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ORIGINAL ARTICLE

Oral immunisation with Taishan *Pinus massoniana* pollen polysaccharide adjuvant with recombinant *Lactococcus lactis*-expressing *Proteus mirabilis* ompA confers optimal protection in mice

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KEYWORDS
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Abstract
Background: *Proteus mirabilis* poses a critical burden on the breeding industry, but no efficient vaccine is available for animals.
Method: A recombinant *Lactococcus lactis* expressing the ompA of *P. mirabilis* was used to develop a vaccine. The mucosal and systemic immune responses of the recombinant vaccine were evaluated in mice after oral immunisation. The inhibition on *P. mirabilis* colonisation of vaccines was also determined. Moreover, Taishan *Pinus massoniana* pollen polysaccharides (TPPPS) were used as adjuvants to examine the immunomodulatory effects.
Results: The pure recombinant *L. lactis* vaccine significantly induced the production of specific IgA and IgG, IL-2, IL-4, IFN-γ, and T lymphocyte proliferation, and the immunised mice exhibited significant resistance to *P. mirabilis* colonisation. Notably, the TPPPS adjuvant vaccines induced higher levels of immune responses than the pure *L. lactis*.
Conclusions: The *L. lactis* as a vaccine vehicle combined with TPPPS adjuvant provides a feasible method for preventing *P. mirabilis* infection.
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Introduction

Proteus mirabilis is a well-known zoonotic pathogen that widely exists in nature[1]; this pathogen occurs at an unprecedented rate in animal and human populations and is a major cause of consternation for public health and veterinary communities. *P. mirabilis* can infect various animals and induce clinical symptoms, such as diarrhoea, sepsis, muscle erosion, and encephalomalacia. The pathogen is also responsible for skin ulcers and causes high mortality and economic losses in aquatic animals.[2] In humans, *P. mirabilis* can cause ascending opportunistic and nosocomial urinary tract infections, which occur in patients with functional or structural abnormalities in the urinary tract.[3] In animal husbandry, *P. mirabilis* leads to serious diarrhoea and death of lambs as well as miscarriage of pregnant sheep in large-scale sheep farms.[4,5] The use of antibiotics in controlling *P. mirabilis* infection has not been recommended because of health hazards to consumers and induced multidrug resistance of pathogenic bacteria. However, few commercial vaccines for animals are available currently. Given the high morbidity rates associated with *P. mirabilis* infections and the limited therapeutic options, scholars have focused on developing a safe and effective vaccine against *P. mirabilis* for the breeding industry.

Mucosa tissues are important for protection of an organism from diseases caused by viral, bacterial, and parasitic pathogens, which invade the body through the mucosal system.[6] However, vaccines administered by parenteral routes generally fail to induce mucosal immune responses. Therefore, oral vaccination can be an efficient approach for interfering the colonisation of enteropathogenic bacteria; this strategy can effectively induce local immune responses at the intestinal mucosa and concurrently elicit systemic immune responses.[7] Nevertheless, orally administered antigens must survive the harsh acidic environment and attack of proteases to interact with the immune tissues of the gut and induce immune responses.[6] *Lactic acid bacteria* (LAB) are traditionally used in food industry and generally regarded as safe for human consumption. *Lactococcus lactis* is a model LAB that has been extensively studied for oral vaccine delivery. *L. lactis* is used to express some bacterial, viral, and parasitic antigens, and the resultant recombinant strains can induce specific mucosal and systemic immune responses upon oral administration.[8–10] In *L. lactis* expression mode, a Nisin-controlled gene expression system (NICE) can transport the foreign protein to the bacterial cell surface; this mode is an effective and multifunctional tool.[11] Thus, oral immunisation with *L. lactis* carriers exhibits potential for prevention of *P. mirabilis* infection.

In this study, we constructed a recombinant *L. lactis* expressing *P. mirabilis* outer membrane protein A (ompA), one of the main protective antigens in *P. mirabilis*.[12] We also evaluated the specific protection conferred by the recombinant strain against bacterial challenge in mice. Administration of a mucosal adjuvant can augment both mucosal and systemic immune responses to vaccine antigens.

Taishan *Pinus massoniana* pollen polysaccharides (TPPPS), a pleiotropic polysaccharide extracted from Taishan *P. massoniana* pollens, has been studied in our laboratory since 2003; TPPPS can be used as an effective adjuvant to improve the immune system and facilitate immune responses.[13,14] In the present study, TPPPS was first used as an oral vaccine adjuvant. The effects of TPPPS on conditioning intestine mucosal immunity were also investigated.

Methods and materials

Ethics statement

The animal procedures used in this study were approved by the Animal Care and Use Committee of Shandong Agricultural University (permit number: 20010510) and performed in accordance with the ''Guidelines for Experimental Animals'' of the Ministry of Science and Technology (Beijing, China).

Bacterial strain, vector, and medium

P. mirabilis strain PM.1 was isolated from a dead lamb with diarrhoea in 2013 (Shandong, China) and then preserved in our laboratory. The plasmid pNZ8149 and *L. lactis* NZ3900 strain were purchased from MoBiTec GmbH (Goettingen, Germany). *Escherichia coli* DH5α and pMD18-T were purchased from Takara Co., Ltd., China. Elliker-medium [yeast extract, 5 g/L; tryptone, 20 g/L; NaCl, 4 g/L; CH_3COONa, 1.5 g/L; L(+) ascorbic acid, 0.5 g/L; agar, 15 g/L; 2 and bromocresol purple 0.5%, pH = 6.8] and M17 broth medium containing 0.5% lactose were purchased from Sigma (Beijing, China). All yeast culture media were prepared in accordance with the manufacturers' guidelines.

Construction of recombinant pNZ-ompA/*L. lactis*

On the basis of the ompA gene sequence of *P. mirabilis* (GenBank Type: RefSeq (Nucleotide) NC_010554.1), a pair of primers (ompA-F: 5′-CCCATGGG TATGATAACGAGGCGTAAAATGAAAAAGACAGCTATCGCATTAGCAG-3′, ompA-R: 5′-CTGCTCTAGATTAGTGACCAGGTTGAACAACAAC-3′) was designed to produce a 1107 bp fragment by polymerase chain reaction (PCR). The PCR product was then digested with the restriction enzymes Nco I and Xba I, and the digested fragment was cloned into plasmid pNZ8149. The resultant plasmid was confirmed by sequencing (Sunny, Shanghai) and transformed into *L. lactis* NZ3900 (named pNZ-ompA/*L. lactis*). The blank pNZ8149 plasmid was transformed into *L. lactis* NZ3900 as negative control and named blank-pNZ/*L. lactis*.

Protein expression, flow cytometry, and immunofluorescence microscopy

Recombinant *L. lactis* NZ3900 cells were cultured in M17 broth medium containing 0.5% lactose as carbon source at 30 °C overnight under anaerobic conditions. Recombinant pNZ-ompA/*L. lactis* cells were diluted by 1:25 to 50 in M17 medium. When the cell density reached 0.4 of OD_{600}, Nisin was added every six hours to ensure continuous induction up to a final concentration of 10 ng/mL. Western blot analyses were performed to identify ompA by using methods described in previous study.[12] The mouse anti-ompA protein monoclonal antibody used in Western blot analysis was prepared in the laboratory.

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The recombinant pNZ-ompA/*L. lactis* and blank-pNZ/*L. lactis* were centrifuged at 5000 × *g* for 10 min at 4 °C and washed thrice with sterile phosphate-buffered saline (PBS) to investigate whether the ompA protein was expressed on the surface of *L. lactis*. The bacteria were then incubated with mouse anti-ompA protein monoclonal antibody at 4 °C overnight, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma, China). The stained cells were analysed by flow cytometry (Guaga Easy Cyte Mini, USA).

For immunofluorescence staining, the recombinant pNZ-ompA/*L. lactis* and blank-pNZ/*L. lactis* were harvested after induction and incubated with mouse anti-ompA monoclonal antibody as the primary antibody followed by FITC-conjugated goat anti-mouse IgG (Sigma, China) as the secondary antibody. The blank-pNZ/*L. lactis* was used as negative control.

Vaccine preparation

The cultured recombinant pNZ-ompA/*L. lactis* was resuspended in sterile PBS at a concentration of 1×10^{11} colony-forming unit (CFU)/mL (100 μL/mouse). The corresponding blank-pNZ/*L. lactis* was treated similarly.

TPPPS was prepared in our laboratory through water extraction and ethanol precipitation.[13] The contents of TPPPS were set at the following three doses: 50 (low), 100 (moderate), and 200 (high) mg/mL in three separate TPPPS adjuvant vaccines. The recombinant pNZ-ompA/*L. lactis* mixed with three doses of TPPPS was separately prepared to obtain the corresponding adjuvant oral vaccine.

Animal experiment

A total of 360 six-week-old SPF BALB/c mice (female; Spirax Ferrer Poultry Co., Ltd, Jinan) were randomly separated into six sterilised isolators (groups I–VI), with 60 mice each. The ambient conditions were set to 20–25 °C and 30–40% relative humidity. Air entering the isolators was filtered. Mice in groups I–VI were inoculated orally with 0.1 mL of low, moderate, and high doses of TPPPS adjuvant recombinant pNZ-ompA/*L. lactis*, pure pNZ-ompA/*L. lactis*, blank-pNZ/*L. lactis*, and PBS. Groups I–VI were named pNZ-ompA-TPPPS (L), pNZ-ompA-TPPPS (M), pNZ-ompA-TPPPS (H), pNZ-ompA, blank-pNZ, and Mock, respectively. The vaccines were inoculated daily at 0–4 dpi (days post the first inoculation). Two booster immunisations were conducted at 10–14 dpi and 24–28 dpi.

At 0, 14, 28, 42, and 56 dpi, three mice in each group were selected randomly to determine the antibody titres and the concentrations of IL-2, IFN-γ, IL-4, and interleukin 10 (IL-10) in serum, as well as T-cell proliferative response (LTRs) and counts of CD4+ and CD8+ T lymphocytes in peripheral blood. The tracheal and intestinal lavage fluids were collected to determine sIgA titres by using methods described in previous study.[15] The animals were starved for 12 h before sampling.

Detection of specific IgG and sIgA antibodies as well as IL-2, IFN-γ, IL-4, and IL-10

Three sera, tracheal, and intestinal lavage fluid samples were randomly collected from each group during sampling. Standard enzyme-linked immunosorbent assay (ELISA) protocol was performed for specific antibody IgG titres in serum and sIgA titres in intestinal and tracheal lavage samples.[16] The concentrations of IL-2, IFN-γ, IL-4, and IL-10 were detected using mouse IL-2, IFN-γ, IL-4, and IL-10 ELISA kits (Langdon Bio-technology Co., Ltd, Shanghai) in accordance with the manufacturer's instructions. Absorbance was determined with a microplate reader at 450 nm.

Counts of CD4+ and CD8+ T lymphocytes in peripheral blood

The fresh anticoagulant-heart blood was collected, and then the lymphocytes were obtained with lymphocyte separation medium (P8620-200, Solarbio, China) after centrifugation at 2000 rpm for 10 min. Then, 10 μL of fluorescein isothiocyanate anti-mouse CD4 antibody (BioLegend, USA) and 10 μL of phycoerythrin anti-mouse CD8 antibody (BioLegend, USA) were decanted into 50 μL of lymphocyte suspension. The mixture was incubated for 20 min at 4 °C.[17] The percentages of CD4+ and CD8+ T lymphocytes were detected through flow cytometry (Guaga Easy Cyte Mini, USA) in accordance with the manufacturer's instructions.

Peripheral blood lymphocyte proliferation

Fresh anti-coagulated peripheral blood samples were collected from the mice selected in each group and used to separate lymphocytes as previously described.[18]

Challenge with *P. mirabilis*

At one day after the last immunisation, 20 mice from each group were challenged orally with LD_{50} of *P. mirabilis*. The number of *P. mirabilis* colonies in the intestine was determined. Faecal excretion of *P. mirabilis* was monitored every two days, and serial dilutions of the samples were plated in blood agar. α-Haemolytic colonies were determined after incubation of the plates for 24 h at 37 °C.

Twenty mice from each group were challenged with 10 LD_{50} *P. mirabilis* two weeks after the last immunisation. Mice were maintained for seven days post challenge, and deaths were recorded every day. The survival status of mice was calculated with the following formula:Survival rate (%) = No. of surviving mice/Total No. × 100

Statistical analysis

Data were presented as mean ± standard deviation (SD), and Duncan's multiple-range test was performed to analyse differences among groups by using SPSS 17.0 software. A *P*-value of < 0.05 was considered statistically significant.

