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Oral immunization with *LacVax*® OmpA induces protective immune response against *Shigella flexneri* 2a ATCC 12022 in a murine model

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ABSTRACT

Shigellosis is an acute invasive disease of the lower intestine, which afflicts millions of people worldwide with an estimated one million fatalities per annum. Despite of extensive research during the last two decades, a vaccine against multi-drug resistant *Shigella* is not yet available in the market. To provide a safe, effective and broad-spectrum vaccine against *Shigella*, we explored food grade bacteria *Lactococcus lactis* (*L. lactis*) for the delivery of conserved antigenic protein; Outer membrane protein A (OmpA) to the mucosal sites for effective elicitation of systemic and mucosal immunity. We have previously confirmed the immunogenic potential of recombinant *L. lactis* expressing OmpA (*LacVax*® OmpA) in BALB/c mice. In the present study, we have characterized the humoral and cellular immune profile of *LacVax*® OmpA and assessed its protective efficacy using a newly developed human like murine shigellosis model. The significant increase in OmpA specific serum IgG, fecal sIgA and a Th1 dominant immune response (indicated by high INF-γ/IL-4 ratio) in *LacVax*® OmpA immunized mice revealed successful activation of humoral and cellular immunity. The *LacVax*® OmpA immunized animals were also protected from human-like shigellosis when challenged with *S. flexneri* 2a ATCC 12022. The antigen specific serum IgG, fecal sIgA, INF-γ and IL-10 levels were found to be the significant correlates of protection. Collectively these results suggest that the *LacVax*® OmpA is a promising prophylactic candidate against shigellosis. However, the protective efficacy of *LacVax*® OmpA in the higher animals would further strengthen its future application in humans.

1. Introduction

Shigella, a Gram negative bacteria, is an important etiological agent responsible for acute invasive bloody dysenteriae, commonly known as shigellosis. This bacillary dysenteriae is responsible for childhood morbidity and mortality and remains a major public health problem [1]. Recurrence of *Shigella* infections in children also results in poor absorption of nutrients in the intestine which can lead to stunted growth, impaired cognitive development and various long-term health problems [2]. The disease burden is maximum in resource-poor settings where as many as 167 million diarrheal episodes are reported. Every year, around 600,000 deaths occur due to shigellosis, of which, 500,000 cases are reported amongst military personnel and travelers from industrialized countries. The major factors responsible for high incidences of shigellosis in developing countries are lack of clean water, poor sanitation and malnutrition [3]. While public health strategies to reduce exposure and transmission are effective, their establishment in many developing countries, especially in the context of conflict or mass displacement of susceptible person remains challenging [4].

To control the *Shigella* infection, antibiotics along with oral rehydration therapies are generally used. However, during the last decade, *Shigella* strains resistant to Ampicillin, Chloramphenicol, Nalidixic Acid, Tetracycline, Trimethoprim Sulfamethoxazole and Ciprofloxacin have been isolated with the increasing frequency in Asia and Africa [5,6]. The rapid emergence of multi-drug resistant *Shigella* spp. and the increasing number of infected persons in developing countries pose an urgent need to develop an effective prophylactic against *Shigella* [7]. Despite of numerous efforts in

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the past few decades, at present there is no licensed vaccine available in the market while several other candidate vaccines are currently at different pre-clinical and clinical stages [3].

Shigella spreads through contaminated food and water and reaches to colon wherein it enters intestinal epithelium preferentially *via* M cells. The internalized bacteria spreads intracellularly resulting in inflammation, followed by cell death and dysenteriae. Hence, the desirable vaccine candidate should elicit efficient systemic as well as mucosal immune response.

In this regard, our group has explored food grade *Lactococcus lactis* as an antigen and DNA vaccine delivery vehicle against enteric pathogens such as *Shigella* [8–11]. We have developed non-invasive and invasive r-*L. lactis* harbouring DNA vaccine reporter plasmid and evaluated DNA delivery potential of these strains *in vitro* as well as *in vivo* [9,11]. Oral immunization with r-*L. lactis* harbouring DNA vaccine reporter plasmid; pPERDBY (*LacVax® DNA-I*) resulted in systemic and mucosal immune responses against model antigen EGFP [12].

The use of food grade *L. lactis* as an antigen (DNA/Protein) delivery vehicle offers several advantages over attenuated pathogens. The immunostimulatory properties, capability to survive and transit through stomach and a GRAS (generally regarded as safe) status have paved the way for its application in mucosal delivery of numerous antigens and therapeutic candidates [13–17]. *L. lactis* as a live vaccine vector has been exploited for the delivery of listeriolysin O (LLO) protein of *Listeria monocytogenes* [13], SARS-coronavirus nucleocapsid protein [18], tetanus toxin fragment C (TTFC) [19] and Cag12 antigen of *Helicobacter pylori* [20]. Heine et al., in 2015 has exploited *L. lactis* to develop non-living bacterium-like particles (BLPs), displaying IpaB and IpaD, and showed the induction of protective immunity against different strains of *Shigella* in adult and infant mice [21].

Using live *L. lactis*, our group has also demonstrated, for the first time, the immunogenic potential of live r-*L. lactis* expressing outer membrane protein A (OmpA) of *Shigella dysenteriae* type-1 (SD-1) in BALB/c mice. The oral immunization of r-*L. lactis*::pSEC:OmpA (*LacVax®* OmpA) resulted in better systemic and mucosal immune response against OmpA than the intranasal route of immunization. In the present study, we have further characterized the immune responses following the oral immunization with *LacVax®* OmpA and defined the correlates of protection. Moreover, using our newly developed in-house shigellosis murine model [22], we have evaluated the protective efficacy of *LacVax®* OmpA. To the best of our knowledge, this is the first report describing the potential application of live *L. lactis* based vaccine delivery platform in providing protective immune response against *S. flexneri*.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture condition

The bacterial strains and plasmids used in the present study are listed in Table 1. Briefly, *S. flexneri* 2a ATCC 12022 was grown in nutrient broth (HiMedia Laboratories, India) at 37 °C at 180 rpm for 14 h. *Lactococcus lactis* NZ9000 and r- *L. lactis*::pSEC:OmpA (*LacVax®* OmpA) strains were grown statically at 30 °C in M17 broth (Difco Laboratories, Franklin Lakes, NJ, USA) supplemented with 0.5% (w/v) sterile glucose (GM17). Chloramphenicol was added at 10 μg/mL concentration for *LacVax®* OmpA.

2.2. Animals

Around 6–8-week-old, pathogen free, female BALB/c mice, weighing 25–30 g, 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. Groups of three mice were housed in polypropylene cages with sterilized bedding under controlled lighting (12-h light, 12-h dark), temperature (25 °C), and relative humidity (55%). The mice were given free access to food and sterilized water. Sterilized water bottles and bedding material were replaced every 3 days. All animal experiments were done in accordance with the institutional animal ethics committee.

2.3. