ARTICLE

In vivo delivery of pPERDBY to BALB/c mice by *LacVax*® *DNA-I* and comparison of elicited immune response with conventional immunization methods

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Abstract

The non-invasive food grade *Lactococcus lactis* (*L. lactis*) represents a safe and attractive alternative to invasive pathogens for the delivery of plasmid DNA at mucosal sites. We have earlier shown the DNA delivery potential of r-*L. lactis* harboring DNA vaccine reporter plasmid; pPERDBY in vitro. In the present work, we examined in vivo delivery potential of food grade non-invasive r-*L. lactis*::pPERDBY (*LacVax*® *DNA-I*) in BALB/c mice. Moreover, using EGFP as a model antigen, we also characterized and compared the immune response elicited by *LacVax*® *DNA-I* with other conventional vaccination approaches using protein and naked DNA immunization. The presence of antigen-specific serum IgG and fecal secretory IgA (sIgA) antibodies demonstrated in vivo DNA delivery and immune elicitation potential of the developed *LacVax*® *DNA-I*. As compared with intramuscular injection, oral delivery of pPERDBY via *L. lactis* resulted in a significantly rapid increase in IgG and higher sIgA titers, indicating the immunogenic and immunostimulatory properties of the *LacVax*® *DNA-I*. The needle-free immunization with *LacVax*® *DNA-I* led to increased production of IL-4, an indicator of Th2 screwed response. To the best of our knowledge, this report for the first time outlines comparison of orally administered *LacVax*® *DNA-I* with other conventional vaccination approaches.

Introduction

Vaccination is one of the most effective ways to prevent infection and disease establishment. Different strategies for vaccine development have been deployed against a wide range of pathogens [1]. Mucosal immunization with plasmid DNA has been studied with great interest in generating antigen-specific systemic as well as mucosal immunity [2].

Different bacterial carriers have been used for the delivery of DNA vaccines to mucosal sites. In the earlier attempts, attenuated invasive pathogens such as *Salmonella, Shigella, Yersinia*, and *Listeria* were used as a vaccine carrier [3]. The adhesive and invasive properties of these bacteria allow efficient DNA delivery to professional and non-professional phagocytes, leading to enhanced expression and presentation of antigen [4]. However, the major risk of reversion to virulent phenotype and associated safety issues discourage the use of attenuated invasive pathogens [3].

The use of food grade *Lactococcus lactis* (*L. lactis*) as a DNA delivery vehicle represents an attractive alternative to attenuated pathogens [3]. The GRAS (generally regarded as safe) status of *L. lactis* has paved the way for its application in oral delivery of antigens and numerous therapeutic molecules [5]. With this background, our group has explored *L. lactis* for the development of a mucosal vaccine against *Shigella*. We have reported efficient expression and delivery of outer membrane protein A (OmpA) of *Shigella dysenteriae* type-1 (SD-1) in *L. lactis* and evaluated its immunogenic potential in BALB/c mice [5, 6].

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In addition, we have also explored the potential use of non-invasive *L. lactis* and invasive *L. lactis* [7] for the delivery of the newly constructed DNA vaccine reporter plasmid; pPERDBY to Caco-2 cells [3]. The *Escherichia coli* (*E. coli*)–*L. lactis* shuttle vector; pPERDBY consists of eukaryotic expression cassette composed of the cytomegalovirus promoter (pCMV), a multiple cloning site fused with enhanced green fluorescent protein gene (MCS-EGFP), and SV40 early mRNA polyadenylation signal, essential for gene expression by eukaryotic host cells. Moreover, the presence of two immunostimulatory CpG motifs strengthens the potential of pPERDBY as a prospective DNA vaccine plasmid. We demonstrated that the non-invasive recombinant *L. lactis* harboring pPERDBY (*LacVax*® *DNA-I*) successfully delivered pPERDBY to Caco-2 as determined by EGFP expression in co-cultured Caco-2 cells [3]. These results indicate the potential of non-invasive *L. lactis* to deliver DNA vaccine plasmid in vivo efficiently.

In the present study, *LacVax*® *DNA-I* was administered orally to BALB/c mice to evaluate its in vivo DNA delivery potential. Moreover, the present study also characterized and compared the immune response elicited by *LacVax*® *DNA-I* with other immunization approaches using EGFP as a model antigen. The systemic and mucosal immune response elicited against EGFP, following *LacVax*® *DNA-I* immunization were compared with purified EGFP-OmpH protein and endotoxin-free plasmid pPERDBY, given intramuscularly to BALB/c mice.

The present article demonstrates the in vivo DNA delivery potential of *LacVax*® *DNA-I* and evaluates its immunogenic response. To the best of our knowledge, this is the first report describing the comparison of immune response elicited by selected antigen when administered via different immunization strategies, i.e. naked DNA vs. purified protein vs. r-*L. lactis* delivering the plasmid DNA in BALB/c mice.

Results

Confirmation of r-*E. coli* BL21 (DE3)::pCOLDII-EGFP-OmpH

The vector maps of pCOLDII-EGFP-OmpH and pPERDBY are shown in Fig. 1a, b respectively. In order to attain high expression and yield of EGFP-OmpH, pCOLDII expression system was used in the present study. Colony PCR of transformants using *egfp*-specific primers resulted in the amplicon of ~750 bp, which indicated the presence of pCOLDII-EGFP-OmpH in transformed r-*E. coli* BL21 (DE3)::pCOLDII-EGFP-OmpH cells. Purified plasmid; pCOLDII-EGFP-OmpH and wild-

type *E. coli* BL21 (DE3) served as a positive and negative control, respectively (Fig. 1c). The presence ~5177 bp band after digestion with *Eco*RV or *Hin*dIII indicated linearized pCOLDII-EGFP-OmpH (Lanes 2 and 3, Fig. 1d). The pCOLDII-EGFP-OmpH when double digested with *Eco*RV and *Hin*dIII resulted in two DNA fragments of 3106 and 2071 bp, confirming the successful construction of r-*E. coli* BL21 (DE3)::pCOLDII-EGFP-OmpH (Fig. 1e).

EGFP-OmpH expression and purification

The expression of EGFP-OmpH was evaluated in uninduced and induced cell fractions of r-*E. coli* BL21 (DE3):: pCOLDII-EGFP-OmpH by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 2a, b). The presence of a ~34.8 kDa band in the induced cells and not in uninduced cells confirmed inducible expression of EGFP-OmpH (Fig. 2c).

After IPTG induction and incubation at low temperature, r-*E. coli* BL21 (DE3) harboring pCOLDII-EGFP-OmpH expressed EGFP-OmpH protein in an insoluble fraction which was then solubilized and subjected to Ni-NTA affinity purification. The presence of a ~34.8 kDa band in lane 5, 6, and 7 (Fig. 2c) confirms EGFP-OmpH expression and purification. The purified protein was then quantified by Bradford assay [8] (data not shown).

Stability of DNA vaccine plasmid in *LacVax*® *DNA-I*

The stability of pPERDBY in our previously constructed DNA vaccine candidate *LacVax*® *DNA-I* [3] was evaluated after repeated sub-culturing. It was found that around 91% and 94% of cells revived and formed colonies on GM17 agar in the absence and presence of chloramphenicol, respectively, suggesting stability of pPERDBY in developed DNA vaccine candidate.

Humoral response after immunization with *LacVax*® *DNA-I* and its comparison with conventional immunization methods

We had previously shown the in vitro delivery of pPERDBY using non-invasive *L. lactis* [3]. To evaluate the in vivo DNA delivery potential of *LacVax*® *DNA-I* and immune response elicitation post its immunization, serum IgG and fecal sIgA levels were analyzed. Moreover, the immune response elicited after *LacVax*® *DNA-I* was compared with conventional methods of immunization, i.e. intramuscular (i.m.) injection of naked DNA and purified protein. Control groups were either immunized with saline (DS) or wild-type *L. lactis* (DW). The serum IgG of all the immunized mice was analyzed on day 0th, 20th, 43rd, and
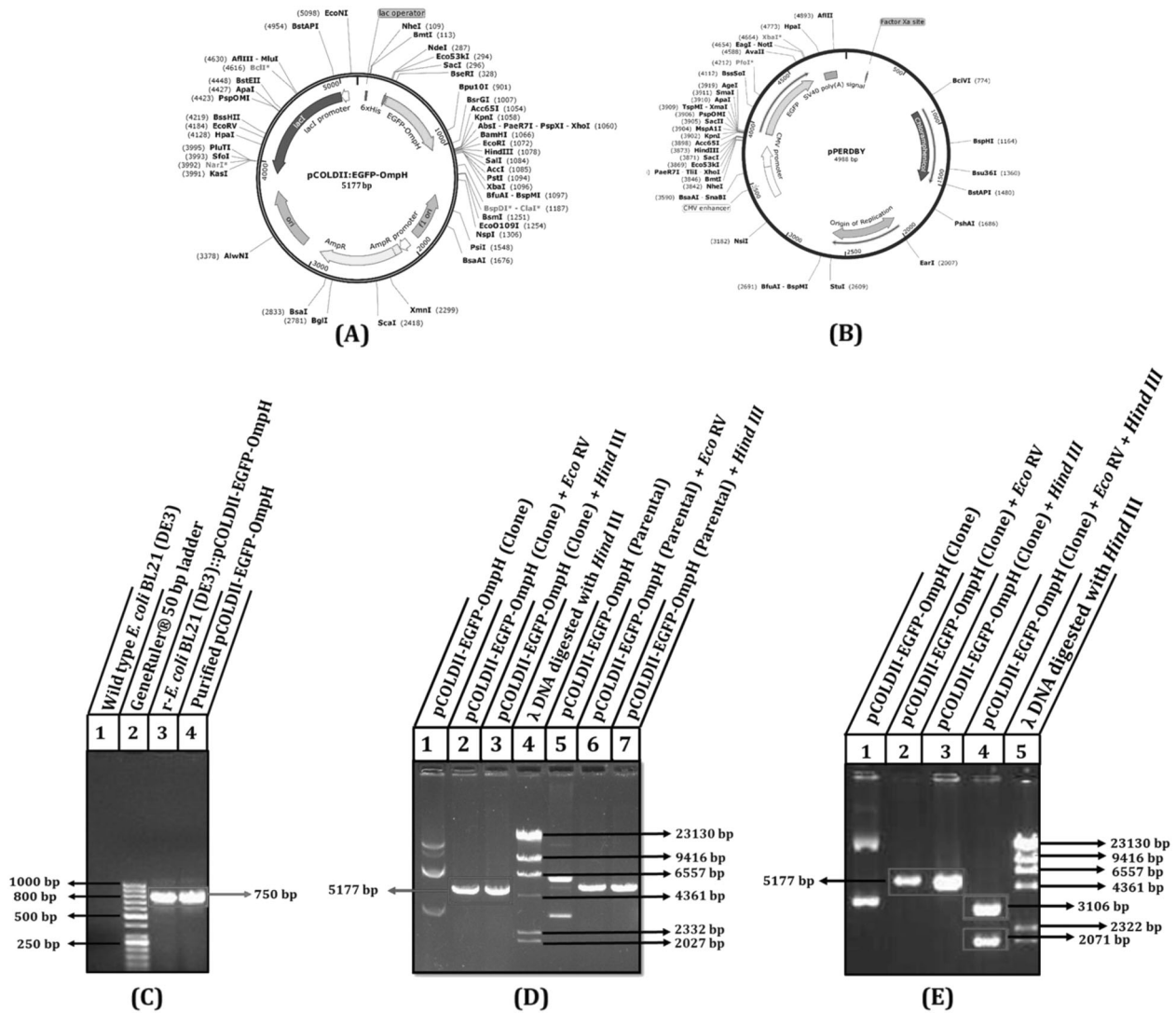
Fig. 1 Confirmation of pCOLDII-EGFP-OmpH. **a** Schematic representation of pCOLDII-EGFP-OmpH vector. **b** Schematic representation of pPERDBY vector. **c** A 1% agarose gel electrophoresis of *egfp*-specific PCR amplicons; Lane-1: no band observed when PCR was performed using plasmid DNA isolated from wild-type *E. coli* BL21 (DE3); Lane 2: GeneRuler® 50 bp ladder from Thermo Fisher Scientific, USA; Lane 3: a 750 bp PCR amplicon obtained using a plasmid template isolated from r-*E. coli* BL21 (DE3)::pCOLDII-EGFP-OmpH; Lane-4: a 750 bp PCR amplicon obtained using purified pCOLDII-EGFP-OmpH (positive control). **d** A 0.8% agarose gel electrophoresis of RE-digested plasmid samples isolated from r-*E. coli* BL21::pCOLDII-EGFP-OmpH; Lane-1: undigested plasmid sample isolated from r-*E. coli* BL21::pCOLDII-EGFP-OmpH; Lane 2: a 5177 bp linearized DNA sample following RE digestion of plasmid sample isolated from r-*E. coli* BL21:pCOLDII-EGFP-OmpH with *Eco*RV; Lane 3: a 5177 bp linearized DNA sample following RE digestion of plasmid sample isolated from r-*E. coli* BL21:pCOLDII-EGFP-OmpH with *Hin*dIII; Lane-4: λ DNA digested with *Hin*dIII; Thermo Fisher Scientific, USA; Lane 5: undigested plasmid pCOLDII-EGFP-OmpH sample received from Dr. Yong-Suk Jang; Lane-6: a 5177 bp linearized DNA sample following RE digestion of received pCOLDII-EGFP-OmpH with *Eco*RV; Lane-7: A 5177 bp linearized DNA sample following RE digestion of received pCOLDII-EGFP-OmpH with *Hin*dIII. **e** A 1% agarose gel electrophoresis of double digested plasmid samples isolated from r-*E. coli* BL21::pCOLDII-EGFP-OmpH; Lane-1: undigested pCOLDII-EGFP-OmpH; Lane 2: a 5177 bp linearized DNA sample following RE digestion of plasmid sample isolated from r-*E. coli* BL21::pCOLDII-EGFP-OmpH with *Eco*RV; Lane 3: a 5177 bp linearized DNA sample following RE digestion of plasmid sample isolated from r-*E. coli* BL21:pCOLDII-EGFP-OmpH with *Hin*dIII; Lane-4: pCOLDII-EGFP-OmpH when digested with *Eco*RV and *Hin*dIII, it results in the generation of two DNA fragments of 3106 and 2071 bp, confirming the integrity of pCOLDII-EGFP-OmpH in r-*E. coli* BL21 (DE3) cells; Lane 5: λ DNA digested with *Hin*dIII; Thermo Fisher Scientific, USA

49th, whereas fecal sIgA was analyzed after the 7th day of the last immunization (day 49th).

As shown in Fig. 3a, b, on day 49, IgG titers of *LacVax*® *DNA-I*-immunized BALB/c mice (Group DR) were higher than that of the DIM group which was immunized intramuscularly with endotoxin-free pPERDBY. However, immunization with purified EGFP-OmpH (Group P) resulted in the highest IgG titers (Fig. 3a). On the other hand, mice immunized with phosphate-buffered saline (PBS) (Group DS) or wild-type *L. lactis* (DW) did not exhibit
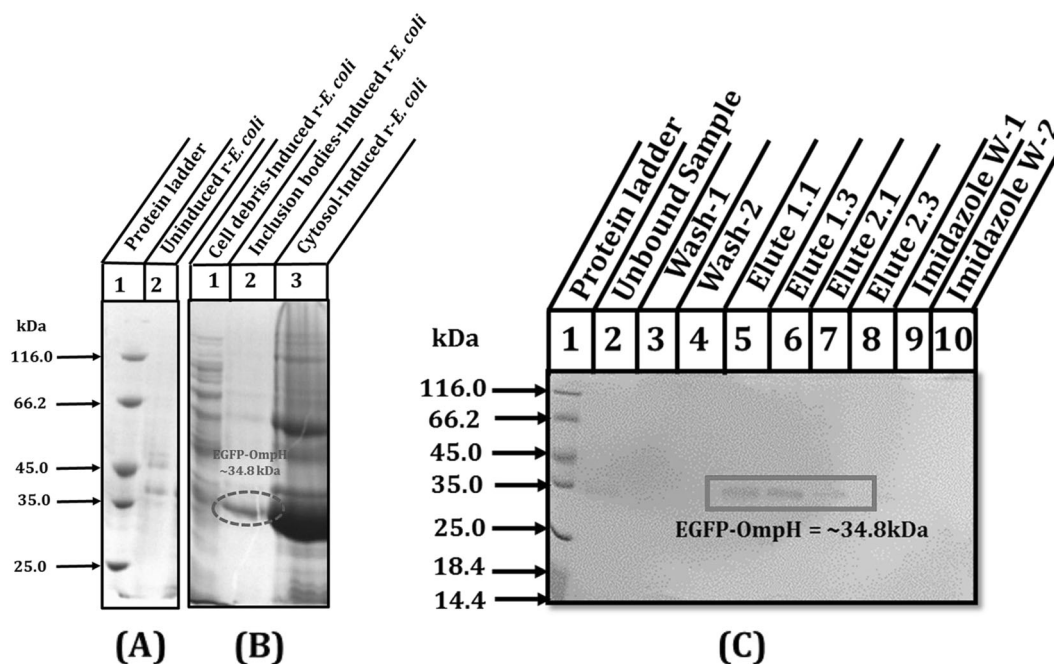
Fig. 2 EGFP-OmpH expression and purification from r-*E. coli* BL21::pCOLDII-EGFP-OmpH. **a** A 10% SDS-PAGE of uninduced r-*E. coli* BL21::pCOLDII-EGFP-OmpH cells: Lane-1: Pierce™ Unstained Protein MW Marker; Thermo Fisher Scientific, USA; Lane 2: uninduced r-*E. coli* BL21::pCOLDII-EGFP-OmpH cells mixed with the SDS-PAGE loading dye (reducing). **b** A 10% SDS-PAGE of different cellular fractions of induced r-*E. coli* BL21:pCOLDII-EGFP-OmpH: Lane-1: bacterial cell debris; Lane 2: solubilized inclusion bodies; Lane 3: cytosolic matter. **c** A 12% SDS-PAGE of purification samples of induced r-*E. coli* BL21:pCOLDII-EGFP-OmpH: Lane-1: Pierce™ Unstained Protein MW Marker; Thermo Fisher Scientific, USA; Lane 2: Unbound protein sample collected after the loading of protein fraction; Lanes 3 and 4: Protein sample fractions collected as a part of column washing steps; Lanes 5–8: Protein sample fractions collected as a part of elution step; Lanes 9 and 10: Samples obtained as a part of final wash step

EGFP-specific antibodies (Fig. 3a). The EGFP-specific serum IgG antibodies increased in Group DR and DIM with each round of immunization (Fig. 3c). Interestingly, the EGFP-specific serum antibodies increased significantly immediately after the third dose of *LacVax*® *DNA-I* (20th day), which was not in the case of naked DNA immunization (DIM group). Also, the production of antigen-specific IgG was rapid and titers were high in *LacVax*® *DNA-I* immunized mice as compared to the DIM group. On the 49th day, i.e., 7 days after the last booster, there were significantly higher EGFP-specific serum IgG antibodies in DR ($P = 0.039$) and DIM ($P = 0.0023$) groups of mice. IgG levels remained constant in control groups DS and DW over the entire dosage regimen (Fig. 3c).

The production of sIgA, a hallmark of mucosal priming, was evident in mice immunized with *LacVax*® *DNA-I*, naked pPERDBY, and purified EGFP-OmpH. Interestingly, antigen-specific fecal sIgA levels of *LacVax*® *DNA-I* immunized animals were similar to that of mice immunized with purified EGFP-OmpH ($P < 0.0001$) and higher than the DIM group of mice which were immunized with naked pPERDBY. As anticipated, control groups: DS and DW, showed an insignificant rise in fecal IgA (Fig. 3d).

Cell mediated immune activation

In an attempt to evaluate and compare the activation of cellular immunity, by different modes of immunization, we have estimated IFN-γ (a representative Th1 cytokine) and IL-4 (a representative Th2 cytokine) production in immunized mice. As evident from Fig. 4a, the IL-4 levels are significantly elevated in the groups P, DR, and DIM as compared to the control groups DS and DW. As shown in Fig. 4b, i.m. injection of pPERDBY resulted in an indicative Th1 cellular response, whereas delivery of the same plasmid by oral route via r-*L. lactis* resulted in an indicative Th2 response. Immunization with purified EGFP-OmpH revealed an indicative Th2 response (Fig. 4b), which can be correlated with significantly higher IgG titers (Fig. 3a) in Group P. The indicative Th2 response in the DR group compared to DIM supports the early rise in IgG levels in the DR group on the 20th day (Fig. 3c).

Integrity of intestinal tissues

To investigate the tissue integrity of orally *LacVax*® *DNA-I* immunized BALB/c mice, we performed H&E staining of the small and large intestine of DR, DW, and DS groups. Of
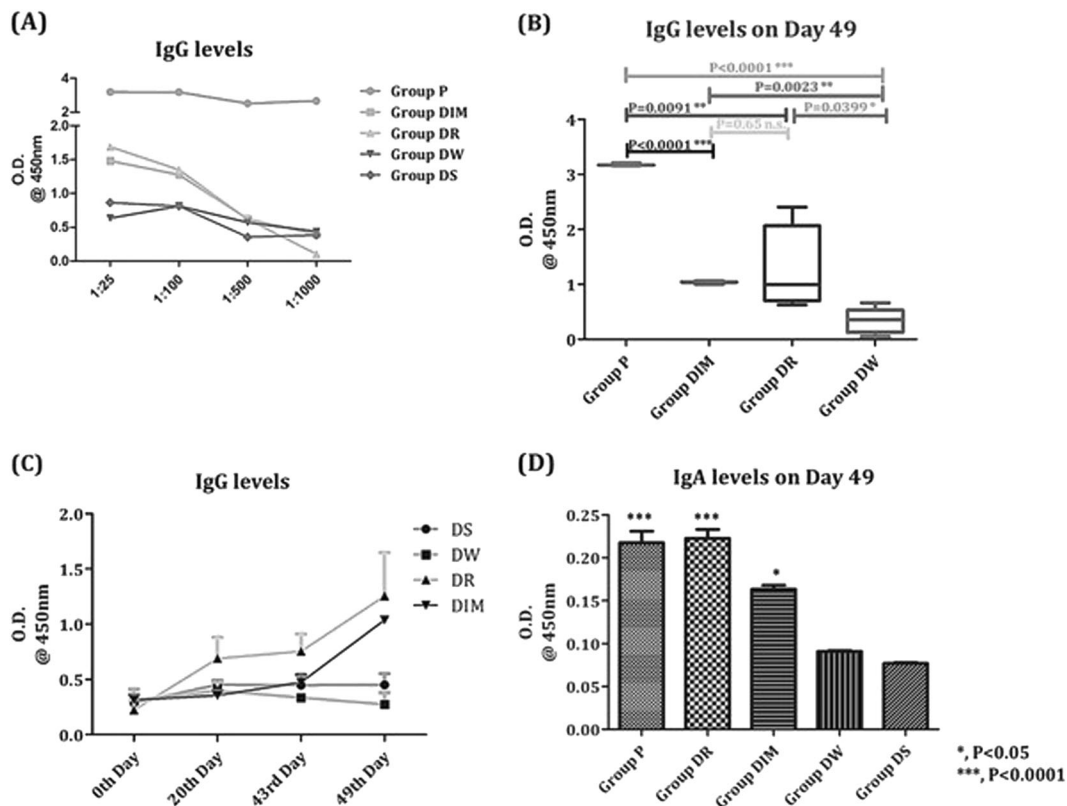
Fig. 3 Humoral responses of immunized animals. **a** Serum IgG levels of all immunized animals on 49th day. **b** Serum IgG levels of *LacVax*® immunized mice (Group DR), pPERDBY injected BALB/c mice (Group DIM), Purified EGFP-OmpH immunized mice (Group P), and wild-type *L. lactis* NZ9000 (Group DW) on day 49th. **c** IgG levels of group DR, DIM, DW, and DS mice at different time intervals. **d** Fecal sIgA levels of all immunized groups on day 49th

note, oral inoculation of *LacVax*® *DNA-I* did not induce inflammatory or structural changes. The physiological characteristics of the DR group were similar to that of control groups: DS and DW (Fig. 4b), indicating the safe profile of *LacVax*® *DNA-I*.

Discussion

A number of enteric pathogens breach host mucosal epithelial barrier and establish infections [9]. To combat against these infectious agents, induction of mucosal immunity in addition to systemic immunity is essential and can be achieved by efficient delivery of antigens or vaccine candidates at the site of infection [1]. Different vaccination approaches such as immunization with naked plasmid DNA and subunit vaccines are in practice. However, these non-living vaccines require larger dose and fail to induce cellular immunity with long lasting humoral immunity that is essential to provide protection against pathogens [10]. Moreover, these immunization procedures are cumbersome and use syringes and needles for vaccine administration [10]. Considering the patient compliance, unsafe usage, cost, and rapid increase in the incidents of cross-

contamination, World Health Organization has accelerated the search for safer and needle-free strategies of immunization [11–13].

In the search for needle-free mucosal vaccination, live bacterial carriers such as attenuated pathogens were exploited for DNA as well as antigen delivery. Such vaccine carriers have minimal virulence but offer high immunogenicity and hence their use was promising in earlier attempts for, e.g., *Salmonella enterica, Shigella flexneri, Listeria monocytogenes*, and *Yersinia enterocolitica*. However, the unmitigated risk of reversion has triggered the hunt for safer yet live vaccine carriers [14]. Food grade bacteria such as *L. lactis* when used as an antigen delivery vehicle provides advantages similar to that of attenuated pathogens and is safe even for immunocompromised individuals and children [15]. Apart from antigen delivery vehicle, *L. lactis* has recently been exploited as a DNA delivery vehicle [3, 4, 16–19].

We had previously shown successful delivery of DNA vaccine reporter plasmid; pPERDBY to Caco-2 cells by non-invasive r-*L. lactis*::pPERDBY and demonstrated EGFP expression in vitro [3]. In the present study, we investigated in vivo DNA delivery potential of the developed non-invasive r-*L. lactis* harboring pPERDBY
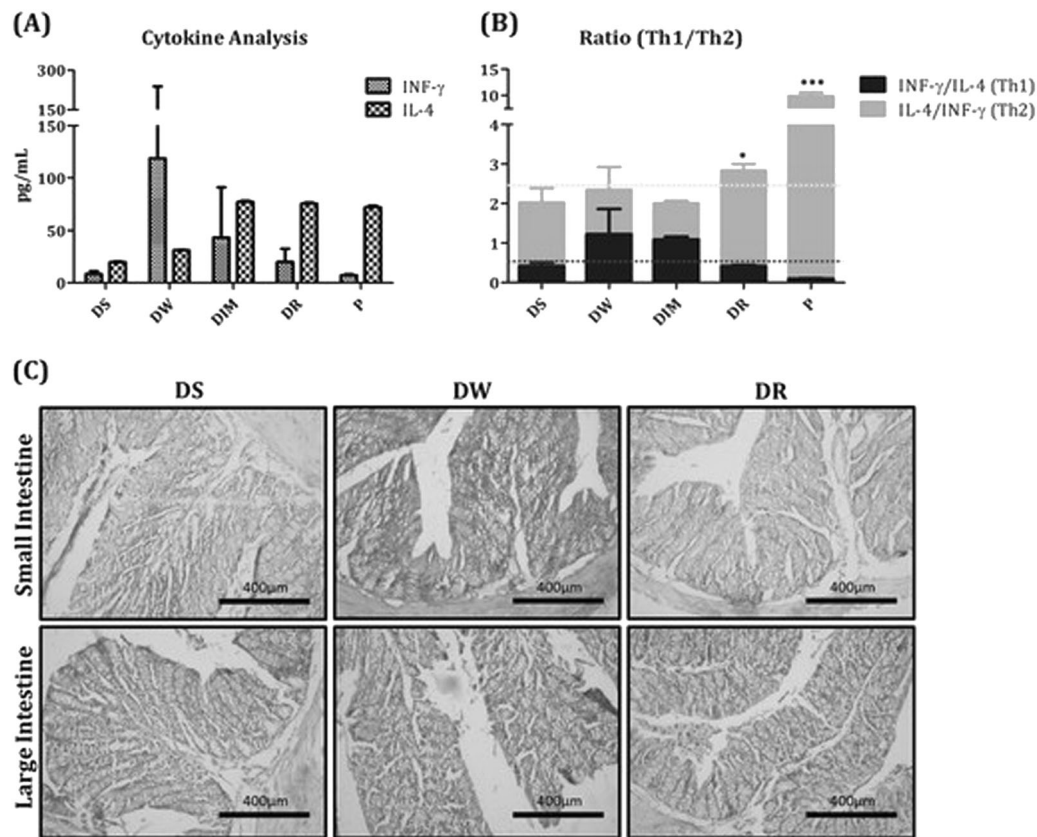
Fig. 4 Cellular immune response of immunized animals and safety profile of *LacVax*®. **a** INF-γ and IL-4 levels in all the immunized animals. **b** INF-γ/IL-4 cytokines ratio (an indicative Th1/Th2 ratio) of all immunized groups. **c** H&E staining of the small intestine and large intestine samples of DS, DW, and DR group

(*LacVax*® *DNA-I*) and evaluated the immunogenic potential of *LacVax*® *DNA-I*, using EGFP as a model antigen. Moreover, the cellular and humoral response elicited following *LacVax*® *DNA-I* immunization were compared with the conventional vaccination approaches.

EGFP has been widely used as an intracellular reporter molecule to assess the gene transfer [20]. Mancha-Agresti et al. [21] in 2016 have shown the expression of EGFP in enterocytes of BALB/c mice following the delivery of pExu:*egfp* by *L. lactis* MG1363 [21]. Moreover, Stripecke et al. in 1999 have reported immunogenic potential of EGFP to induce cellular and humoral immunity in BALB/c mice [22, 23] and rhesus macaque monkey [24]. Hence, in the present study, EGFP was employed to assess gene delivery and to compare and characterize immune response following alternate vaccination strategies.

Oral administration of purified naked plasmid DNA harboring GFP or GFP variants result in no *gfp* expression at mRNA and protein levels [25–27], indicating complete degradation of plasmid administered via oral route. In contrast, serum IgG, fecal sIgA, and cellular responses achieved with oral administration of our *LacVax*® *DNA-I* suggest not only protection of the plasmid; pPERDBY from degrading enzymes but also indicate efficient delivery of the

plasmid DNA to epithelial cells and antigen-presenting cells (APCs). These findings reinforce the potential of *LacVax*® *DNA-I* in surviving the enzymatic environment and low pH of the stomach.

Moreover, oral inoculation of *LacVax*® *DNA-I* resulted in an increase in EGFP-specific IgG and sIgA. The re-stimulation of splenocytes of *LacVax*® *DNA-I* immunized animals with EGFP-OmpH resulted in an indicative Th2 cytokine response. Previous studies by J-M Chatel et al. demonstrated the delivery of BLG cDNA using non-invasive *L. lactis* to BALB/c mice. However, they failed to detect fecal sIgA, IgG, and cytokine secretion after re-stimulation with purified BLG in vitro [28]. The probable reasons for the observed differences could be: (a) the differences in the backbone of the plasmids used in both the studies, (b) the large size of the plasmid used; pLIG-BLG1 (10.9 kb) [17, 28], whereas our constructed pPERDBY is one of smallest DNA vaccine reporter plasmid (~4.9 kb) [3]. The small plasmid size facilitates better transformation efficiency [29], which induces a strong immune response, (c) The antigen in both the studies was different, attributing towards the observed immune differences.

Our hypothesis of immune elicitation following the in vivo delivery of pPERDBY by food grade lactic acid bacteria
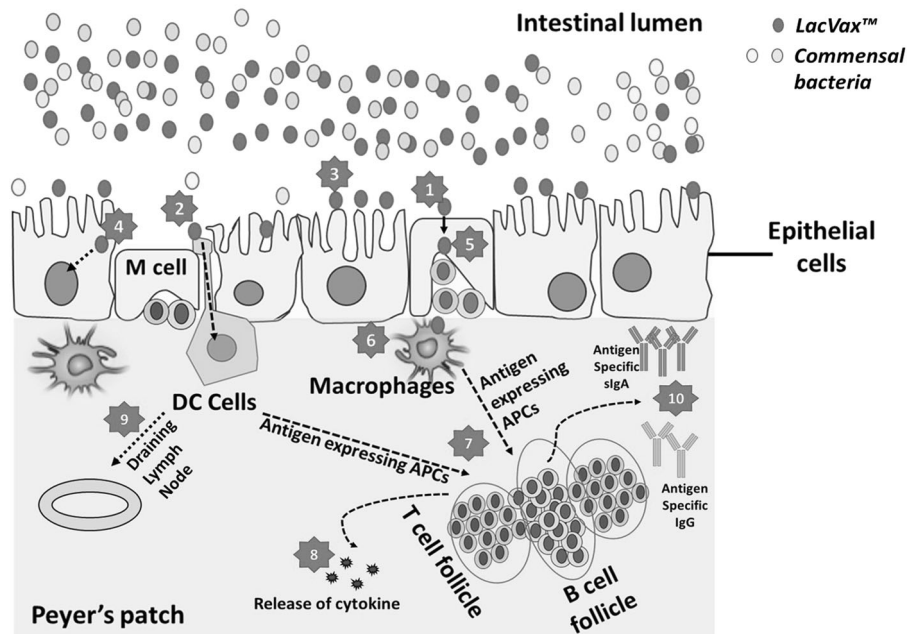
Fig. 5 Hypothesis of in vivo DNA delivery and immune stimulation by *LacVax*®. (1) Entry of *LacVax*® inside M cells; (2) sampling of antigen (*LacVax*®) by DC cells; (3) adhesion of *LacVax*® to epithelial cells; (4) endocytosis of *LacVax*® inside epithelial cells and DNA delivery to nucleus; (5) transfer of *LacVax*® to B cells and/or T cells via M cell pocket; (6) antigen uptake (*LacVax*®) by macrophages; (7) antigen presentation to T cells and B cell follicles by antigen loaded APCs; (8) release of cytokines by antigen stimulated T cells; (9) movement of antigen captured DC cells towards draining lymph nodes; (10) production of IgG and sIgA by stimulated B cells

(LAB) is adhesion of *L. lactis* to epithelial cells or M cells [30], resulting in the entry of *L. lactis* inside epithelial cells or M cells [3, 27, 31]. The internalized bacteria is being lysed inside epithelial cells or gets translocated via M cells. The M cell translocated bacteria may get engulfed and lysed by antigen-presenting cells (APCs) [32]. The plasmids released from the lysed bacteria enter into the nucleus of epithelial cells or APCs, resulting in gene expression and antigen presentation (Fig. 5) [2]. The proposed hypothesis is strengthened by other researchers and astonishing observation that certain LAB strains and commensal bacteria such as *Enterobacter cloacea* are known to penetrate dendritic cells and able to reside within the cells for several days [3, 28, 33–35]. The outcomes and the hypothesis of the present study are in line with the recent findings of Mancha-Agresti et al., 2016, who could successfully show EGFP expression in the enterocytes of BALB/c mice following the delivery of pExu:*egfp* using non-invasive *L. lactis*. However, they have not evaluated the immunogenic response elicited against the model antigen EGFP [21].

Although, the i.m. injection of plasmid pPERDBY resulted in gradual increase in EGFP-specific IgG, the significant increase in IgG levels was evident only on the last booster dose (day 49th). In contrast, oral immunization with *LacVax*® *DNA-I* resulted in significant rise in IgG antibodies immediately after the third dose (20th day). The observed differences in the immune response between the two groups could be due to the differences in the route of immunization and the effective antigen concentration, presented to the immune system. Moreover, i.m. injection of naked DNA failed to produce significant sIgA, indicating the shortcoming of intramuscularly injected DNA vaccines. These findings are in accordance with the previous studies which demonstrated the weak potential of intramuscularly injected plasmid DNA in evoking mucosal immunity [2, 36, 37]. Interestingly, i.m. injection of pPERDBY resulted in an indicative Th1 response, whereas delivery of the same plasmid via *L. lactis* resulted in an indicative Th2 response, attributing immunomodulatory adjuvant properties of *L. lactis* [38, 39]. Moreover, the observed differences in cytokine profile of DS and DW groups also support the immunostimulatory properties exerted by wild-type *L. lactis* upon repeated administration to BALB/c mice. The high ratio of Th2/Th1 response in DR mice can be correlated with a high and early rise in IgG of the DR group.

Though protein immunization resulted in highest IgG, IgA titers and an indicative Th2 cellular response, poor stability of protein vaccine candidate, batch to batch variability and inability to induce an indicative Th1 immune response, discourage its application as a subunit vaccine for enteric pathogens [10]. Moreover, protein immunization via the oral route results in antigen degradation and weak immune stimulation [40]. Remarkably, the *LacVax*® *DNA-I* immunization could achieve similar sIgA response and better Th1 response as compared to protein immunization, reinforcing the potential of *LacVax*® *DNA-I* as an oral mucosal vaccine carrier.