Results

Construction of recombinant pNZ-ompA/*L. lactis* and ompA protein expression in vitro

The recombinant plasmid pNZ-ompA was first constructed. The insertion of the ompA gene into the pNZ8149 plasmid was confirmed by restriction enzyme digestion (Fig. 1A). Analysis of the phenotypic screening of recombinant *L.*

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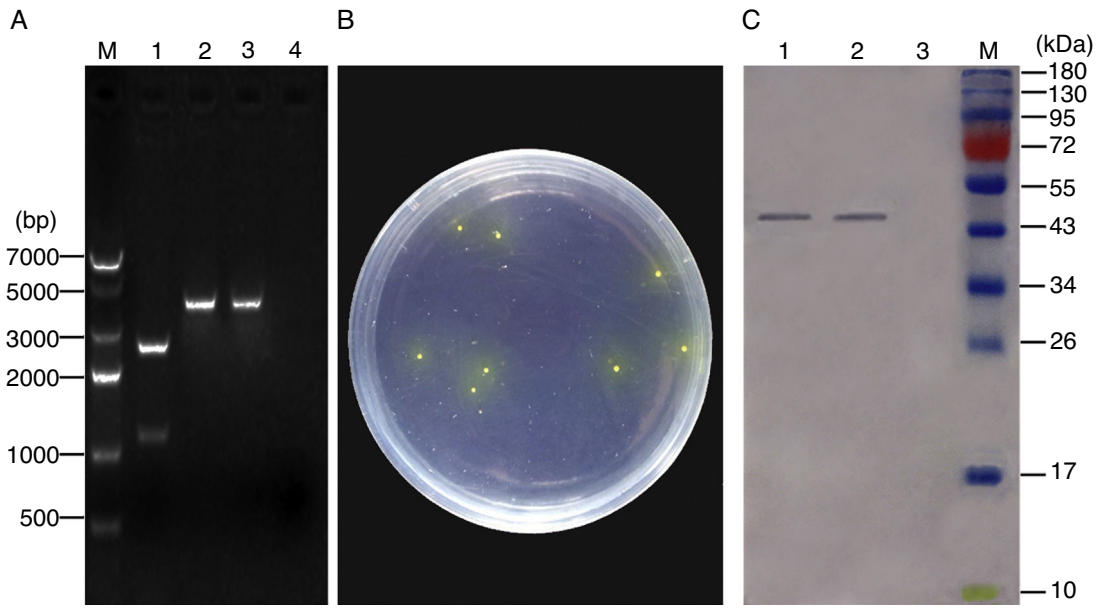

Figure 1 Identification of ompA gene expression in the recombinant *L. lactis*. (A) Identification of the recombinant pNZ-ompA plasmid by digestion with restriction enzymes. M: DNA 7000 bp ladder; 1: the recombinant pNZ-ompA plasmid digested with NcoI and XbaI. The digested *ompA* gene at the bottom (1107 bp); the backbone of the pNZ8149 plasmid at the top (2548 bp); 2: the recombinant pNZ-ompA plasmid digested by single NcoI enzyme (3655 bp); 3: the recombinant pNZ-ompA plasmid digested by single XbaI (3655 bp). (B) Phenotypic screening of recombinant pNZ-ompA/*L. lactis* utilising lactose in Elliker medium. The recombinant pNZ-ompA/*L. lactis* presented yellow colony. (C) Detection of ompA expression by Western blot assay. The mouse anti-ompA monoclonal antibody was used in this assay. M: Pageruler pre-stained protein ladder; the predicted bands (approximately 45 kDa) of the ompA protein were detected in lanes 1–2. No band was shown in Lane 3 carrying a control protein β-actin.

lactis utilising lactose in Elliker medium showed that the recombinant pNZ-ompA/*L. lactis* presented yellow colonies (Fig. 1B). The molecular size of the target protein was approximately 45 kDa. Western blot analysis indicated the lack of the clear band in blank-pNZ/*L. lactis* used as control (Fig. 1C).

To determine whether the ompA protein was expressed on the surface of *L. lactis*, we performed flow cytometric analysis. The results showed that the fluorescence intensity significantly increased in the recombinant pNZ-ompA/*L. lactis* (Fig. 2A). Immunofluorescence analysis further showed that the recombinant pNZ-ompA/*L. lactis* was immunostained positive for ompA (Fig. 2B), but blank-pNZ/*L. lactis* did not (Fig. 2C). Hence, the constructed recombinant pNZ-ompA/*L. lactis* could effectively display the ompA protein on the *L. lactis* surface.

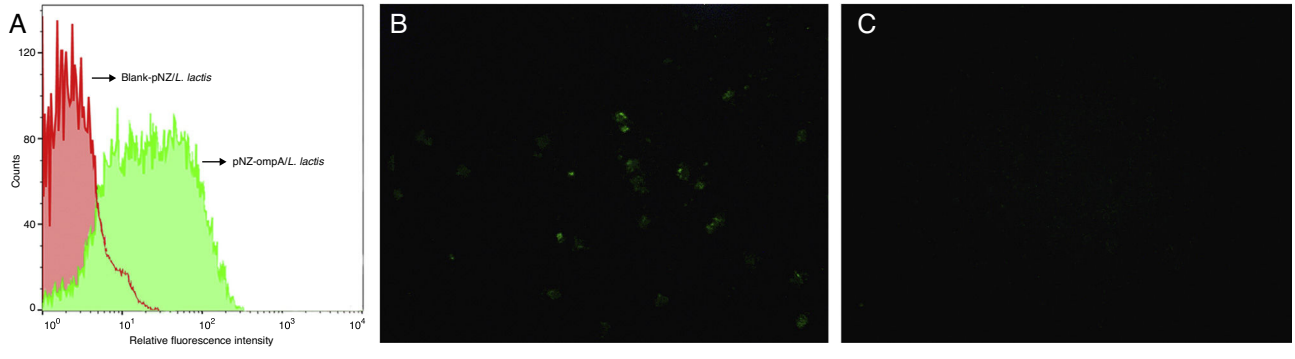

Figure 2 Flow cytometric and immunofluorescence analysis of the *L. lactis* expressing ompA. (A) Flow cytometric analysis of the *L. lactis* expressing ompA. The mouse anti-ompA monoclonal antibody was used in this assay. The recombinant pNZ-ompA/*L. lactis* showed a significant increase of fluorescence intensity (green); the blank-pNZ/*L. lactis* showed negative fluorescence (red). (B and C) Immunofluorescence analysis of the *L. lactis* expressing ompA. The mouse anti-ompA monoclonal antibody was used in this assay. The recombinant pNZ-ompA/*L. lactis* showed positive green fluorescence on the cells (B). No fluorescence was shown in the blank-pNZ/*L. lactis* (C).

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TPPPS promoted mucosal and humoral immune responses induced by oral immunisation of the recombinant pNZ-ompA/*L. lactis*

Mucosal antibodies play an important role in protection against pathogens. In this study, sIgA responses in intestinal and tracheal lavage fluid samples were analysed by ELISA. The anti-ompA sIgA levels in the pNZ-ompA group are significantly higher than those in the control groups inoculated with blank-pNZ/*L. lactis* or PBS in the intestine and trachea at 28–56 dpi (Fig. 3A and B; $P < 0.05$). Notably, a significantly higher anti-ompA sIgA response was detected in groups pNZ-ompA-TPPPS (L), (M), and (H) than that in group pNZ-ompA. The group pNZ-ompA-TPPPS (H) produced the highest anti-ompA sIgA titres in the intestine and trachea at 28–42 dpi ($P < 0.05$).

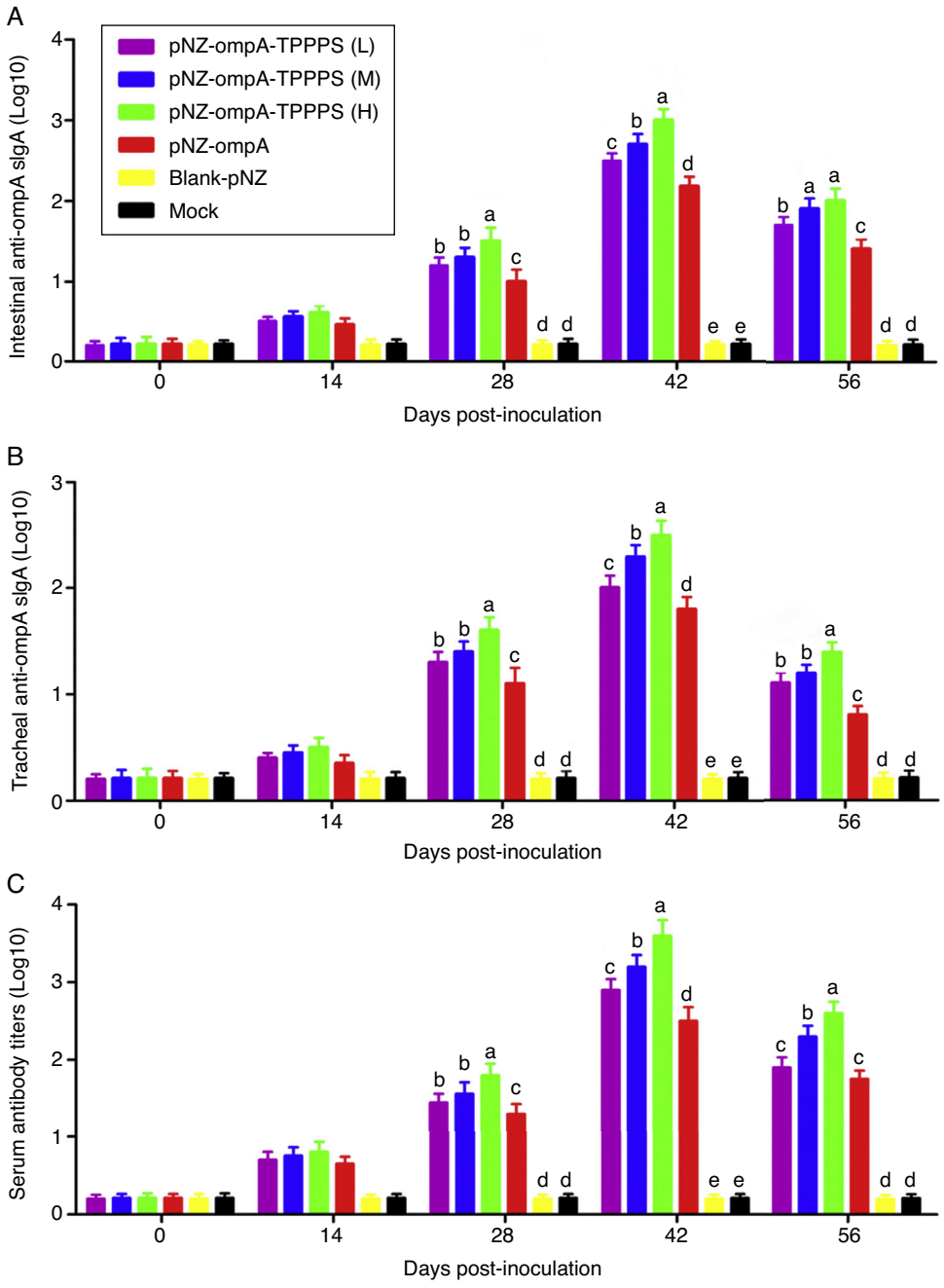

Figure 3 Changes in the intestinal and tracheal specific sIgA and serum antibody titres of the mice inoculated with oral vaccines. Mice in six groups were inoculated with 50, 100, and 200 mg/mL of TPPPS adjuvant recombinant pNZ-ompA/*L. lactis*, pure recombinant pNZ-ompA/*L. lactis*, blank-pNZ/*L. lactis*, and PBS respectively at 0–4, 10–14, and 24–28 dpi. Intestinal and tracheal lavage fluids and serums were collected at 0, 14, 28, 42, and 56 dpi. The specific sIgA and serum antibody titres were then determined by indirect ELISA. All values shown are the means ± SD of three independent experiments. Different superscripts indicate a significant difference ($P < 0.05$).

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Anti-ompA IgG responses were evaluated in serum samples. The results showed that the anti-ompA IgG titres in mice vaccinated with pNZ-ompA are significantly higher than those in the control groups inoculated with blank-pNZ/*L. lactis* or PBS at 28–56 dpi (Fig. 3C; $P < 0.05$). Mice in groups pNZ-ompA-TPPPS (L), (M), and (H) exhibited significantly higher levels of anti-ompA IgG than those that received pure pNZ-ompA at 28–42 dpi compared with the TPPPS adjuvant formulations ($P < 0.05$). Group pNZ-ompA-TPPPS (H) showed increased IgG production compared with pNZ-ompA-TPPPS (L) and (M) at 28–56 dpi ($P < 0.05$).

These results showed that the constructed recombinant pNZ-ompA/*L. lactis* can elicit both ompA-specific mucosal and systemic antibody responses via oral inoculation. Additionally, TPPPS promoted antibody titres induced by pNZ-ompA/*L. lactis* in mice, especially at a concentration of 200 mg/mL.

TPPPS promoted cell-mediated immune responses to the recombinant pNZ-ompA/*L. lactis*

Cytokines IL-2, INF-γ, IL-4, and IL-10 in serum were determined to characterise cellular immune responses induced by oral immunisation with the recombinant pNZ-ompA/*L. lactis*. Immunised mice in group pNZ-ompA produced significantly higher IL-2, INF-γ, and IL-4 levels than those inoculated with blank-pNZ/*L. lactis* or PBS at 28–56 dpi (Fig. 4A–C; $P < 0.05$). Moreover, IL-2, INF-γ, and IL-4 secretion was significantly enhanced in groups pNZ-ompA-TPPPS (L), (M), and (H) compared with that in group pNZ-ompA without TPPPS ($P < 0.05$). Notably, group pNZ-ompA-TPPPS (H) showed increased IL-2, INF-γ, and IL-4 levels compared with groups pNZ-ompA-TPPPS (L) and (M) ($P < 0.05$). However, IL-10 production in the six groups showed no significant differences (Fig. 4D; $P > 0.05$).

The ratio of lymphocyte proliferation is commonly used to evaluate cellular immunity.[19] In the present study, we found that mice in group pNZ-ompA showed significantly higher LTRs than control mice that received blank-pNZ/*L. lactis* or PBS at 14–56 dpi (Fig. 5A; $P < 0.05$). By contrast, the LTRs in mice immunised with TPPPS adjuvant pNZ-ompA are higher than those immunised with pNZ-ompA alone at 28–56 dpi ($P < 0.05$). The LTRs were the highest in mice immunised with pNZ-ompA-TPPPS (H) but were not significantly different from those in groups pNZ-ompA-TPPPS (L) and (M) ($P > 0.05$). The number of CD4+ and CD8+ T lymphocytes in serum directly reflects immune function in animals.[20] Overall, the counts of T lymphocytes showed similar trends to that of LTRs (Fig. 5B and C). In these two detection indices, the optimal effects were obtained when at 200 mg/mL TPPPS was used.

Inhibition of *P. mirabilis* colonisation and infection of BALB/c mice after vaccination with the recombinant pNZ-ompA/*L. lactis*

To evaluate the resistance of pNZ-ompA/*L. lactis* on *P. mirabilis* colonisation, we challenged the mice with *P. mirabilis* 1 d after the last immunisation. *P. mirabilis* colonisation was significantly reduced in the small intestine in group pNZ-ompA compared with those in groups blank-pNZ and Mock (Fig. 6A; $P < 0.05$). Interestingly, the administration of TPPPS adjuvant pNZ-ompA conferred improved inhibition on *P. mirabilis* colonisation than pNZ-ompA administration alone ($P < 0.05$). Group pNZ-ompA-TPPPS (H) showed the highest resistance to *P. mirabilis* colonisation ($P < 0.05$).

Furthermore, we examined the protection of the recombinant pNZ-ompA/*L. lactis* on mice challenged with *P. mirabilis*. As shown in Fig. 6B, the protection rate in mice reached 100% in group pNZ-ompA-TPPPS (H), 85% in group pNZ-ompA-TPPPS (M), 75% in group pNZ-ompA-TPPPS (L), and 70% in pure pNZ-ompA ($P < 0.05$). No mouse survived in groups blank-pNZ and Mock at four days post-challenge. Hence, the pure recombinant pNZ-ompA/*L. lactis* protects mice against *P. mirabilis* challenge limitedly. However, addition of 200 mg/mL TPPPS to pNZ-ompA/*L. lactis* can completely protect mice against *P. mirabilis* infection.

Discussion

P. mirabilis is an enteric pathogenic bacteria that frequently causes animal infections and is thus considered as pathogenic diarrheagenic bacteria.[21] Protective immunity against *P. mirabilis* mainly depends on specific mucosal immune responses induced by intestinal submucosal lymphoid tissues.[22] For mucosal vaccination, LAB are widely used as live vaccine vehicles against various microbes because they present heterologous epitopes, thereby facilitating recognition by the immune system and mediating an immunoadjuvant effect with some of its components.[23] In the present study, we constructed *L. lactis* harbouring the recombinant pNZ8149-ompA plasmid, which expressed exogenous *P. mirabilis* ompA protein based on a Nisin-controlled gene expression system. We also evaluated the immunogenicity of this recombinant pNZ-ompA/*L. lactis* in mice. TPPPS was first used as adjuvant to examine its immune enhancement effects on the oral vaccine. Our results demonstrated that oral immunisation with the recombinant pNZ-ompA/*L. lactis* can elicit both mucosal sIgA and systemic IgG immune responses. Furthermore, TPPPS adjuvant increased immunity levels induced by the recombinant pNZ-ompA/*L. lactis*.

Several expression systems are available for regulated and constitutive expression in *L. lactis*[24]; NICE system is the most often used. Nisin-regulated gene expression in *L. lactis* exhibits numerous characteristics: (1) overexpression of homologous and heterologous genes for functional studies to obtain large quantities of specific gene products; (2) metabolic engineering; (3) expression of prokaryotic and eukaryotic membrane proteins; and (4) protein secretion and anchoring in the cell envelope.[11] This expression system is highly versatile and exhibits potential in pharmaceutical, medical, and food technology fields.[25] In the past 10 years, some immune functional proteins expressed by the NICE system in *L. lactis* can be transported to the bacterial cell surface; consequently, both mucosal and systemic immune responses in the body conferred protection against pathogens.[26]

The recombinant pNZ-ompA/*L. lactis* can also significantly increase the production of IL-2, IFN-γ, and IL-4 in mice. IL-2, and IFN-γ, which belong to the Th1 cell cytokine

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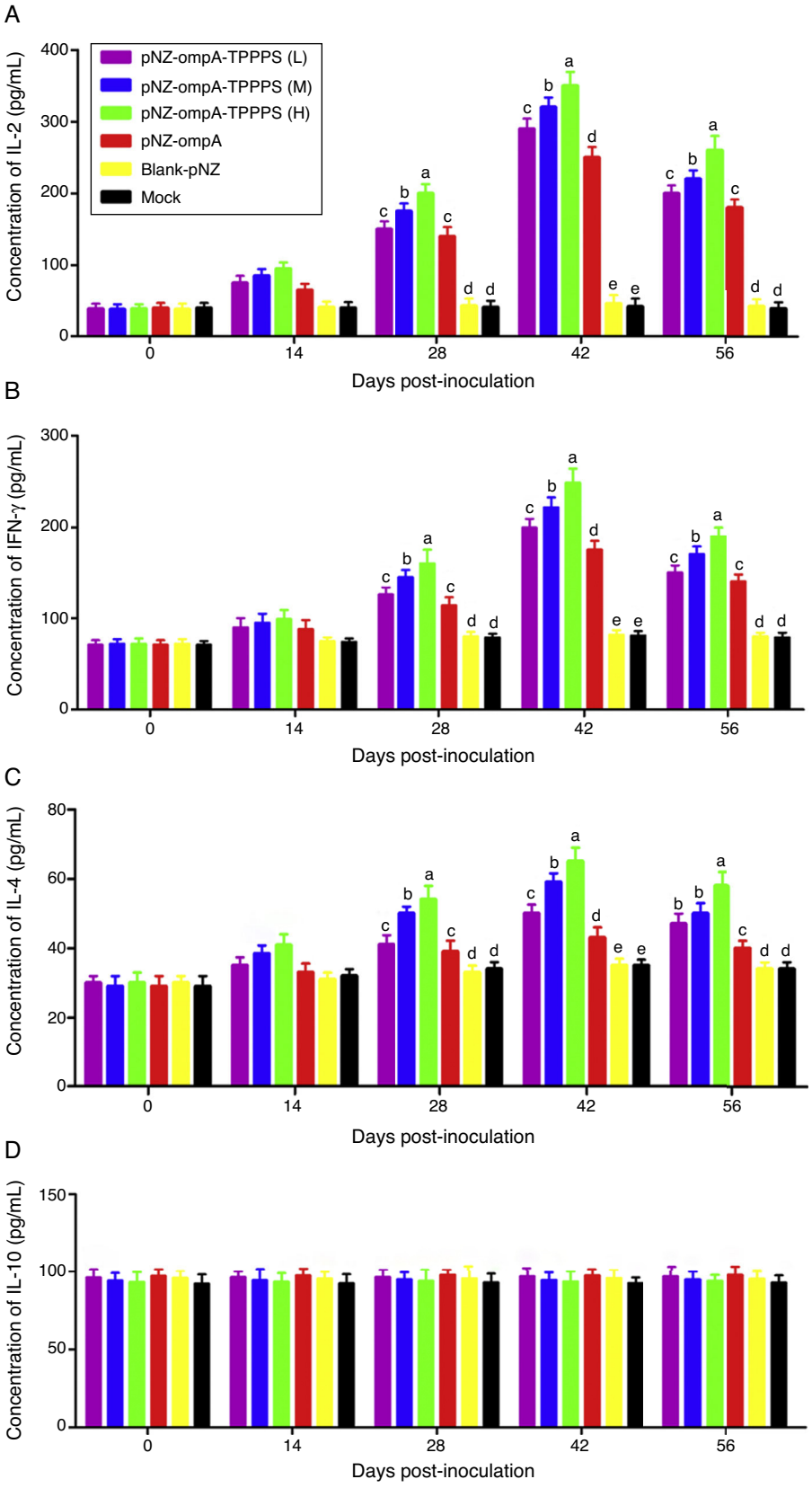

Figure 4 Changes in cytokines of the mice inoculated with vaccines. Mice in six groups were inoculated with 50, 100, and 200 mg/mL of TPPPS adjuvant recombinant pNZ-ompA/*L. lactis*, pure recombinant pNZ-ompA/*L. lactis*, blank-pNZ/*L. lactis*, and PBS respectively at 0–4, 10–14, and 24–28 dpi. Serum was collected at 0, 14, 28, 42, and 56 dpi. IL-2 (A), IFN-γ (B), IL-4 (C) and IL-10 (D) were detected by using the mouse IL-2, IFN-γ, IL-4 and IL-10 ELISA kits. All values shown are the means ± SD of three independent experiments. Different superscripts indicate a significant difference ($P < 0.05$).

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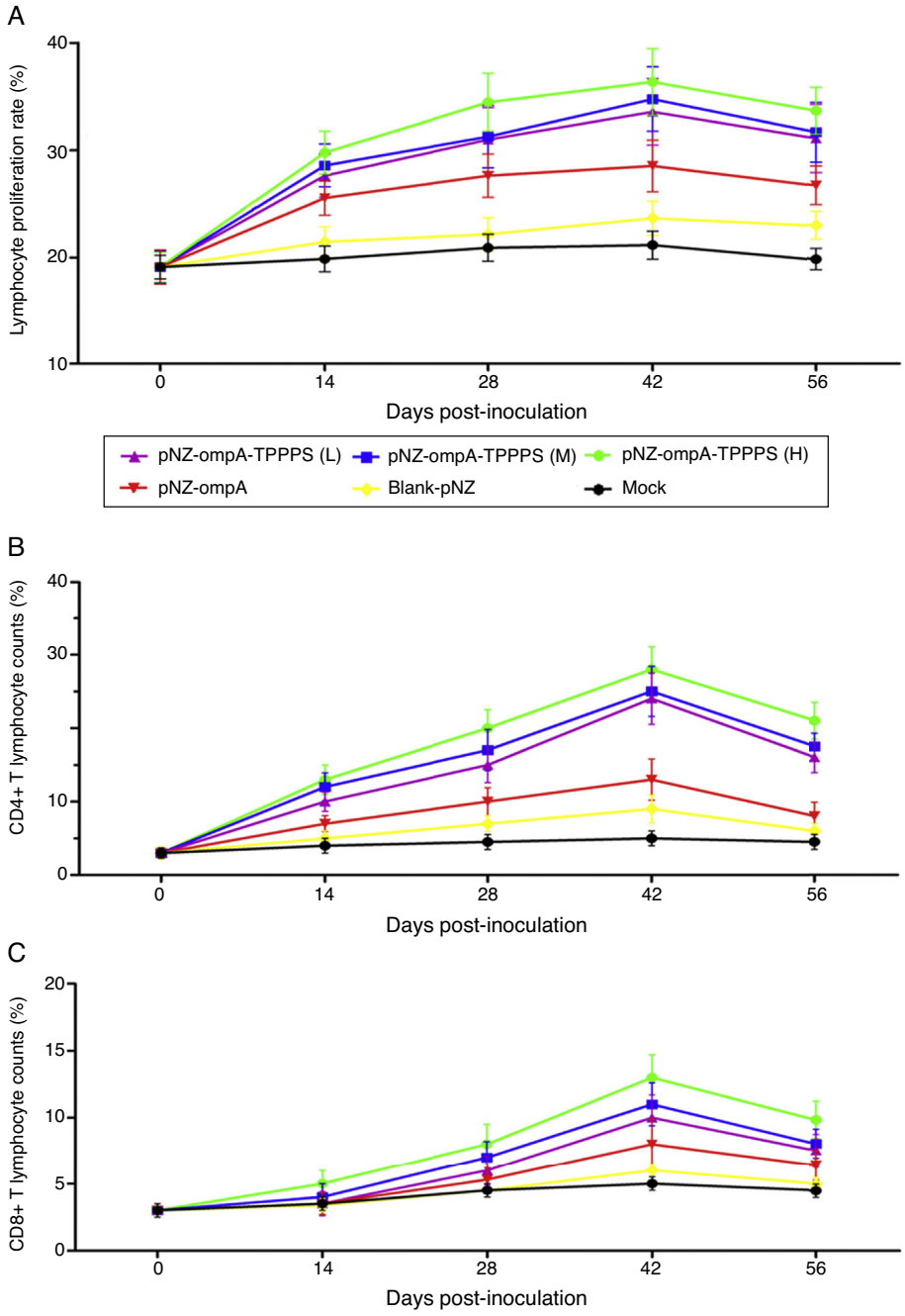

Figure 5 Changes in LTRs, CD4+, and CD8+ T lymphocytes in mice inoculated with vaccines. Mice in six groups were inoculated with 50, 100, and 200 mg/mL of TPPPS adjuvant recombinant pNZ-ompA/*L. lactis*, pure recombinant pNZ-ompA/*L. lactis*, blank-pNZ/*L. lactis*, and PBS respectively at 0–4, 10–14, and 24–28 dpi. Serum was collected at 0, 14, 28, 42, and 56 dpi. Then the LTRs, and the percentages of CD4+ and CD8+ T lymphocytes were detected by flow cytometry. An asterisk indicates that the value of the corresponding group was significantly different from that of the control group ($P < 0.05$).

family, play an important role in mediating cytotoxic effects and local inflammatory responses, assisting antibody generation, and participating in cellular immune responses.[27] IL-4, as a Th2 cell cytokine, mainly promotes B cell proliferation and mediates humoral immune responses. However, we observed no significant differences on secretion of IL-10, which inhibits pro-inflammatory cytokine production and prevents macrophage apoptosis and tissue damage.[28] This result demonstrated that a mixed Th1/Th2-based cell-mediated immune response was upregulated, and IL-10-mediated immunosuppression can be avoided through the common mucosal immune system during oral mouse immunisation with the recombinant pNZ-ompA/*L. lactis*.

Adjuvants are widely applied to enhance the immunogenicity of oral vaccines.[29] Taishan *P. massoniana* pollens have been used as traditional medicine for thousands of years in China and are considered effective adjuvants for improving the immune system and facilitating immune

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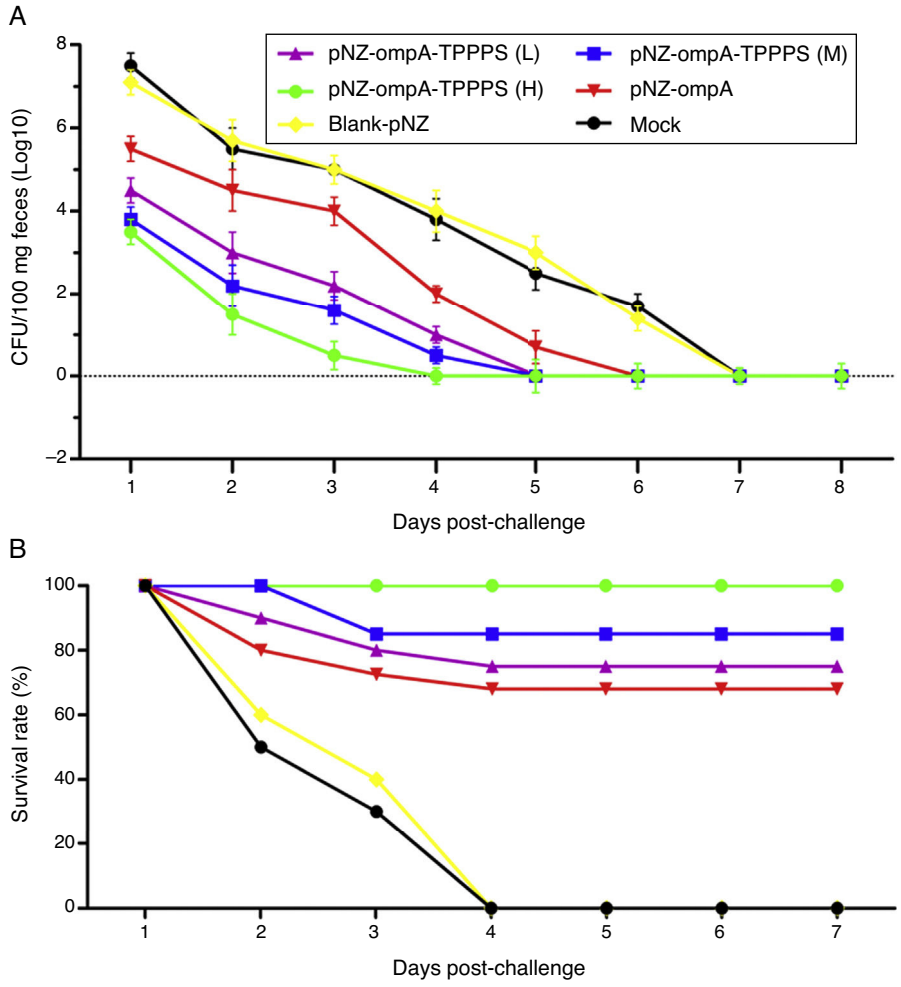

Figure 6 Intestinal colonisation and protective rates of *P. mirabilis*-challenged mice. Mice in six groups were inoculated with 50, 100, and 200 mg/mL of TPPPS adjuvant recombinant pNZ-ompA/*L. lactis*, pure recombinant pNZ-ompA/*L. lactis*, blank-pNZ/*L. lactis*, and PBS, respectively, at 0–4, 10–14, and 24–28 dpi. (A) At 29 dpi, 20 mice were challenged by oral inoculation of LD_{50} of *P. mirabilis*. Faecal shedding was monitored daily by determining the CFU of *P. mirabilis* in samples. (B) Two weeks after the last inoculation, 20 mice from each group were challenged with oral inoculation of 10 LD_{50} of *P. mirabilis*. The survival status of mice was calculated with the following formula: Survival rate (%) = No. of surviving mice/Total No. × 100.

responses in our laboratory.[13,14] TPPPS contains three kinds of polysaccharides (named TPPPS1–3), and each component is composed of different monosaccharides and shows different dominant activities in anti-oxidant, anti-virus, and immunomodulation; hence, TPPPS exhibit synergistic effects on facilitating the immune function of organisms.[30] As such, the use of TPPPS as adjuvant presented satisfactory effects on improving the immune responses of the recombinant pNZ-ompA/*L. lactis* oral vaccine by enhancing both mucosal and systemic immunity.

In conclusion, we demonstrated that orally-administered recombinant pNZ-ompA/*L. lactis* survives the transit of the upper gastrointestinal tract as well as expresses and secretes heterologous proteins, which induced specific mucosal and system immune responses against *P. mirabilis*. Moreover, TPPPS adjuvant presented good immune-enhancing effects on orally administered pNZ-ompA/*L. lactis*. This study presents the potential of the recombinant pNZ-ompA/*L. lactis* combined with TPPPS adjuvant on preventing *P. mirabilis* infection.

Ethical disclosures

Confidentiality of data. The authors declare that they have followed the protocols of their work centre on the publication of patient data and that all the patients included in the study have received sufficient information and have given their informed consent in writing to participate in that study.

Right to privacy and informed consent. The authors declare that no patient data appears in this article.

Protection of human subjects and animals in research. The authors declare that the procedures followed were in accordance with the regulations of the responsible Clinical Research Ethics Committee and in accordance with those of the World Medical Association and the Helsinki Declaration.

