. I-SceI cleaves the pL-S-donor plasmid resulting in generation of the linear DNA fragment for λ-Red mediated recombination to generate the recombinant *E. coli* O142(yaiT:: LTA1-STa13-STb-LTA2-LTB-STa13-STb).
Panel C. PCR reaction for the verifying of the recombinant *E. coli* strain *ER-T* by using the primers yaiT-L-arm and P2. M: molecular size marker; N: PCR negative control; 1: PCR product of *E. coli* O142: △STa; 2: PCR product of O142(yaiT::PRPL-Kil); 3: PCR product of *ER-T*.
Panel D. Detection of the LTA1-STa13-STb-LTA2-LTB-STa13-STb fusion protein in the Western blot assay. anti-His (ZSGB-BIO Co.; 1:500) were used as primary antibody and secondary antibody by (HRP)-conjugated goat anti-mouse IgG (ZSGB-BIO Co.; 1:2000), development following the manufacturer's instructions of ECL Plus Reagent Kit (7 Sea Biotech, China) M: Protein marker; 1: *E. coli* O142: △STa as the negative control; 2: *E. coli ER-T*.

the toxicity of *ER-T* had been reduced or eliminated (Fig. 3, panel A). The results of *in vitro* cytotoxicity assay showed that supernatant of *ER-T* cannot cause ZYM-DIEC02 cells to die, but supernatant of *E. coli* O142 (STa), 274-A (LT) and 344-C (STb) induce cell death (Fig. 3, panel B). The tolerance test indicated that *ER-T* tolerates well gastric acid pH 2.5 to 4.5, intestinal juice, and bile 0.05–0.3% (Fig. 3, panels C–E). During the first 3 days of the rearing period, the feed intake decrease. Over the whole period, the feed intake and weight were similar in each group (Fig. 3, panel F). The stability curves of *ER-T* in the growth phases were similar and no significant differences between them were found, indicating that insertion of the chimeric gene did not impact the growth of the bacterial cell (Fig. 3, panel G). Large doses of *ER-T* did not affect the weight gain of mice (Fig. 3, panel H). We checked the stability of *ER-T*, and the results indicated that all the colonies analysed presented the expected bands. Sequencing showed that chimeric gene LTA1-STa_{13}-STb-LTA2-LTB-STa_{13}-STb is not lost after 100 generations (data not shown). EM images revealed that insertion of the LTA1-STa_{13}-STb-LTA2-LTB-STa_{13}-STb fusion gene did not affect the *E. coli* O142: ΔSTa fimbriae expression (Fig. 3, panel I). The colonization assay demonstrated that *ER-T* had a higher binding capacity in the intestinal tract of mice (Fig. 3, panel J).

Systemic and mucosal immunogenicity elicited by recombinant E. coli

After oral immunization recombinant, bacteria can enhance the serum IgG and faecal IgA. High levels of anti-F41, anti-LTA, anti-LTB, anti-STa and anti-STb IgG antibodies were detected after 7, 14, 14, 21 and 21 days in the serum samples respectively (Fig. 4A–E). Forty-two days after immunization, *ER-T* (Fig. 4F–I) specific IgG antibodies were revealed in milk, spleens, mesenteric lymph nodes and intestinal mucus samples. By contrast, in the control groups there was no enhancement of specific IgG antibody responses. Similar findings were revealed on the secreted IgA antibody responses in faeces of mice after being administered orally with *ER-T* (Fig. 5A–I). These results show that the recombinant *E. coli* has the capability to elicit both systemic and mucosal antibody responses.

Lymphocyte proliferation responses and cytokines assay

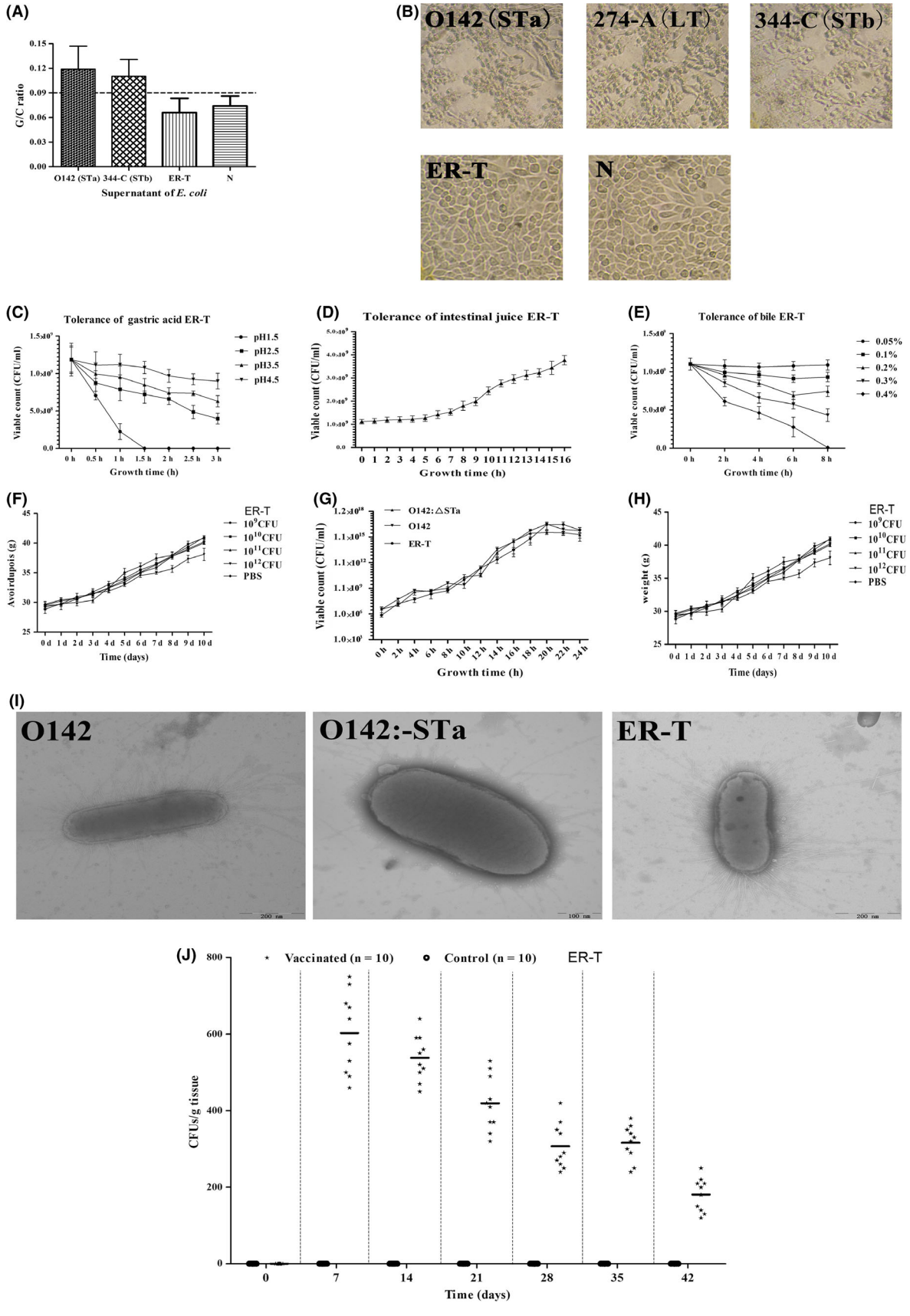
The results indicated that the splenic and mesenteric lymphocytes from mice immunized orally with *ER-T* showed significant responses compared with the PBS group by an MTT assay. For splenic lymphocyte proliferation, splenocyte, mesenteric lymphocyte from different groups of immunized mice were cultured *in vitro*. The higher level of proliferation was demonstrated in *ER-T* group for STa, LT_{192}-STa_{13}, STb, LT_{192}-STb, LT with the mean SI of 2.38, 2.34, 2.40, 2.11 and 2.31 (Fig. 6, panel A). In addition, the group of *ER-T* (Fig. 6, panel B) induced higher mesenteric lymphocyte proliferation responses (SI of STa 2.59, LT_{192}-STa_{13} 2.85, STb 2.58, LT_{192}-STb 2.93, LT 2.79) than control and O142:△STa.

Mice vaccinated with *ER-T* had splenic lymphocytes IFN-γ (mean 43.55 ± 12.63), and neither were different from those found in splenic lymphocytes from both control groups (Fig. 6, panel C). In contrast, the groups vaccinated with *ER-T* (mean 313.89 ± 33.21) had notably higher splenic lymphocytes IL-4 than the groups control (Fig. 6, panel D). When the IL-4 to IFN-γ ratio was analysed, it was found that the oral immunization groups had 7 times more IL-4 than IFN-γ, which is a clear indicator of a polarized Th2 immune response.

