Diarrhea induced by F4 enterotoxigenic *Escherichia coli* (ETEC) in neonatal and weaned piglets results in severe economic losses due to the high mortality caused by infections [24]. F4 fimbriae are a primary virulence factor of ETEC and enable the bacteria to attach to F4-specific receptors (F4R) on the brush border of intestinal enterocytes [18]. Consequently, ETEC produces enterotoxins and causes piglet diarrhea. The F4 fimbriae of ETEC are composed of a repeating major subunit, FaeG and some minor subunits [23]. Since FaeG has both adhesive and antigenic properties, the development of anti-adhesive vaccines to prevent ETEC infections has long been pursued by researchers [16, 17, 22].

An effective vaccine is crucial for neonatal and weaned piglets to evoke protective immunity against ETEC. Oral administration of antigens can induce intestinal mucosal immune responses to produce an abundance of secretory IgA (sIgA) antibodies that play an important role [7, 19]. Therefore, oral immunization is thought to an effective measure against ETEC infections during the postweaning period [22]. However, parenteral vaccination could be more appropriate for suckling piglets, because of mucosal immune system difficulties stimulated during the suckling period by the oral route [21]. Therefore, different immune strategies should be taken according to the stage of piglet weaning.

*Lactococcus lactis* is a Gram-positive lactic acid bacterium that is used to produce fermented foods and generally recognized as safe (GRAS). *L. lactis* is an attractive candidate for the production and delivery of heterologous proteins to the mucosal immune system [3]. Nonpathogenic and noninvasive *L. lactis* may be a good alternative to attenuated pathogens as a vaccine vector [4]. We have previously shown that FaeG could be produced intracellularly in *L. lactis* [11]. Then, we further constructed secretory vector (pNZ8112-faeG) for expressing FaeG extracellularly. The aim of the present study was to detect the immunogenicity of rFaeG and investigate the levels of immune responses in BALB/c mice immunized with *L. lactis* NZ9000 (pNZ8112-faeG) via the subcutaneous and oral routes. Moreover, immune doses of recombinant *L. lactis* were also evaluated through oral immunization.

**MATERIALS AND METHODS**

Preparation of recombinant *L. lactis* for immunization: *L. lactis* NZ9000 and plasmid pNZ8112 with the usp45 signal sequence were obtained from NIZO food research [25]. The construction of recombinant *L. lactis* that harbors the expression vector pNZ8112-FaeG has been previously described [11]. Recombinant FaeG could be detected in the cytoplasm of *L. lactis*, but not in the supernatant of the culture.

*L. lactis* NZ9000 (pNZ8112-FaeG) was grown at 30°C in M17 medium (Difco, Becton Dickinson, Franklin Lakes, NJ, U.S.A.) supplemented with 0.5% (w/v) glucose without agitation. Overnight cultures of recombinant *L. lactis* were used to inoculate fresh medium at a dilution of 1:25. The strains were grown until OD$_{600}$=0.4 and induced with 10 ng/ml of nisin (Sigma-Aldrich, St. Louis, MO, U.S.A.) for 3
Table 1. The groups and dosages received by mice via the oral or subcutaneous route