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Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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References

1. Xue C, Budrene EO, Othmer HG. Radial and spiral stream formation in *Proteus mirabilis* colonies. PLoS Comput Biol. 2011;7:e1002332.
2. Wang R-J, Xiong X-J. Isolation, identification and drug sensitivity tests of *Proteus mirabilis* from rotten-skin disease of *Rana spinosa*. Freshw Fish. 2012;4:007.
3. Ronald A. The etiology of urinary tract infection: traditional and emerging pathogens. Am J Med. 2002;113:14–9.
4. Blatny JM, Ertesvåg H, Nes IF, Valla S. Heterologous gene expression in *Lactococcus lactis*; expression of the *Azotobacter vinelandii* algE6 gene product displaying mannuronan C-5 epimerase activity. FEMS Microbiol Lett. 2003;227:229–35.
5. Vu T, Farish S, Jenkins M, Kelly H. A meta-analysis of effectiveness of influenza vaccine in persons aged 65 years and over living in the community. Vaccine. 2002;20:1831–6.
6. Wen L-J, Hou X-L, Wang G-H, Yu L-Y, Wei X-M, Liu J-K, et al. Immunization with recombinant *Lactobacillus casei* strains producing K99, K88 fimbrial protein protects mice against enterotoxigenic *Escherichia coli*. Vaccine. 2012;30:3339–49.
7. Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. Nat Rev Immunol. 2006;6:148–58.
8. Wells J. Mucosal vaccination and therapy with genetically modified lactic acid bacteria. Annu Rev Food Sci Technol. 2011;2:423–45.
9. Gao S, Li D, Liu Y, Zha E, Zhou T, Yue X. Oral immunization with recombinant hepatitis E virus antigen displayed on the *Lactococcus lactis* surface enhances ORF2-specific mucosal and systemic immune responses in mice. Int Immunopharmacol. 2015;24:140–5.
10. Ahmed B, Loos M, Vanrompay D, Cox E. Mucosal priming of the murine immune system against enterohemorrhagic *Escherichia coli* O157: H7 using *Lactococcus lactis* expressing the type III secretion system protein EspB. Vet Immunol Immunopathol. 2013;152:141–5.
11. Mierau I, Kleerebezem M. 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. Appl Microbiol Biotechnol. 2005;68:705–17.
12. Zhang Y, Yang S, Dai X, Liu L, Jiang X, Shao M, et al. Protective immunity induced by the vaccination of recombinant *Proteus mirabilis* OmpA expressed in *Pichia pastoris*. Protein Expr Purif. 2015;105:33–8.
13. Wei K, Sun Z, Yan Z, Tan Y, Zhu X, Wang X, et al. Effects of Taishan *Pinus massoniana* pollen polysaccharide on immune response of rabbit haemorrhagic disease tissue inactivated vaccine and on production performance of Rex rabbits. Vaccine. 2011;29:2530–6.
14. Cui G, Zhong S, Yang S, Zuo X, Liang M, Sun J, et al. Effects of Taishan *Pinus massoniana* pollen polysaccharide on the subunit vaccine of *Proteus mirabilis* in birds. Int J Biol Macromol. 2013;56:94–8.
15. Wang Z, Gao J, Yu Q, Yang Q. Oral immunization with recombinant *Lactococcus lactis* expressing the hemagglutinin of the avian influenza virus induces mucosal and systemic immune responses. Future Microbiol. 2012;7:1003–10.
16. Li K, Gao H, Gao L, Qi X, Gao Y, Qin L, et al. Development of an indirect ELISA for serological detection of reticuloendotheliosis virus using the gp90 protein expressed in *Pichia pastoris*. J Virol Methods. 2012;180:43–8.
17. Wang C, Liu L, Zhang Z, Yan Z, Yu C, Shao M, et al. Immunological and protective effects of *Bordetella bronchiseptica* subunit vaccines based on the recombinant N-terminal domain of dermonecrotic toxin. Int Immunopharmacol. 2015;28:952–9.
18. Zhang Y, Yang S, Zhao X, Yang Y, Li B, Zhu F, et al. Immune enhancement of Taishan *Robinia pseudoacacia* polysaccharide on recombinant *Proteus mirabilis* OmpA in chickens. Int Immunopharmacol. 2014;22:236–41.
19. Asherson G, Ferluga J, Janossy G. Non-specific cytotoxicity by T cells activated with plant mitogens in vitro and the requirement for plant agents during the killing reaction. Clin Exp Immunol. 1973;15:573.
20. Parnes JR. Molecular biology and function of CD4 and CD8. Adv Immunol. 1989;44:265–311.
21. dos Reis RS, Horn F. Enteropathogenic *Escherichia coli*, *Samonella*, *Shigella* and *Yersinia*: cellular aspects of host-bacteria interactions in enteric diseases. Gut Pathog. 2010; 2:1.
22. Zhang Y, Pacheco S, Acuna CL, Switzer KC, Wang Y, Gilmore X, et al. Immunoglobulin A-deficient mice exhibit altered T helper 1-type immune responses but retain mucosal immunity to influenza virus. Immunology. 2002;105:286–94.
23. Ferreira D, Darrieux M, Silva D, Leite L, Ferreira J, Ho P, et al. Characterization of protective mucosal and systemic immune responses elicited by pneumococcal surface protein PspA and PspC nasal vaccines against a respiratory pneumococcal challenge in mice. Clin Vaccine Immunol. 2009;16:636–45.
24. de Vos WM. Gene expression systems for lactic acid bacteria. Curr Opin Microbiol. 1999;2:289–95.
25. Le Loir Y, Nouaille S, Commissaire J, Bretigny L, Gruss A, Langella P. Signal peptide and propeptide optimization for heterologous protein secretion in *Lactococcus lactis*. Appl Environ Microbiol. 2001;67:4119–27.
26. Xu Y-G, Cui L-C, Ge J-W, Tang L-J, Zhao L-L, Li Y-J. The immune response induced in mice orally immunized with *Lactobacillus casei* expressing porcine parvovirus vp2 protein. Sci Agric Sin. 2008;3:032.
27. Wang Z-H, Cao X-H, Du X-G, Feng H-B, Zeng X-Y. Mucosal and systemic immunity in mice after intranasal immunization with recombinant *Lactococcus lactis* expressing ORF6 of PRRSV. Cell Immunol. 2014;287:69–73.
28. Beamer GL, Flaherty DK, Assogba BD, Stromberg P, Gonzalez-Juarrero M, de Waal Malefyt R, et al. Interleukin-10 promotes *Mycobacterium tuberculosis* disease progression in CBA/J mice. J Immunol. 2008;181:5545–50.
29. Jackson RJ, Fujihashi K, Xu-Amano J, Kiyono H, Elson C, McGhee J. Optimizing oral vaccines: induction of systemic and mucosal B-cell and antibody responses to tetanus toxoid by use of cholera toxin as an adjuvant. Infect Immun. 1993;61: 4272–9.
30. Yang S, Wei K, Jia F, Zhao X, Cui G, Guo F, et al. Characterization and biological activity of Taishan *Pinus massoniana* pollen polysaccharide in vitro. PLOS ONE. 2015;10:e0115638.

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