Table 1 Bacterial strains and plasmids used in the study

Strains or plasmids	Characteristics	Reference
Strains		
E. coli BL21 (DE3)	F⁻ *omp*T *hsdS*(rb⁻mb⁻) *gal dcm* λ(DE3) *nin*5 *lac*UV-T7	Lab Source
r- *E. coli* BL21 (DE3):: pCOLDII:EGFP-OmpH	Amp^r, *E. coli* BL21(DE3) harboring pCOLDII:EGFP-OmpH plasmid, lac operon	This work
L. lactis NZ9000	*L. lactis* subsp. *cremoris* (derivative strain of MG1363, carrying nisRK genes on the chromosome)	Gift from Dr. Luis Bermudez-Humaran, INRA, France
LacVax® *DNA-I* (r-*L. lactis::* pPERDBY)	Cm^r, NZ9000 harboring pPERDBY	[3]
Plasmids		
pCOLDII-EGFP-OmpH	Amp^r, His-tagged *egfp*, lac operon, EGFP is fused with a small peptide, outer membrane protein H (OmpH) of ~2.1 kDa. OmpH is M cell ligand, used for targeted mucosal delivery	Gift from Dr. Yong-Suk Jang
pPERDBY	Cm^r, P$_{CMV}$ fused to *egfp* gene, *E. coli*-LAB shuttle vector. GenBank Accession No.: KR072508.1	[3]

Chloramphenicol (Cm) was used at 10 μg/ml for *E. coli* and *L. lactis*. Ampicillin (Amp) at 100 μg/ml was used for *E. coli*

In conclusion, the developed non-invasive *LacVax*® *DNA-I* delivery platform is not only capable of delivering pPERDBY but could also elicit systemic and mucosal immune response. The *LacVax*® *DNA-I*, as a vaccine delivery platform, offers several benefits such as low production cost, safe profile, and needle-free administration which is convenient for mass immunization [41]. Furthermore, the immunostimulatory adjuvant properties of *L. lactis* help to counteract vaccine loss and make up for the lower immunogenicity of non-living DNA vaccines [2, 10, 42]. Moreover, the present work for the first time outlines the immune comparison of orally administered *LacVax*® *DNA-I* with conventional vaccination approaches. Efficient antigen delivery at the intestinal tissue is one of the major hurdles in the mucosal vaccine development.

The promising findings of the present work demonstrate the potential of *LacVax*® *DNA-I*-based platform in targeting antigens of enteric pathogens at various mucosal sites. However, further studies involving detailed characterization of Th1 and Th2 cytokines and in vivo challenge are warranted to establish the effectiveness of *LacVax*® *DNA-I* as a vaccine delivery platform. Currently, we are evaluating the immunogenic potential of *LacVax*® *OmpA-I*, harboring *ompA* gene of SD-1 in BALB/c mice in line to provide candidate *Shigella* vaccine.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in the study are listed in Table 1. *E. coli* DH5α and BL21 (DE3) strains were grown in Luria-Bertani (LB) medium at 37 °C with vigorous shaking. *L. lactis* NZ9000 was grown statically at 30 °C in M17 medium (Difco Laboratories, USA) supplemented with 0.5% glucose (GM17). Antibiotics were added at the indicated concentration as necessary: Chloramphenicol (Cm), 10 μg/ml for *E. coli* and *L. lactis* and Ampicillin (Amp), 100 μg/ml for *E. coli*.

Construction of recombinant *E. coli* BL21 (DE3):: pCOLDII-EGFP-OmpH strain

Chemical (CaCl$_2$) competent cells of *E. coli* BL21 (DE3) were prepared and transformed with pCOLDII-EGFP-OmpH, received as a gift sample from Dr. Yong-Suk Jang [43], by heat-shock method as described by Sambrook and Russel [44]. Transformants were selected on Luria Agar (LA) plates containing 100 μg/ml of Ampicillin. The plasmid was purified from r-*E. coli* culture using GeneJet Plasmid Miniprep Kit (Thermo Fischer Scientific) according to the manufacturer's protocol. The presence of *egfp* was detected using the following oligonucleotides: EGFP-Forward: 5′-AGCGTCGACATGGTGAGCAAGGGCG-3′ and EGFP-Reverse: 5′-ACGGATATCGGTTTCACGCGC AGGTA-3′. Restriction enzymes (REs), *i.e.*, *Eco*RV and *Hin*dIII and polymerase chain reaction (PCR) components (*Taq* polymerase, dNTPs, MgCl$_2$, and *Taq* polymerase buffer) were used as recommended by the manufacturer (Thermo Scientific, USA).

Protein expression and purification

Recombinant *E. coli* BL21 (DE3) cells harboring pCOLDII-EGFP-OmpH were used to express EGFP-OmpH. Briefly, r-*E. coli* BL21 (DE3) strain was grown at 37 °C at 180 revolutions per minute (rpm) until an optical density of 600 nm (OD$_{600}$) was reached to 0.4–0.6. This followed by refrigerating the culture at 15 °C for 30 min. The culture

was then induced with 0.4 mM isopropyl-β-D-1-thioga-lactopyranoside (IPTG) and incubated at 15 °C for 24 h with continuous shaking at 180 rpm. To ensure the induction of EGFP-OmpH expression, induced and uninduced cells were subjected to 12% SDS-PAGE.

Recombinant His-tagged EGFP-OmpH was purified by nickel affinity chromatography as described by Yagnik et al. [5]. Briefly, induced recombinant cells were sonicated in lysis buffer (8 M Urea; 0.1 M NaH_2PO_4; 0.01 M Tris-Cl; pH 8.0) and centrifuged at 10,000 g for 30 min. The lysate was then mixed with 50% Ni-NTA slurry (4:1) and kept at 4 °C for 16 h. Ni-NTA slurry was loaded on the column and the EGFP-OmpH protein was eluted using acidic elution buffer (8 M Urea; 0.1 M NaH_2PO_4; 0.01 M Tris-Cl; pH 4.5) after washing with wash buffer (8 M Urea; 0.1 M NaH_2PO_4; 0.01 M Tris-Cl; pH 6.3 and 5.9). Affinity purified fraction was dialyzed in the phosphate-buffered saline (PBS; 10 M Na_2HPO_4; 137 M NaCl; 2.7 M KCl and 1.8 M K_2HPO_4) and quantified by Bradford assay [8]. Different fractions of purified protein were then subjected to 12% SDS-PAGE.