Toxin-neutralizing ability in vitro

We used ZYM-DIEC02 cells to detect the neutralizing efficacy of serum, intestinal mucus, splenocyte lysate and mesenteric lymphocyte lysate from orally inoculated mice. Results showed that the samples (from the immunized mice) had effective neutralization LT toxin, in serum (1:32), splenocyte lysate (1:16), intestinal mucus (1:32) and mesenteric lymphocytes lysate (1:32) (Fig. 7,

Fig. 3. Feasibility of recombinant *E. coli ER-T* for oral vaccine candidate.
Panel A. Suckling mice assay for identifying the toxicity of *ER-T*. The toxicity of *ER-T* was eliminated.
Panel B. ZYM-DIEC02 cells inoculated with supernatant of *E. coli*. a: cells inoculated with supernatant of O142 showed significant cell death; b: cells inoculated with supernatant of 274-A showed significant cell death; c: cells inoculated with supernatant of 344-C showed significant cell death; d: cells inoculated with supernatant of *ER-T* grew normally; e: non-treated cells grew normally.
Panel C. The gastric acid tolerance of *ER-T*, from using the plate method to enumerate the amounts of *ER-T* that survived in different pH levels of gastric acid.
Panel D. The intestinal juice tolerance of *ER-T*, from using the plate method to enumerate the amounts of *ER-T*.
Panel E. The bile tolerance of *ER-T*, from using the plate method to enumerate the amounts of *ER-T* that survived in different pH concentrations of bile.
Panel F. The daily appetite of mice orally fed with *ER-T*.
Panel G. The growth curve of *ER-T*. Fed *ER-T* was determined by the number of CFU at the time points indicated.
Panel H. The change of avoirdupois in mice orally fed with *ER-T*.
Panel I. Electron microscope observations of the structure of fimbriae of *E. coli* O142, O142: △STa, *ER-T*, (magnification 30 000×).
Panel J. Colonization efficacy of *ER-T* in intestinal tracts of mice. Mean values are shown, and error bars represent standard deviations.



panel A–D). Likewise, the immunized mice exhibited higher STa toxin-neutralizing activity in serum (1:16), splenocyte lysate (1:16), intestinal mucus (1:32) and mesenteric lymphocytes lysate (1:32) (Fig. 7, panel E–H). Moreover, samples from the immunized mice showed the STb toxin neutralization potential in serum (1:16), splenocyte lysate (1:16), intestinal mucus (1:32) and mesenteric lymphocytes lysate (1:32) (Fig. 7, panel I–L).

Toxin-neutralizing activity in vivo

Recombinant *E. coli ER-T* used as immunoprophylactic had the ability to induce neutralizing antibodies against STa and STb. When comparing the samples of *ER-T* for the neutralizing efficiency to STa toxins, results indicated that STa toxin antibodies were in serum (1:5), splenocyte lysate (1:5), intestinal mucus (1:2.5) and mesenteric lymphocyte lysate (1:5) (Fig. 8, panel A–D). Likewise,

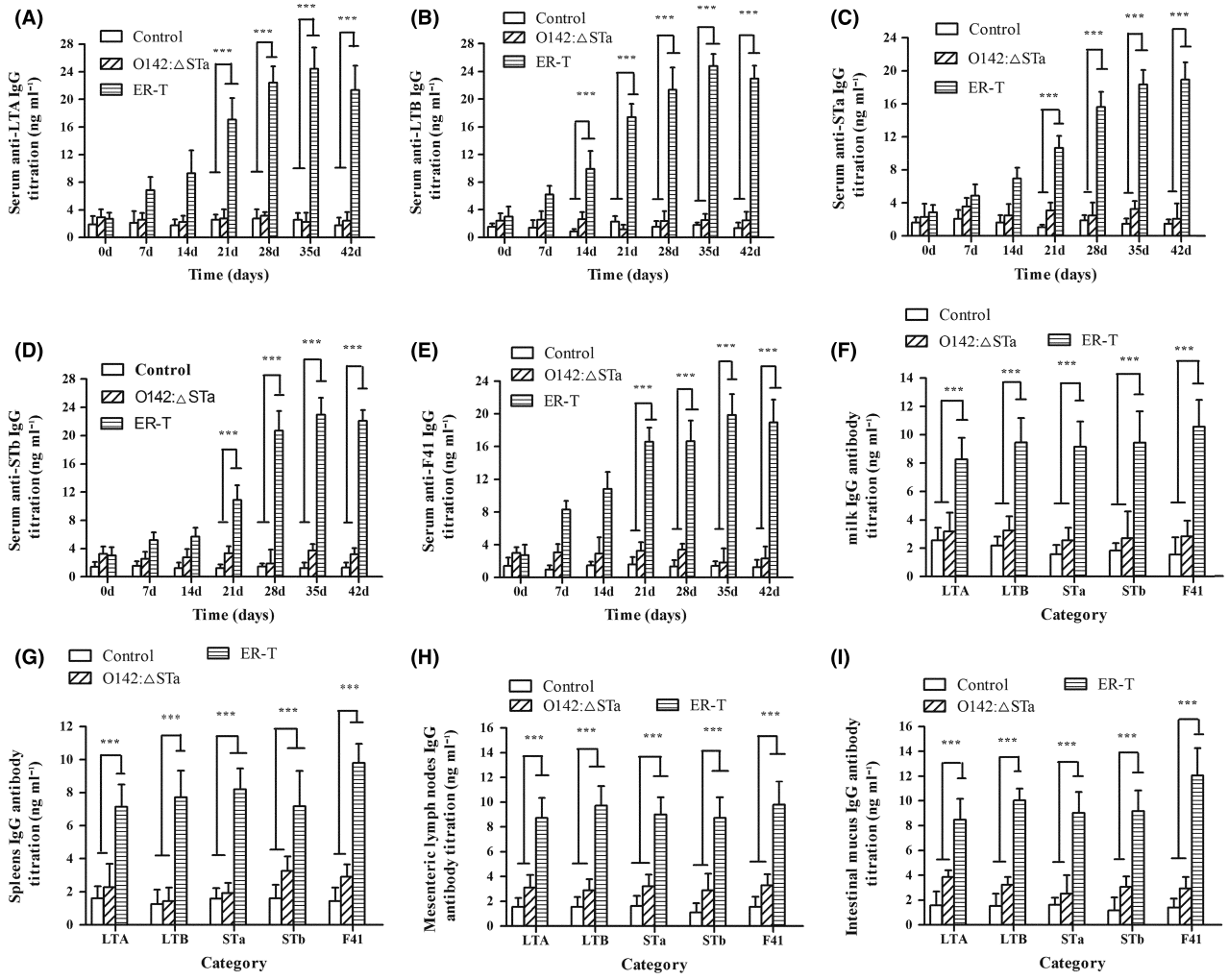


Fig. 4. ELISA analysis of sera IgG from mice inoculated intragastrically with ER-T.
A. IgG-specific of anti-LTA in the serum.
B. IgG-specific of anti-LTB in the serum.
C. IgG-specific of anti-STa in the serum.
D. IgG-specific of anti-STb in the serum.
E. IgG-specific of anti-F41 in the serum.
F. IgG-specific in the milk samples.
G. IgG-specific in the spleen samples.
H. IgG-specific in the mesenteric lymph node samples.
I. IgG-specific in the intestinal mucus samples. Because LTA1-STa13-STb-LTA2-LTB-STa13-STb insertion causes ER-T systemic antibody responses have a slight advantage. Error bars represent standard deviations, and mean values are shown. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

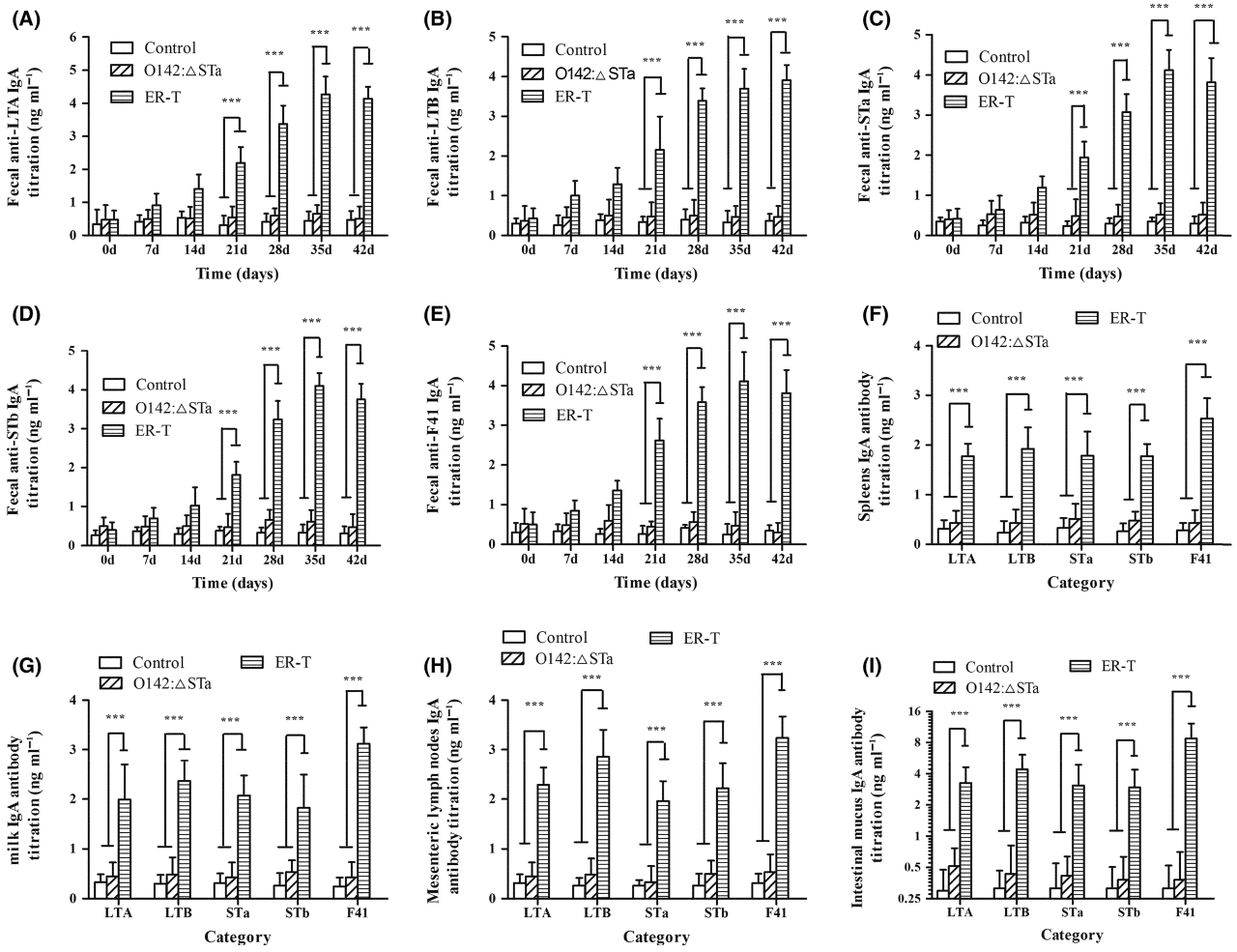


Fig. 5. ELISA analysis of faecal IgA from mice inoculated intragastrically with ER-T.
A. IgA-specific of anti-LTA in the faecal.
B. IgA-specific of anti-LTB in the faecal.
C. IgA-specific of anti-STa in the faecal.
D. IgA-specific of anti-STb in the faecal.
E. IgA-specific of anti-F41 in the faecal.
F. IgA-specific in the spleen samples.
G. IgA-specific in the milk samples.
H. IgA-specific in the mesenteric lymph node samples.
I. IgA-specific in the intestinal mucus samples. ER-T mucosal antibody responses are stronger by inserting foreign genes into pseudogenes. Error bars represent standard deviations, and mean values are shown. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

the samples from the immunized mice group of *ER-T* showed the neutralization ability to STb toxin in serum of (1:2.5), splenocyte lysate (1:7.5), intestinal mucus (1:2.5) and mesenteric lymphocyte lysate (1:2.5) (Fig. 8, panel E–H).

Protection efficacy of maternal antibody

The protective efficacy of suckling mice intaking milk from the immunized pregnant mice challenged with STa or STb toxin was investigated. After being administered orally with STa toxin (1:15 diluted) the *ER-T* G/C ratios, outlined below (*ER-T* G/C = 0.083 ± 0.005), significantly below that of the mice of the control group (Fig. 9, panel A). Likewise, after challenge with STb toxin (1:15 diluted), *ER-T* G/C ratios outlined below (*ER-T* G/C = 0.080 ± 0.005), which is lower than control (1:15 diluted G/C = 0.104 ± 0.005) and O142:△STa (1:15 diluted G/C = 0.094 ± 0.006) (Fig. 9, panel B).

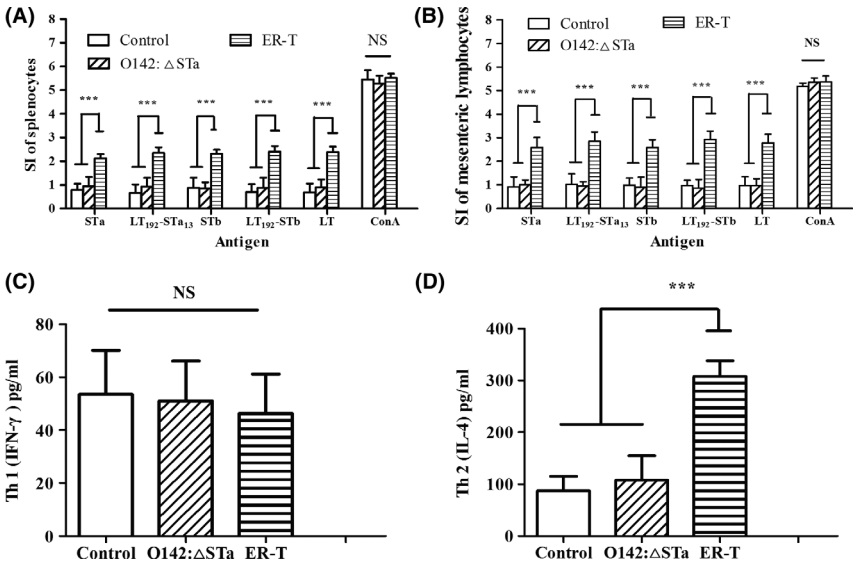


Fig. 6. Characterization of lymphocyte and cytokines proliferation responses in mice by orally administered ER-T.
A. Splenocyte samples stimulated with different purified antigens by MTT method.
B. Mesenteric lymphocyte samples stimulated with different purified antigens by MTT method.
C. The levels of IFN-γ concentration in the culture supernatant were measured by ELISA.
D. The levels of IL-4 concentration in the culture supernatant were measured by ELISA. Error bars represent standard deviations, and mean values are shown. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Discussion

Porcine ETEC-associated diarrhoea, especially PWD and neonatal piglet diarrhoea, remains a major problem for swine producers around the world, but an effective vaccine against neonatal piglet diarrhoea and PWD is lacking (Ruan and Zhang, 2013). Due to the vast majority of piglet diarrhoea being caused by ETEC expressing K88ac or F18 fimbriae in North America (Zhang *et al*., 2007), abundantly expressed CFA/I, 987P, K99, F18 and/or K88ac fimbriae vaccines were developed. However, in China, pregnant sows are often vaccinated with inactivated vaccines containing fimbriae antigens to protect their offspring from intestinal infection with ETEC. In a recent epidemiological survey, the fimbriae were not frequently associated with ETEC for suckling pigs with diarrhoea in China. Likewise, low proportions of adhesin-positive *E. coli* strains have been reported in previous studies in the Netherlands (Guinee and Jansen, 1979), Japan (Nakazawa *et al*., 1987) and Sweden (Soderlind *et al*., 1988). Therefore, efficient ETEC vaccines should work against enterotoxins (Ruan and Zhang, 2013).

Oral subunit or killed vaccine antigen delivery through the digestive tract is subject to digestive degradation. Therefore, oral vaccination must maintain the integrity of the antigen during delivery to the intestinal tract. Recently, extensive work on a live vehicle vaccine system presented evidence that recombinant attenuated *E. coli* is an efficient carrier for antigens by the oral route (Liu *et al*., 2014). In our study, we build oral vaccine strains obtained from attenuated wild-type ETEC strain. Moreover, recombinant bacteria achieve ephemeral colonization in the host by F41 fimbriae to overcome host defences, such as the gastrointestinal mucus barrier and intestinal peristalsis cleaning mechanism (Pacheco *et al*., 2012). Orally administered *ER-T* can deliver antigens to the immune system for a prolonged period. In addition, we demonstrated that a single oral dose of recombinant *E. coli* was without significant reactogenicity or toxicity at dosages up to 10^9 CFU. Recombinant *E. coli* containing LTA1-STa$_{13}$-STb-LTA2-LTB-STa$_{13}$-STb operon have been stably maintained for over 100 generations in wild-type *E. coli*. Another aspect of this research is that it is the first report of oral polyvalent vaccine fusion of all swine ETEC enterotoxins simultaneously. Enterotoxins are an important virulence factor of swine ETEC strains and have been frequently identified in pigs with ETEC diarrhoea disease (Zhang *et al*., 2006). The most significant contributors to piglet ETEC diarrhoea were LT, STa and STb.

STa is composed of only 19 amino acids (2 KDa), so the immunogenicity is poor, and it can cause diarrhoea unless inactivated or modified STa peptides pSTa (A13Q) reduction toxicity (Zhang *et al*., 2010). Recent studies indicate that STa (A13Q) fuses to LT became immunogenic (Zhang *et al*., 2013), resulting in LT-STb fusion antigen can stimulate body to produce antibodies against LT and STa toxins (Liu *et al*., 2011).

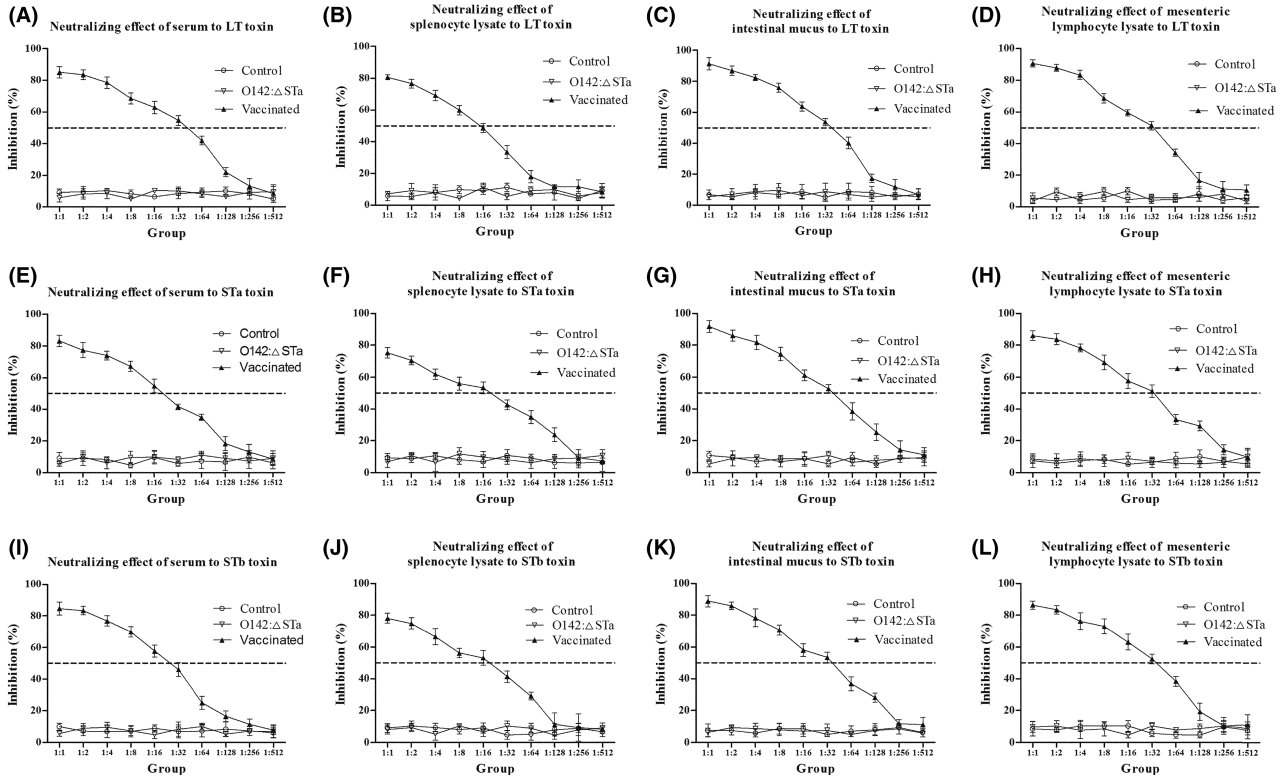


Fig. 7. *In vitro* neutralization assays of samples from group ER-T neutralized to LT, STa and STb toxin.
A–D. Serum, splenocyte lysate, intestinal mucus and mesenteric lymphocyte lysate from immunized mice showed neutralization efficiency to LT toxin when compared with that from control mice.
E–H. Serum, splenocyte lysate, intestinal mucus and mesenteric lymphocyte lysate from immunized mice showed neutralization efficiency to STa toxin when compared with that from control mice.
I–L. Serum, splenocyte lysate, intestinal mucus and mesenteric lymphocyte lysate from immunized mice showed neutralization efficiency to STb toxin when compared with that from control mice. The ratios on the figure are the dilution gradient of toxins, the immune mice produced antibodies that protected ZYM-DIEC02 cells (cell death < 50%), the control group did not protect ZYM-DIEC02 cells.

STb positive isolates from diarrhoeic piglets were more prevalent than STa in Canada (Harel *et al*., 1991), Poland (Osek and Truszczynski, 1992) and Spain (Blanco *et al*., 1997) respectively. STb consisting of 40 amino acids (5.2 KDa) was too short for induced anti-STb immunity in hosts (Sears and Kaper, 1996). Moreover, a study demonstrated that STb coupled to an appropriate carrier molecule had the advantages of reducing toxicity and inducing neutralizing antibodies (Dubreuil *et al*., 1996).

LT consisted of a toxic A1 subunit (LTA1), non-toxic A2 subunit (LTA2) and five identical polypeptide chains of B subunit (LTB) (Spangler, 1992) and was capable of enhancing systemic and mucosal antibodies. Insertion of exogenous genes between LTA1 and LTA2 shows that reduce the toxicity of LTA1 and preserve the adjuvant function of LTA (Zhang and Sack, 2015). LTB is a potent immunogen and possess adjuvant properties (Millar *et al*., 2001; Sanchez and Holmgren, 2005), and binding to specific receptors on the host cell membrane then toxin entry into host cells (Salimian *et al*., 2010). In addition, LT facilitates the immunogenicity of STa and STb in vaccines immunization against bacterial infections (Norton *et al*., 2012). Therefore, using STa or STb fusion at the C terminus of LTB subunits would generate quadruple or quintuple anti-ST fusion antigens (Ruan *et al*., 2011). Thus, LT plays a key role in the prevention of enteric infection. Moreover, previous vaccines were mainly targeted at LT-STa or against LT-STb, so diarrhoea caused by enterotoxins (LTB, STa, STb) could not be completely prevented (Zhang *et al*., 2013; Ruan *et al*., 2014), to prevent enterotoxin-induced diarrhoea in piglets, two vaccines need to be used simultaneously. In this study, increasing immunogenicity of enterotoxins (STa, STb) a mature STb peptide fused to a full-length of porcine STa_{A13Q} toxoid generated STa_{13}-STb fusion antigen. However, the preliminary results of this laboratory show that STa_{13} can poison its own cells after four

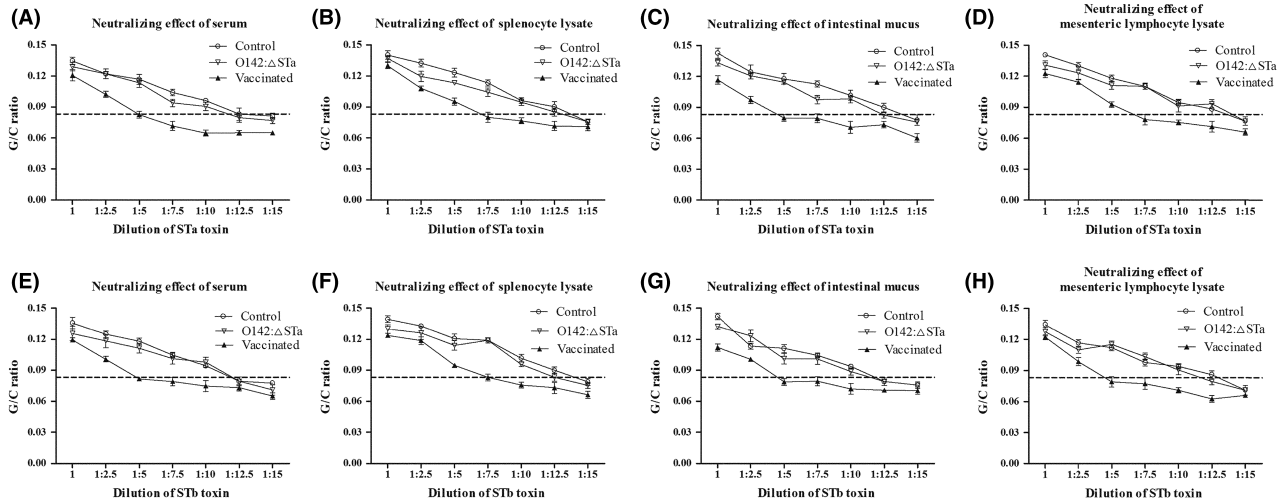


Fig. 8. Enterotoxins neutralization assay with suckling mice, samples from ER-T mix with serially diluted toxins STa and STb respectively.
A. Serum neutralization of STa toxin activity compared with control and *E. coli* O142: △STa mice.
B. Splenocyte lysate neutralization of STa toxin activity compared with control and *E. coli* O142: △STa mice.
C. Intestinal mucus neutralization of STa toxin activity compared with control and *E. coli* O142: △STa mice.
D. Mesenteric lymphocyte lysate neutralization of STa toxin activity compared with control and *E. coli* O142: △STa mice.
E. Serum neutralization of STb toxin activity compared with control and *E. coli* O142: △STa mice.
F. Splenocyte lysate neutralization of STb toxin activity compared with control and *E. coli* O142: △STa mice.
G. Intestinal mucus neutralization of STb toxin activity compared with control and *E. coli* O142: △STa mice.
H. Mesenteric lymphocyte lysate neutralization of STb toxin activity compared with control and *E. coli* O142: △STa mice. Samples from control and *E. coli* O142: △STa suckling mice resisted 1:15 dilution of the toxin, ER-T resistant to 1:5 dilution of toxins. Experimental results show that recombinant ER-T has certain ability to protect suckling mice. Error bars represent standard deviations, and mean values are shown.

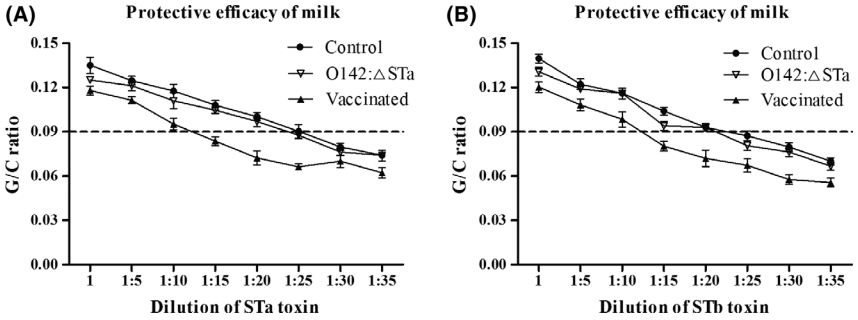


Fig. 9. *In vivo* neutralization assays using milk-immunized suckling mice *ER-T* challenge with STa and STb toxin in serial dilutions respectively.
A. The group of *ER-T* G/C ratios challenged with STa toxin negative value at 1:20 dilution below than that of control.
B. The group of *ER-T* G/C ratios challenged with STb toxin negative value at 1:15 dilution below than that of control. Error bars represent standard deviations, and mean values are shown.

times tandem repeat (STa_{13}-STa_{13}-STa_{13}-STa_{13}) (data not published). Therefore, to reduce the toxicity a point insertion was used. Connecting STa_{13}-STb to the 3′ end of the full-length porcine LT_{R192G} operon, constitutes LTB-STa_{13}-STb. Between LTA1 and LTA2 inserted STa_{13}-STb then form LTA1-STa_{A13Q}-STb-LTA2. Finally, LTA1-STa_{13}-STb-LTA2-LTB-STa_{13}-STb is formed. Since the native promoter of LT is preserved, the expression of the fusion protein does not require an inducer. Fusion antigen LTA1-STa_{13}-STb-LTA2-LTB-STa_{13}-STb was bicistronic like LT, LTA1-STa_{13}-STb and LTB-STa_{13}-STb were expressed in the cytosol of the bacteria. The A2 region of LT A subunit complexes with LT B subunit forming a structure of A:B5 when secreted to the external of live bacteria under the guidance of signal peptide. When SDS-PAGE carried out, subunits A and B were separated by heating. Previously, we inserted STa_{13}-STb-His after LTA1 and STa_{13}-STb-His downstream of LTB, respectively, therefore two bands in WB experiment 17 and 35 kDa.

In addition, previous LT pathogenesis studies have indicated that the LTB amino acids 82-97 were binding

regions of the GM1 receptor; we also retained GM1 binding activity as suggested. Data from this study indicated that *ER-T* expression fusion gene LTA1-STa_{13}-STb-LTA2-LTB-STa_{13}-STb elicits specific immune response anti-LT, anti-STa and anti-STb in a mouse model by the oral route. We chose the location of pseudogene *yaiT* gene, inserted by IS element, in addition far away from the promoter and terminator. Homologous recombination systems were used to insert exogenous genes on pseudogenes. Then, the influence on *ER-T* of biological activity and growth was tested (Echols *et al*., 2002; Balakirev and Ayala, 2003).

IgA production exceeds all the other immunoglobulins at the mucosal surface. Thus, IgA plays a significant role in defending against the invasion of pathogens. Our results indicated faecal *ER-T* sIgA levels against LTA (day 21, $P < 0.001$), LTB (day 14, $P < 0.001$), STa (day 21, $P < 0.05$), STb (day 21, $P < 0.01$) and F41 (day 21, $P < 0.01$). Likewise, ELISA titres of IgG display statistically significant differences in spleens, milk, mesenteric lymph nodes and intestinal mucus of mice immunized with *ER-T*. In addition, serum IgG and faecal sIgA titres from mice after immunization with the vaccine *ER-T* were conspicuously higher than those of the control group in both experiments ($P < 0.05$). This indicates oral administration could trigger immune responses not only mucosa but also systemically. The lymphocyte proliferation assay indicated that SI ratios of splenocytes and mesenteric lymphocytes cell proliferative responses were significantly higher in the *ER-T* immunized groups than in controls ($P < 0.05$; Fig. 6, panels A and B). For this study, within the *ER-T* groups of mice immunized by oral administration, results suggested that LTA1-STa_{13}-STb-LTA2-LTB-STa_{13}-STb fusion antigen can induce sufficient cellular immune responses. Immunized mice had higher levels of IL-4 than IFN-γ. The IFN-γ effects are partially blocked by IL-4 in infectious diseases (Myers *et al*., 1992), suggesting that recombinant strains induced Th2-preferred responses. *in vitro* and *in vivo* neutralization assays were performed for evaluating the sufficient neutralizing antibody titres by *ER-T*. Results showed that after LT, STa and STb enterotoxin incubation with serum, splenocyte lysate, intestinal mucus and mesenteric lymphocyte lysate samples from the immunized mice had the capability of preventing infection of ZYM-DIEC02 cells *in vitro*. Furthermore, these samples obviously neutralized the biological activity for STa and STb enterotoxins in suckling mice.

Experimental procedures

Bacterial strains, plasmids and cells

The strains used in this study are listed in Table 1. *E. coli* O142 was deposited in the Chinese Veterinary Culture Collection Center (Beijing, China) (CVCC accession no. C83920). *E. coli* C83903 was purchased from the Chinese Institute of Veterinary Drug Control (Beijing, China). Strain LT_{192}-STa_{13} fused LT_{192} (GenBank accession no.

Table 1. *E. coli* strains used and constructed in this study

Strain	Relevant properties	Reference or source
O142	Cattle ETEC field isolate, F41/STa	CVCC: C83920
274-A	Porcine ETEC field isolate, LT/K88, harboring LT as the only toxin	This laboratory
344-C	Porcine ETEC field isolate, STb/paa, harbor the STb as the only toxin	This laboratory
C83903	Porcine ETEC field isolate, K88/LT/STb/EAST1	China Institute of Veterinary Drug Control
ZYM-DIEC02	Swine small intestine cell lines	This laboratory
O142: △STa	STa gene deleted in O142	This laboratory
O142 (yaiT: PRPL-Kil)	PRPL-Kil cassette inserted into the yaiT gene to construct the double selection platform: grows normally at 30°C but is killed at 43°C by the expression of Kil	This study

Table 2. Plasmids used and constructed in this study

Plasmid	Relevant properties	Reference or source
pDOC-K	Kanamycin cassette flanked with FRT sites, MCS and I-SceI sites	Lee *et al*. (2009)
pDOC-C	MCS flanked with I-SceI sites	Lee *et al*. (2009)
pACBSCE	I-SceI and λ-Red protease under control of arabinose promoter, p15A ori	Lee *et al*. (2009)
pEASY-Blunt-Simple	TA cloning vector	Beijing TransGen Biotech, China
pKil	PRPL-Kil cassette cloned into the MCS1 of pDOC-K at BamH I and Kpn I sites	This laboratory
pKil-donor	The left side (yaiT-L) and right side (yaiT-R) of the insertion site cloned into pKil	This study
pL-S	LTA1-STa_{13}-STb-LTA2-LTB-STa_{13}-STb cassette cloned into the MCS of pDOC-C at Sal I and Xho I	This study
pL-S-Donor	yaiT-L-arm and yaiT-R-arm cloned into pL-S by BamH I/Sal I, and Xho I/Spe I, respectively	This study

Table 3. PCR primers used for constructing the double selection platform

Primer	Sequence (5′–3′)	Description
yaiT1 (T1)	GGATCCTAACGGAAGCAAGTGGGTTGGTCAG	Anneals to 5′ end of yaiT-L gene, with *BamH* I site
yaiT2 (T2)	GTCGACATCAGCCCCCCACCCAGTAGATT	Anneals to 3′ end of yaiT-L gene, with *Sal* I site
yaiT3 (T3)	CTCGAGTACAACCTGTACGCCAATACTATCAC	Anneals to 5′ end of yaiT-R gene, with *Xho* I site
yaiT4 (T4)	ACTAGTGTTGCGTTGTCGATACGAACTTTG	Anneals to 3′ end of yaiT-R gene, with *Spe* I site

CP002732.1) with (GenBank accession no. V00612.1) and Strain LT_{192}-STb (GenBank accession no. AY028790.1) have been previously described (Liu *et al*., 2015a,2015b). The plasmids constructed and used are listed in Table 2. Plasmids pDOC-K, pDOC-C and pACBSCE were kindly provided by Prof. David J. Lee (School of Biosciences, University of Birmingham, UK).

Construction and characterization of the double selection platform

A double selection platform was established using gene doctoring method and λ-red method (Datsenko and Wanner, 2000; Lee *et al*., 2009). Restructuring strategy flow chart of *E. coli* O142: △STa double selection platform is displayed in the online Resource Fig. 1, panel A. Briefly, use primer T1 paired with T2 to clone the left homologous arms from *E. coli* O142:ΔSTa, use primer T3 paired with T4 to clone the right homologous arms from *E. coli* O142:ΔSTa (the primers T1, T2, T3 and T4 are listed in Table 3), construct pKil-donor plasmid, insert the left fragment and right fragment into the pKil plasmid. Transform pACBSCE plasmid and pKil-donor plasmid into *E. coli* O142:ΔSTa, subsequently, homologous recombination is induced by L-arabinose, then generated O142 (*yaiT*-Kil) double selection platform. Use PCR primers (T1 and T4) to verify that pKil-donor fragment was assembled on the platform, the platform screening capability validation by 30 and 43°C, as previously described (Liu *et al*., 2015a,2015b).

Construction and characterization of the recombinant E. coli

Merges chimeric gene produced the LTA1-STa13-STb-LTA2-LTB-STa13-STb by splicing overlap extension (SOE) PCR (Fig. 2, panel A). The first PCR, using LT_{192}-STa_{13} (constructed and preserved in our laboratory) as the template and primer P1 paired with P3, generated a fragment including the native LT gene promoter, full-length LT_{192} genes and the 3′ end of the STa_{13} gene (26 bp). The second PCR, using P2 and P4 primer and LT_{192}-STb (constructed and preserved in our laboratory) as the template, yield the fragment consisted of the 3′ end of the STa_{13} gene (13 bp), the STb, the 6 × His gene and the native LT gene terminator. In the third step, we used a splicing overlap extension (SOE) PCR, connected the DNA fragment from the first and second PCR step and generated the fusion gene LT_{192}-STa_{13}-STb cassette. In the fourth PCR, we used LT_{192}-STa_{13}-STb as the template, primer P5 with P6, and generated STa_{A13Q}-STb-6 × His gene and A1 gene (1500 bp), named STa-STb-6 × His. In the fifth PCR, we use the same template as in the fourth, primer P1 with P7, and generated A1 gene (1500 bp), named A1. In the sixth PCR by primer P1 paired with P8, we used STa-STb-6 × His and A1 as the template, and generated the fragment consisting of the LT_{192} A1 subunit and the STa_{A13Q}-STb-6 × His (1700 bp), named LTA1-STa-STb-6 × His. In the seventh PCR, we used LT-STa_{13}-STb as the template by primer P9 with P2, and

Table 4. PCR primers used for constructing the recombinant *E. coli*

Primer	Sequence (5′–3′)	Description
P1	GTCGACGGCGCTGATATCACGATTAGCCT	Anneals to the left side of native LT promoter, with *Sal* I sites, cloned into pDOC-C
P2	CTCGAGAAGCTTGCCCCCCAGCCTA	Anneals to the right side of native LT terminator, with *Xho* I sites, cloned into pDOC-C
P3	CGGGTACCGAGCTCG*ATAACATCCAG CACACTGAGGATTAC*	3′ end of STa_{13} (26 bp, no stop codon) + 5′ linker (15 bp)
P4	TGCTGGATGTTAT*CGAGCTCGGTACCCGGGGAT*	3′ end of STa_{13} (13 bp, no stop codon) + 5′ linker (20 bp)
P5	GGTTGTGGAAATTCATCAGGAACAATCACAAACA CATTTTACTGCTGTGAACTTTGTTG	3′ end of LT-A1 (30 bp) + 5′ STa13(29 bp)
P6	CAGATTCTGGGTCTCCTCATTACAAGTATCACCATG ATGATGATGATGGTG*GCATCCTT*	5′ end of LT-A2 (33 bp) + 6*his (13 bp) + 3′ end of STb (8 bp)
P7	TGTGATTGTTCCTGATGAATTTCCACAACC	3′ end of LT-A1 (30 bp)
P8	CAGATTCTGGGTCTCCTCATTACAAGTATCACC	5′ end of LT-A2 (33 bp)
P9	GGTGATACTTGTAATGAGGAGACCCAGAATCTG	5′ end of LT-A2 (33 bp)

generated a fragment including the LTA2 subunit, the LTB subunit and the STa_{13}-STb-6 × His chimeric gene, named A2-LTB-STa-STb-6 × His. Subsequently, the eighth SOE PCR connected the LTA1-STa-STb-6 × His and A2-LTB-STa-STb-6 × His fragment and produced fusion genes (LTA1-STa_{13}-STb-LTA2-LTB-STa_{13}-STb cassette). The primers are listed in Table 4.

The LTA1-STa_{13}-STb-LTA2-LTB-STa_{13}-STb cassette was inserted into the pDOC-C plasmid by restriction endonucleases, producing the pL-S plasmid, employing left side and right side PCR products ligated into pL-S plasmid and generating *yaiT*(pL-S-Donor) plasmid respectively. Then, for pACBSCE plasmid, pL-S-Donor plasmid co-transformation selection platforms, an analogous protocol was used for homologous recombination as mentioned above, to yield the recombinant *E. coli* O142(*yaiT*::LTA1-STa_{13}-STb-LTA2-LTB-STa_{13}-STb), named as *ER-T* (Fig. 2, panel B). Recombinant *E. coli* strains were identified by PCR, using primers L-arm paired with P2 respectively. The primers are listed in Table 3. The sequences of the respective fusion genes were confirmed by sequencing. Moreover, recombinant *E. coli* expressions of LTA1-STa_{13}-STb-LTA2-LTB-STa_{13}-STb fusion protein separated by 12% SDS-PAGE, examined with anti-His, were used as primary antibody and secondary antibody by (HRP)-conjugated goat anti-mouse IgG, as previously described (Liu *et al*., 2015a,2015b).

Feasibility analysis

Recombinant *E. coli* strains were further evaluated for feasibility as an oral vaccine by six different assays. First, the residual enterotoxicity in recombinant *E. coli* assay by suckling mouse and ZYM-DIEC02 cell. Briefly, 4-day-old suckling mice administered orally with Soybean trypsin inhibitor (2 mg ml^{-1}; Solarbio, Beijing, China), and 100 μl of culture supernatant was inoculated intragastrically. The mice were killed after 4 h, G/C (the weight ratio of gut to the remaining carcass) ratios of ≥ 0.090 were confirmed positive for toxicity of STa or STb as previously described (Frantz and Robertson, 1981). In the ZYM-DIEC02 cell assay, using 100 μl of culture supernatant incubation with ZYM-DIEC02 cells in 5% CO_2 at 37°C for 24 h, showed the change in the cells as previously described (Feng *et al*., 2013). Second, we analysed the survival characteristics of recombinant *E. coli* in the simulation environment of the gastrointestinal tract. Briefly, recombinant *E. coli* was cultured in lysogeny broth (LB) medium, supplemented with gastric acid (1.5, 2.5, 3.5 or 4.5 of pH), and intestinal juice and bile (0.05%, 0.1%, 0.2%, 0.3% and 0.4%). After shaking at 37°C for 8 h, bacteria survival rate was determined by the plate method of enumerated intermittently, as previously described (Liu *et al*., 2015a,2015b). Third, we measured the influence of oral administration on feed intake and body weight. Briefly, groups of 10 six-week-old female BALB/c mice (Liaoning Changsheng Biotechnology Co., Ltd., China), received doses of 10^9 CFU, 10^{10} CFU, 10^{11} CFU and 10^{12} CFU for 3 days, and were observed as previously described (You *et al*., 2011). Fourth, the growth curves of the recombinant *E. coli* were determined. Briefly, isolate single clone of *ER-T* from LB agar medium then inoculate into LB medium, at intervals of 2 h, take sample from LB medium, use the plate method to produce growth curves, as previously described (Yang *et al*., 2015). Growth curves of *E. coli* O142, O142: ΔSTa, *ER-T*. Fifth, analyse the structural stability of *ER-T*, continuous subcultures *ER-T* for 100 generations, then the 100 generations *ER-T* are plated on LB agar medium, randomly selected several colonies, and identified using PCR primers L-arm paired with P2, as described above (Nguyen *et al*., 2005).Sixth, recombinant *E. coli* was examined via electron microscope (EM). Briefly, recombinant *E. coli* was in static culture for 3 days, placed onto Formvar carbon-coated copper grids (200 mesh), then stained with 2% potassium-phosphotungstic acid (pH 6.8) and observed by EM as previously described (Torres *et al*., 2004). Seventh, recombinant *E. coli* intestinal colonization was evaluated. Briefly, six-week-old BALB/c mice were orally administered nalidixic acid-resistant (Nal) recombinant *E. coli ER-T* 10^9 CFU. Subsequently, faeces samples were collected at 7 day intervals and incubated on LB_{Nal} agar plates, the assessed by PCR as described above (Huang *et al*., 2013).

Oral immunization of mice

A total of 80 six-week-old BALB/c mice were split into eight groups (A, B, C, D, G1 female BALB/c mice, G2, G3 and G4 male BALB/c mice), with 10 mice per group. *ER-T* was grown in improved MINCA medium for 24 h at 37°C and harvested by centrifugation. Resuspension of *ER-T* centrifugal sedimentation in milk arrived at concentration of approximately 1×10^{10} CFU ml^{-1}. Mice in group A and B were orally administered with 10^9 CFUs of *ER-T* with a 1.5-inch, 20 gauge ball-tip needle. Mice in group C and D received 10^9 CFUs of O142: △STa. G1, G2, G3 and G4 as control groups received doses of 0.1 ml of milk. All groups were inoculated on a single day (Time 0); then, identical booster doses were administered on 14 and 28 days later. At 21 days, groups B coupled with G2, D coupled with G4, G1 coupled with G3, as previously described (Liu *et al*., 2015a,2015b).

ELISA analysis for antibody levels

IgG and IgA antibodies were measured by enzyme-linked immunosorbent assay (ELISA). On days 0, 7, 14,

21, 28, 35 and 42, mice were killed and samples harvested as previously described (Liu *et al*., 2014). Briefly, the plates were coated with purified lymphotoxin alpha (LTA) recombinant protein, lymphotoxin beta (LTB) recombinant protein, F41 fimbriae, 4 × STa recombinant protein and MBP-STb recombinant protein at 50, 50, 200, 100 and 200 ng per well, respectively, then treated samples with serial dilution with PBS as the primary antibodies. HRP-conjugated goat anti-mouse IgA (Sigma-Aldrich, St. Louis, MO, USA) or IgG (ZSGB-BIO Co., Beijing, China) were used for bound antibodies detection as previously described (Jiang *et al*., 2014).

Lymphocyte proliferation assay and Cytokine assay

Forty-two days after the first immunization, mice mesenteric lymphocytes and splenic lymphocytes were collected and measured by MTT assay, as previously described (Liu *et al*., 2015b). Briefly, the lymphocytes were incubated in 96-well plates at 5×10^5 lymphocytes cells/well for 100 μl then stimulated *in vitro* with LT (10 μg ml^{-1}), STa (10 μg ml^{-1}), STb (10 μg ml^{-1}), LT_{192}-STa_{13} (10 μg ml^{-1}) proteins and LT_{192}-STb (10 μg ml^{-1}) proteins for 100 μl respectively. Meanwhile, concanavalin A (con A) (10 μg ml^{-1}) was used as a positive control and a black controls were without proteins. The plates were kept at 37°C in 5% CO_2 for 72 h and then pulsed with MTT (10 mg ml^{-1}) per well for 4 h. The absorbance was measured with a spectrophotometer at 570 nm. The stimulation indices (SI) were calculated by the following formula: $SI_{proliferation} = OD_{sample}/OD_{normal}$. Th1 cells produce IFN-γ, and Th2 cells produce IL-4. We used mice cytokine ELISA-kits (IFN-γ and IL-4 kits; JRDUN, Shanghai, China) following the manufacturer's instructions to investigate the role of IFN-γ (Th1) and IL-4 (Th2) cytokines in the spleen of mice.

In vitro *neutralization assays*

We used ZYM-DIEC02 cell to assay the neutralizing ability of mice inoculated intragastrically with *ER-T*. Serum, intestinal mucus, splenocyte lysate and mesenteric lymphocyte lysate samples were collected from mice. Filter-sterilized supernatant of enterotoxins LT, STa, STb, then serial dilutions respectively. Further, incubated enterotoxins with equal volume of mice samples at 37°C for 2 h, after that toxin-antibody mixture was added to the ZYM-DIEC02 cells culture plate 0.1 ml per well. After 24 h, crystal violet staining was carried out and normal cells were counted, as previously described (Guan *et al*., 2015).

In vivo *neutralization test*

To assess the neutralization activity of antibodies, we used suckling mice (suckling mice from G1 coupled with G3, aged 4 days). Serial dilutions of enterotoxins STa were mixed with samples (samples from immunization mice), as above. Suckling mice were administered orally with STa toxin-antibody mixture 0.1 ml per mice. The *in vivo* neutralization of STb: fist orally soybean trypsin inhibitor, then received equal volume of STb toxin-antibody mixture. The negative control used the samples from the control group and received equal volume of STa, STb toxin-antibody mixture respectively. Four hours after oral inoculation, the mice were killed and samples harested, as previously described (You *et al*., 2011).

Challenge for maternal antibody in suckling mice

The protective efficacy evaluation of maternal antibody uses suckling mice with milk from pregnant mice. Briefly, four 4-day-old mice per group received serial dilutions enterotoxins (STa or STb) 100 μl. After 4 h, the animals were killed and the G/C ratio was calculated as previously described (Norton *et al*., 2012).

Statistical analysis

The aim of this study was to estimate the recombinant *E. coli ER-T*, statistical analysis (ANOVA) was performed by SPSS version 19.0 (SPSS Statistics, Chicago, IL, USA) and Graphpad Prism 5.0 (Graphpad software, San Diego, CA, USA), and are represented by asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$), P values of < 0.05 were considered to be statistically significant.

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Weikun Guan conceived the experiment(s), Ni Feng conducted the experiment(s) and Weikun Guan analysed the results. All authors reviewed the manuscript.

Conflict of interest

None declared.

Ethics statement

The present study did not involve endangered or protected species. The animal study complied with the Animal Welfare Act by following the NIH guidelines (NIH Pub. No. 85-23, revised 1996), and the protocols were approved and supervised by the Animal Care and Use Committee of Yichun University.

References

Ascon, M.A., Hone, D.M., Walters, N., and Pascual, D.W. (1998) Oral immunization with a *Salmonella typhimurium*

vaccine vector expressing recombinant enterotoxigenic *Escherichia coli* K99 fimbriae elicits elevated antibody titers for protective immunity. *Infect Immun* **66:** 5470–5476.

Balakirev, E.S., and Ayala, F.J. (2003) Pseudogenes: are they "junk" or functional DNA? *Annu Rev Genet* **37:** 123–151.

Bischoff, K.M., White, D.G., McDermott, P.F., Zhao, S., Gaines, S., Maurer, J.J., and Nisbet, D.J. (2002) Characterization of chloramphenicol resistance in beta-hemolytic *Escherichia coli* associated with diarrhea in neonatal swine. *J Clin Microbiol* **40:** 389–394.

Blanco, M., Blanco, J.E., Gonzalez, E.A., Mora, A., Jansen, W., Gomes, T.A., *et al*. (1997) Genes coding for enterotoxins and verotoxins in porcine *Escherichia coli* strains belonging to different O:K: H serotypes: relationship with toxic phenotypes. *J Clin Microbiol* **35:** 2958–2963.

Charles, I., and Dougan, G. (1990) Gene expression and the development of live enteric vaccines. *Trends Biotechnol* **8:** 117–121.

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97:** 6640–6645.

Dubreuil, J.D., Letellier, A., and Harel, J. (1996) A recombinant *Escherichia coli* heat-stable enterotoxin b (STb) fusion protein eliciting neutralizing antibodies. *FEMS Immunol Med Microbiol* **13:** 317–323.

Echols, N., Harrison, P., Balasubramanian, S., Luscombe, N.M., Bertone, P., Zhang, Z., and Gerstein, M. (2002) Comprehensive analysis of amino acid and nucleotide composition in eukaryotic genomes, comparing genes and pseudogenes. *Nucleic Acids Res* **30:** 2515–2523.

Feng, Y., Liu, W., and Shi, D. (2013) Effectiveness of egg yolk antibody against shiga toxin II variant toxicity in vitro and in vivo. *Curr Microbiol* **67:** 448–453.

Field, M., Graf, L.H. Jr, Laird, W.J., and Smith, P.L. (1978) Heat-stable enterotoxin of *Escherichia coli*: in vitro effects on guanylate cyclase activity, cyclic GMP concentration, and ion transport in small intestine. *Proc Natl Acad Sci USA* **75:** 2800–2804.

Frantz, J.C., and Robertson, D.C. (1981) Immunological properties of *Escherichia coli* heat-stable enterotoxins: development of a radioimmunoassay specific for heat-stable enterotoxins with suckling mouse activity. *Infect Immun* **33:** 193–198.

Guan, W., Liu, W., Bao, J., Li, J., Yuan, C., Tang, J., and Shi, D. (2015) Analysis and application of a neutralizing linear epitope on liable toxin B of enterotoxin *Escherichia coli*. *Appl Microbiol Biotechnol* **99:** 5985–5996.

Guinee, P.A., and Jansen, W.H. (1979) Detection of enterotoxigenicity and attachment factors in *Escherichia coli* strains of human, porcine and bovine origin; a comparative study, *Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. Erste Abteilung Originale. Reihe A: Medizinische Mikrobiologie und Parasitologie* **243:** 245–257.

Harel, J., Lapointe, H., Fallara, A., Lortie, L.A., Bigras-Poulin, M., Lariviere, S., and Fairbrother, J.M. (1991) Detection of genes for fimbrial antigens and enterotoxins associated with *Escherichia coli* serogroups isolated from pigs with diarrhea. *J Clin Microbiol* **29:** 745–752.

Harvey, R.B., Andrews, K., Droleskey, R.E., Kansagra, K.V., Stoll, B., Burrin, D.G., *et al*. (2006) Qualitative and quantitative comparison of gut bacterial colonization in enterally and parenterally fed neonatal pigs. *Curr Issues Intest Microbiol* **7:** 61–64.

Huang, X.Z., Zhu, L.B., Li, Z.R., and Lin, J. (2013) Bacterial colonization and intestinal mucosal barrier development. *World J Clin Pediat* **2:** 46–53.

Jertborn, M., Ahren, C., Holmgren, J., and Svennerholm, A.M. (1998) Safety and immunogenicity of an oral inactivated enterotoxigenic *Escherichia coli* vaccine. *Vaccine* **16:** 255–260.

Jiang, X., Yu, M., Qiao, X., Liu, M., Tang, L., Jiang, Y., *et al*. (2014) Up-regulation of MDP and tuftsin gene expression in Th1 and Th17 cells as an adjuvant for an oral *Lactobacillus casei* vaccine against anti-transmissible gastroenteritis virus. *Appl Microbiol Biotechnol* **98:** 8301–8312.

Kotton, C.N., and Hohmann, E.L. (2004) Enteric pathogens as vaccine vectors for foreign antigen delivery. *Infect Immun* **72:** 5535–5547.

Lasaro, M.O., Luiz, W.B., Sbrogio-Almeida, M.E., and Ferreira, L.C. (2005) Prime-boost vaccine regimen confers protective immunity to human-derived enterotoxigenic *Escherichia coli*. *Vaccine* **23:** 2430–2438.

Lee, D.J., Bingle, L.E., Heurlier, K., Pallen, M.J., Penn, C.W., Busby, S.J., and Hobman, J.L. (2009) Gene doctoring: a method for recombineering in laboratory and pathogenic *Escherichia coli* strains. *BMC Microbiol* **9:** 252.

Liu, M., Ruan, X., Zhang, C., Lawson, S.R., Knudsen, D.E., Nataro, J.P., *et al*. (2011) Heat-labile- and heat-stable-toxoid fusions (LTR(1)(9)(2)G-STaP(1)(3)F) of human enterotoxigenic *Escherichia coli* elicit neutralizing antitoxin antibodies. *Infect Immun* **79:** 4002–4009.

Liu, W., Yuan, C., Meng, X., Du, Y., Gao, R., Tang, J., and Shi, D. (2014) Frequency of virulence factors in *Escherichia coli* isolated from suckling pigs with diarrhoea in China. *Vet J* **199:** 286–289.

Liu, W., Li, J., Bao, J., Li, X., Guan, W., Yuan, C., *et al*. (2015a) Simultaneous oral immunization of mice with live attenuated *Escherichia coli* expressing LT192-STa 13 and LT 192-STb fusion immunogen, respectively, for polyvalent vaccine candidate. *Appl Microbiol Biotechnol* **99:** 3981–3992.

Liu, W., Yuan, C., Bao, J., Guan, W., Zhao, Z., Li, X., *et al*. (2015b) Generation of an attenuated strain oral vaccine candidate using a novel double selection platform in *Escherichia coli*. *Appl Microbiol Biotechnol* **99:** 855–867.

Millar, D.G., Hirst, T.R., and Snider, D.P. (2001) *Escherichia coli* heat-labile enterotoxin B subunit is a more potent mucosal adjuvant than its vlosely related homologue, the B subunit of cholera toxin. *Infect Immun* **69:** 3476–3482.

Myers, K.J., Sprent, J., Dougherty, J.P., and Ron, Y. (1992) Synergy between encephalitogenic T cells and myelin basic protein-specific antibodies in the induction of experimental autoimmune encephalomyelitis. *J Neuroimmunol* **41:** 1–8.

Nakazawa, M., Sugimoto, C., Isayama, Y., and Kashiwazaki, M. (1987) Virulence factors in *Escherichia coli* isolated from piglets with neonatal and post-weaning diarrhea in Japan. *Vet Microbiol* **13:** 291–300.

Nataro, J.P., and Kaper, J.B. (1998) Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* **11:** 142–201.

Nguyen, H.D., Nguyen, Q.A., Ferreira, R.C., Ferreira, L.C., Tran, L.T., and Schumann, W. (2005) Construction of plasmid-based expression vectors for *Bacillus subtilis* exhibiting full structural stability. *Plasmid* **54:** 241–248.

Nijsten, R., London, N., van den Bogaard, A., and Stobberingh, E. (1996) Antibiotic resistance among *Escherichia coli* isolated from faecal samples of pig farmers and pigs. *J Antimicrob Chemother* **37:** 1131–1140.

Norton, E.B., Lawson, L.B., Mahdi, Z., Freytag, L.C., and Clements, J.D. (2012) The A subunit of *Escherichia coli* heat-labile enterotoxin functions as a mucosal adjuvant and promotes IgG2a, IgA, and Th17 responses to vaccine antigens. *Infect Immun* **80:** 2426–2435.

Osek, J., and Truszczynski, M. (1992) Occurrence of fimbriae and enterotoxins in *Escherichia coli* strains isolated from piglets in Poland. *Comp Immunol Microbiol Infect Dis* **15:** 285–292.

Pacheco, A.R., Curtis, M.M., Ritchie, J.M., Munera, D., Waldor, M.K., Moreira, C.G., and Sperandio, V. (2012) Fucose sensing regulates bacterial intestinal colonization. *Nature* **492:** 113–117.

Ruan, X., and Zhang, W. (2013) Oral immunization of a live attenuated Escherichia coli strain expressing a holotoxin-structured adhesin-toxoid fusion (1FaeG-FedF-LTA (2):5LTB) protected young pigs against enterotoxigenic *E. coli* (ETEC) infection. *Vaccine* **31:** 1458–1463.

Ruan, X., Liu, M., Casey, T.A., and Zhang, W. (2011) A tripartite fusion, FaeG-FedF-LT(192)A2:B, of enterotoxigenic *Escherichia coli* (ETEC) elicits antibodies that neutralize cholera toxin, inhibit adherence of K88 (F4) and F18 fimbriae, and protect pigs against K88ac/heat-labile toxin infection. *Clin Vaccine Immunol* **18:** 1593–1599.

Ruan, X., Robertson, D.C., Nataro, J.P., Clements, J.D., Zhang, W. and Group, S.T.T.V.C. (2014) Characterization of heat-stable (STa) toxoids of enterotoxigenic *Escherichia coli* fused to double mutant heat-labile toxin peptide in inducing neutralizing Anti-STa antibodies. *Infect Immun* **82:** 1823–1832.

Salimian, J., Salmanian, A., Khalesi, R., Mohseni, M., and Moazzeni, S. (2010) Antibody against recombinant heat labile enterotoxin B subunit (rLTB) could block LT binding to ganglioside M1 receptor. *Iran J Microbiol* **2:** 120–127.

Sanchez, J., and Holmgren, J. (2005) Virulence factors, pathogenesis and vaccine protection in cholera and ETEC diarrhea. *Curr Opin Immunol* **17:** 388–398.

Sears, C.L., and Kaper, J.B. (1996) Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. *Microbiol Rev* **60:** 167–215.

Smith, M.G., Jordan, D., Chapman, T.A., Chin, J.J., Barton, M.D., Do, T.N., *et al*. (2010) Antimicrobial resistance and virulence gene profiles in multi-drug resistant enterotoxigenic *Escherichia coli* isolated from pigs with post-weaning diarrhoea. *Vet Microbiol* **145:** 299–307.

Soderlind, O., Thafvelin, B., and Mollby, R. (1988) Virulence factors in *Escherichia coli* strains isolated from Swedish piglets with diarrhea. *J Clin Microbiol* **26:** 879–884.

Spangler, B.D. (1992) Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiol Rev* **56:** 622–647.

Tang, L., and Li, Y. (2009) Oral immunization of mice with recombinant *Lactococcus lactis* expressing porcine transmissible gastroenteritis virus spike glycoprotein. *Virus Genes* **39:** 238–245.

Torres, A.G., Kanack, K.J., Tutt, C.B., Popov, V., and Kaper, J.B. (2004) Characterization of the second long polar (LP) fimbriae of Escherichia coli O157:H7 and distribution of LP fimbriae in other pathogenic *E. coli* strains. *FEMS Microbiol Lett* **238:** 333–344.

Trevisi, P., Colombo, M., Priori, D., Fontanesi, L., Galimberti, G., Calo, G., *et al*. (2015) Comparison of three patterns of feed supplementation with live Saccharomyces cerevisiae yeast on postweaning diarrhea, health status, and blood metabolic profile of susceptible weaning pigs orally challenged with *Escherichia coli* F4ac. *J Anim Sci* **93:** 2225–2233.

Wang, X.M., Jiang, H.X., Liao, X.P., Liu, J.H., Zhang, W.J., Zhang, H., *et al*. (2010) Antimicrobial resistance, virulence genes, and phylogenetic background in *Escherichia coli* isolates from diseased pigs. *FEMS Microbiol Lett* **306:** 15–21.

Yang, J.K., Xiong, W., Xu, L., Li, J., and Zhao, X.J. (2015) Constitutive expression of Campylobacter jejuni truncated hemoglobin CtrHb improves the growth of *Escherichia coli* cell under aerobic and anaerobic conditions. *Enzyme Microb Technol* **75–76:** 64–70.

You, J., Xu, Y., He, M., McAllister, T.A., Thacker, P.A., Li, X., *et al*. (2011) Protection of mice against enterotoxigenic *E. coli* by immunization with a polyvalent enterotoxin comprising a combination of LTB, STa, and STb. *Appl Microbiol Biotechnol* **89:** 1885–1893.

Zhang, W., and Sack, D.A. (2015) Current progress in developing subunit vaccines against enterotoxigenic *Escherichia coli*-associated diarrhea. *Clin Vaccine Immunol* **22:** 983–991.

Zhang, W., Berberov, E.M., Freeling, J., He, D., Moxley, R.A., and Francis, D.H. (2006) Significance of heat-stable and heat-labile enterotoxins in porcine colibacillosis in an additive model for pathogenicity studies. *Infect Immun* **74:** 3107–3114.

Zhang, W., Zhao, M., Ruesch, L., Omot, A., and Francis, D. (2007) Prevalence of virulence genes in *Escherichia coli* strains recently isolated from young pigs with diarrhea in the US. *Vet Microbiol* **123:** 145–152.

Zhang, W., Zhang, C., Francis, D.H., Fang, Y., Knudsen, D., Nataro, J.P., and Robertson, D.C. (2010) Genetic fusions of heat-labile (LT) and heat-stable (ST) toxoids of porcine enterotoxigenic *Escherichia coli* elicit neutralizing anti-LT and anti-STa antibodies. *Infect Immun* **78:** 316–325.

Zhang, C., Knudsen, D.E., Liu, M., Robertson, D.C., Zhang, W. and Group, S.T.T.V.C. (2013) Toxicity and immunogenicity of enterotoxigenic *Escherichia coli* heat-labile and heat-stable toxoid fusion 3xSTa(A14Q)-LT(S63K/R192G/L211A) in a murine model. *PLoS One* **8:** e77386.