<table>
<thead>
<tr>
<th>Administration route</th>
<th>Group</th>
<th>Immunogen</th>
<th>Dosage and adjuvant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraocular lavage</td>
<td>Group 1 (blank control)</td>
<td>PBS</td>
<td>0.1 ml PBS</td>
</tr>
<tr>
<td></td>
<td>Group 2 (vector control)</td>
<td>L. lactis NZ9000 (pNZ8112)</td>
<td>2.8 × 10^10 CFU</td>
</tr>
<tr>
<td></td>
<td>Group 3 (low dose)</td>
<td>L. lactis NZ9000 (pNZ8112-faeG)</td>
<td>2.8 × 10^9 CFU</td>
</tr>
<tr>
<td></td>
<td>Group 4 (high dose)</td>
<td>L. lactis NZ9000 (pNZ8112-faeG)</td>
<td>2.8 × 10^11 CFU</td>
</tr>
<tr>
<td></td>
<td>Group 5 (low dose + CTB)</td>
<td>L. lactis NZ9000 (pNZ8112-faeG)</td>
<td>2.8 × 10^9 CFU + 10 µg CTB</td>
</tr>
<tr>
<td></td>
<td>Group 6 (high dose + CTB)</td>
<td>L. lactis NZ9000 (pNZ8112-faeG)</td>
<td>2.8 × 10^11 CFU + 10 µg CTB</td>
</tr>
<tr>
<td>Subcutaneous injection</td>
<td>Group 7 (blank control)</td>
<td>PBS</td>
<td>0.1 ml PBS</td>
</tr>
<tr>
<td></td>
<td>Group 8 (vector control)</td>
<td>L. lactis NZ9000 (pNZ8112)</td>
<td>2.8 × 10^8 CFU + FCA or IFA</td>
</tr>
<tr>
<td></td>
<td>Group 9 (recombinant strain)</td>
<td>L. lactis NZ9000 (pNZ8112-faeG)</td>
<td>2.8 × 10^8 CFU + FCA or IFA</td>
</tr>
</tbody>
</table>

hr. Cells were centrifuged at 3, 000 × g for 20 min at 4°C, washed three times with 0.01 M sterile phosphate-buffered saline (PBS) solution and suspended in PBS at a final concentration of 2.8 × 10^10 CFU per 0.2 ml or 2.8 × 10^11 CFU per 0.2 ml for oral immunization and 2.8 × 10^9 CFU per 0.1 ml for subcutaneous immunization.

**Immunization of mice:** Female six-week-old BALB/c mice were purchased from the Department of Experimental Animals of Fudan University (Shanghai, China). All experiments on mice were approved by the Institutional Animal Care Committee. The mice were separated into groups of eight and immunized with recombinant L. lactis either orally or subcutaneously. The immune doses received by each group of eight mice are presented in the Table 1. The mice were administered 2.8 × 10^10 CFU (low dose) or 2.8 × 10^11 CFU (high dose) recombinant L. lactis either orally on days 0, 1, 2, 28, 29, 30 and 35 or 2.8 × 10^9 recombinant L. lactis subcutaneously on days 0, 14 and 28. The mice in the control groups were administered L. lactis NZ9000 or PBS. Cholera toxin B (CTB) and complete or incomplete Freund’s adjuvant (FCA, FIA) were used as oral and injectable adjuvants for improving immune response.

**Sample collection:** To detect F4-specific antibody responses, blood samples and feces of the mice were collected on days 0, 14, 28, 42 and 49. Sera of all mice were obtained and stored at −20°C until use. Fecal pellets from the oral groups were extracted to measure sIgA, and 100 mg of samples were suspended with 1 ml PBS containing 1% BSA and 1 mM phenylmethylsulfonyl fluoride. After incubation for 16 hr at 4°C, fecal samples were centrifuged at 16,000 × g for 15 min, and the supernatants were stored at −20°C until further analyses [15].

**ELISA for F4-specific serum IgG and fecal sIgA:** F4-specific serum IgG and fecal sIgA were determined using an indirect ELISA. The wells of 96-well microtiter plates were coated overnight with purified F4 fimbrin at a concentration of 0.01 mg/ml at 4°C [10]. Sera were added in a twofold dilution series starting at 1/20 dilution or fecal extracts at 1/10 dilution. Goat anti-mouse IgG antibodies or rabbit anti-mouse IgA antibodies conjugated to horseradish peroxidase (SouthernBiotech, Birmingham, AL, U.S.A.) were added respectively. 3,3',5,5'-Tetramethylbenzidine (Amresco, Solon, OH, U.S.A.) was used as the substrate. The cut-off value was calculated as the mean OD_{450} value of all serum (dilution 1/20) or fecal extracts (dilution 1/10) at day 0 plus three times the standard deviation. End-point titers were calculated as the inverse of the highest dilution with an OD_{450} higher than the cut-off value.

**ELISPOT assay:** At 14 days post final immunization, the mesenteric lymph nodes (MLN), spleen (SP) and Peyer’s patches (PP) of mice (n=3) from each group were removed and disrupted by sterile gauze as previously described [12, 14]. The results are expressed as the number of antigen-specific ASCs per 10^7 cells.

**Statistical analysis:** Statistical analyses (using SPSS 11.5) for specific antibody titers were performed using a general linear model (repeated measures analysis of variance). Comparisons of multiple groups were carried out by a one-way ANOVA and the Duncan nonparametric pairwise comparison test. A significance level of P<0.05 was selected.

**RESULTS**

**F4-specific serum IgG antibody responses via the oral and subcutaneous routes:** Four groups (Groups 3, 4, 5 and 6) of mice were orally administered recombinant L. lactis, and weak F4-specific IgG responses were initially induced at 14 days post primary immunization (dppi) and then gradually increased (Fig. 1). They peaked (mean log2 titers of 8.16, 9.48, 9.65 and 9.48, respectively) one week after the last immunization (42 dppi). No increase in specific IgG responses was observed at 49 dppi. In fact, Group 4 showed a decline. The low-dose group showed significantly higher F4-specific serum IgG titers than the control groups at 28, 35, 42 and 47 dppi (P<0.05).

Among the four orally administered recombinant L. lactis groups, the low-dose group obtained the lowest F4-specific serum IgG titers, except at 35 dppi, while the specific IgG titer in the high-dose group was significantly higher than that in the low-dose group at 42 dppi (P<0.05). Coadministering a low dose + CTB resulted in higher IgG titers than those in the low-dose group, except at 35 dppi, and a significant increase at 42 dppi (P<0.05). In addition, coadministering a high dose + CTB resulted in higher IgG titers at 35 and 49 dppi than those in the high-dose group. No significant differences, however, were observed between the groups. The specific IgG titer in the high-dose + CTB group was significantly higher than that in the low-dose + CTB group.
IMMUNIZATION OF MICE WITH *L. LACTIS* EXPRESSING FAEG

For mice subcutaneously immunized (Fig. 2), F4-specific serum responses were observed at 14 dppi, and the specific IgG titers were significantly higher than those in the control groups at 28, 35, 42 and 49 dppi (*P* < 0.05). Furthermore, the IgG titers in the immune group reached a mean log₂ titer of 11.49 at 49 dppi.

FaeG-specific fecal sIgA antibody responses via the oral route: Weak sIgA antibody responses occurred in the low-dose, low-dose + CTB, high-dose and high-dose + CTB groups (Groups 3, 4, 5 and 6) at 28 dppi or at 35 dppi (Fig. 1). The four groups then showed a gradual increase in F4-specific sIgA titers, reaching mean log₂ titers of 4.65, 4.82, 4.99 and 4.99 respectively at 49 dppi. The sIgA titer in the low-dose group was significantly higher than that in the control groups at 49 dppi (*P* < 0.05). The high-dose group showed higher sIgA titers than the low-dose group at 35, 42 and 49 dppi.

After the mice were co-administered either the low- or high-dose recombinant *L. lactis* with the same dose of CTB, the specific sIgA titers in the high-dose + CTB group were still higher than those in the low-dose + CTB group, reaching statistically significant levels at 42 dppi (*P* < 0.05). In addition, the high-dose + CTB group showed improved immune responses and higher sIgA titers as compared with the high-dose group from 28 dppi to 42 dppi. Although the low-dose + CTB group showed increased sIgA titers at 28 and 49 dppi as compared with the low-dose groups, no significant differences were found among the groups.

DISCUSSION

F4 fimbriae play an important role in piglet diarrhea from ETEC infections, and the major F4 fimbrial subunit, FaeG, is considered an alternative to the purified F4 fimbriae.
against diarrhea in piglets [1]. Oral immunization of weaned piglets with rFaeG has been reported to induce a protective mucosal immune response against ETEC infections [22]. In this study, subcutaneously immunizing mice with L. lactis NZ9000 (pNZ8112-faeG) together with FCA or IFA could induce a significant F4-specific IgG response and a higher number of specific IgG ASCs in the spleen. This indicates that rFaeG expressed in L. lactis possessed good immunogenicity and could activate a systemic immune response via subcutaneous vaccination. It has been reported that the immune efficacy of rFaeG expressed in bacteria is related to a higher F4-specific antibody titer than that in transgenic plants [9]. The FaeG produced in transgenic barley grains with the same adjuvant induced F4-specific IgG (at 56 dppi; log₂ titer 8.0) [8], while a stronger immune efficacy of rFaeG expressed in L. lactis was found in this study (at 49 dppi; log₂ titer 11.49). However, FaeG produced in transgenic tobacco showed a strong F4-specific IgG response that peaked at 10^4 (log₂ titer 13.29) [6]. These results indicated that the different immune efficacy of rFaeG may lie in expression and delivery systems related to the conformation and immunogenicity of rFaeG. Therefore, it is important to select an effective delivery system and further improve the immunogenicity of the recombinant protein.

Oral immunization of mice with recombinant L. lactis NZ9000 (pNZ8112-faeG) was sufficient to induce an F4-specific sIgA response and significantly increase specific serum IgG and ASCs in the SP, MLN and PP. The F4 fimbrial adhesion of ETEC was expressed in E. coli Nissle 1917 on the bacterial surface, and oral application of recombinant E. coli resulted in significant IgG serum titers but not IgA titers against K88 in feces, which was considered to induced mucosal tolerance in mice [13]. The results of the present study clearly prove that L. lactis is a potential bacterial vector, and the appropriate stimulation of inductive tissues by recombinant L. lactis induced F4-specific mucosal and systemic responses in mice following oral immunization without oral tolerance. On the one hand, the high-dose group showed a slightly higher sIgA and significantly enhanced IgG titer at 42 dppi as compared with the low-dose group and then showed a decline in IgG titer at 49 dppi. These results indicate that the dose of recombinant L. lactis is proportionally related to the level of the oral immune responses and that a high dose leads to high antibodies titers, which might perhaps decline more rapidly than those in the low-dose and high-dose + adjuvant CTB groups after reaching the maximum at 42 dppi. But, the optimal immune dose via oral immunization requires a further study for improving specific mucosal immune response [20].

Fig. 2. F4-specific serum IgG titers (mean log₂ titers + SEM) of mice via the subcutaneous route at 0, 14, 28, 35, 42 and 49 dppi. Group 7 (blank control, ◊), Group 8 (vector control, □) and Group 9 (recombinant strain, ○). There are significant differences (P<0.05) between the recombinant strain group and control groups ◊, □ (d). Black arrow: Subcutaneous immunization.
In order to enhance the immune response, mice were immunized with different doses of recombinant \textit{L. lactis} + adjuvant CTB by oral coadministration. The results indicate that oral coadministration of recombinant \textit{L. lactis} with CTB enhanced F4-specific immune responses, but only high doses + CTB showed a significantly higher sIgA at 42 dpdi and IgG at 35 dpdi. It was reported that CTB used as a mucosal adjuvant could increase antigen-specific sIgA and systemic IgG antibody responses by the mucosal route [2]. The present study showed that CTB simply added as a mucosal adjuvant needs a high dose of antigen, but the role of CTB was not very effective when admixed with recombinant \textit{L. lactis}. Co-expression of CTB with antigens could be a better means of enhancing mucosal immunization responses via the oral route [5].

In the present study, subcutaneous vaccination in mice with \textit{L. lactis} NZ9000 (pNZ8112-faeG) induced an F4-specific systemic immune response, and oral administration showed an F4-specific mucosal and systemic immune response, even though the specific immune response was weak. The results suggest promising ways of delivering rFaeG expressed in \textit{L. lactis} to the immune sites to induce F4-specific immune responses via oral or parenteral vaccination.

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