Stability of DNA vaccine candidate

Stability of pPERDBY in our previously constructed recombinant *L. lactis* NZ9000 (*LacVax*® *DNA-I*) [3] was evaluated as described by Hongying et al. [45], with minor modifications. A frozen glycerol stock of r-*L. lactis* NZ9000::pPERDBY was diluted (1:25) in GM17 broth and grown to an OD_{600} of 0.9. The preliminary culture was further sub-cultured at least 25 times in GM17 broth and grown at 30 °C statically. Both the cultures were plated on GM17 agar with or without chloramphenicol (10 μg/ml) and incubated at 30 °C for 16 h. The numbers of colony-forming units (CFU) recovered on GM17 agar plates with or without chloramphenicol were compared with the CFU count of *LacVax*® *DNA-I* at the time of glycerol stock preparation in order to evaluate the stability of pPERDBY in r-*L. lactis* NZ9000. The percentage stability was calculated using the following equation:

$$\text{Stability}(\%) = \frac{\text{CFU count of } LacVax^® DNA-I \text{ at the time of revival}}{\text{CFU count of } LacVax^® DNA-I \text{ at the time of glycerol stock preparation}} \times 100$$

Animal immunization

Six- to eight-week-old female BALB/c mice, weighing 25–30 g, were used in the study. Pathogen-free BALB/c mice were procured from Mahaveera Enterprises, Hyderabad, India and housed in B. V. Patel PERD Centre's animal house in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. All animal experiments were done in accordance with the institutional animal ethics committee.

Animals were randomly divided into different groups by a technician who was blind to the study design. However, the investigators were not blinded to the group allocation or analysis. Groups of six mice were immunized as described in Table 2. For DNA immunization, 30 μL of purified endotoxin-free plasmid pPERDBY (0.83 μg/μl) was administered intramuscularly. For protein immunization, purified EGFP-OmpH (3 μg/33 μl) was emulsified with two volumes of Freund's complete adjuvant and for subsequent immunizations with Freund's incomplete adjuvant and administered intramuscularly. Saline and wild-type *L. lactis* NZ9000 receiving groups were used as controls in the present study. The *LacVax*® *DNA-I* and wild-type *L. lactis* NZ9000 cultures were grown as described in the Materials and methods section and resuspended to the 10^8 CFU per 100 μl for oral immunization.

Enzyme-linked immunosorbent assay (ELISA) for the detection of EGFP-specific antibodies

Antigen-specific serum IgG and fecal secretory IgA (sIgA) antibodies were detected by ELISA as described by Yagnik et al. [5], with minor modifications. Briefly, test wells of 96-well microtiter plates (Medisorp, Thermo Scientific Nunc) were coated with 300 ng per well of purified EGFP-OmpH whereas 100 μl of PBS was added to the blank wells. Plates were then incubated at 2–8 °C overnight followed by decanting the unbound antigen. Free sites were blocked for 3 h with 250 μl of 2% bovine serum albumin in PBS at 37 °C. Serum sample (100 μl; 1:100 dilution) or homogenized fecal sample (100 μl; 1:5 dilution) was added and incubated for 2 h at 37 °C. After incubation, wells were washed thrice with 100 μl of wash solution [PBS containing 0.02% (v/v) Tween 20]. For detection of bound antibodies, 100 μl of HRP-conjugated goat anti-mouse antibodies were added at 1:1000 dilution and incubated for 2 h at 37 °C. After incubation, wells were washed thrice with 100 μl of wash solution. Bound antibodies were revealed with TMB (3,3′,5,5′-tetramethylbenzidine) substrate solution in sterile water and incubated in dark at 37 °C for 15 min. The reaction was stopped by the addition of 50 μl of 2 M H_2SO_4. Absorbance at 450 nm was read with a Multiskan GO microplate spectrophotometer (Thermo Scientific, USA).

Cytokine profiling

All mice were sacrificed on the seventh day of the last booster dose and spleens were removed. Splenocytes were isolated as described previously by Pore et al. [46] and Ahmed et al. [40], with minor modifications. Briefly, the single cell suspension was prepared from isolated spleen in Hanks balanced salt solution medium using a 40 μm nylon cell strainer. After erythrocytes lysis, cells were

Table 2 Details of immunization

Antigen	Group symbol	Route of immunization	Dose description	Per dose concentration	Solvent/supplement	Dosage regimen (day)	Blood withdrawal (day)	Fecal sample collection (day)
LacVax® DNA-I (r-*L. lactis*::pPERDBY)	DR	Oral	Bacteria	10^8 CFU/500 µL	PBS	Six doses at the interval of seven days	0, 20, 43, and 49	0, 20, 43, and 49
Naked plasmid (pPERDBY)	DIM	Intramuscular (i.m.)	Plasmid DNA	25 µg/30 µL	Tris-EDTA			
Purified EGFP-OmpH Protein	P	Intramuscular (i.m.)	Protein	3 µg/100 µL	Freud's complete/ incomplete adjuvant			
Wild-type *L. lactis* NZ9000	DW	Oral	Bacteria	10^8 CFU/500 µL	PBS			
Saline	DS	Oral	Phosphate-buffered saline (PBS)	500 µL	PBS			

resuspended in RPMI 1640 medium at a concentration of $3 \times 10^6/0.5$ mL and cultured in a 24-well plate. Cells were stimulated with 3 µg EGFP-OmpH protein for 24 h. Thereafter, supernatants were collected and analyzed for quantification of IL-4 and INF$^-\gamma$ using mouse Th1/Th2 ELISA Ready-SET-Go® kit (eBioscience), according to the manufacturer's instructions.

Histopathological staining and examination

In order to determine the safety profile of orally administered *LacVax® DNA-I*, the intestinal tissues of *LacVax® DNA-I* immunized mice were collected and compared with the intestinal tissues of saline and wild-type *L. lactis*-immunized animals. All the animals were immunized as described in Table 2. After the seventh day of the last booster dose, animals were euthanized and small and large intestine from the DS, DW, and DR group were excised. Tissues were subjected to hematoxylin and eosin (H&E) staining. The stained tissue samples were examined under the light microscope (Olympus Model IX51).

Statistical analysis

Statistical analyses were performed using GraphPad Prism (trial version 5). One-way ANOVA with post hoc Bonferroni's multiple comparison test was used to determine the statistical significance of cytokine levels and sIgA among experimental groups at different time intervals. Two-way ANOVA with post hoc Bonferroni's multiple comparison test was used to determine the significant differences in IgG levels of different groups at different time intervals. Data are presented as the mean ± standard error of mean (SEM). *P*-values of <0.05 were considered as significant.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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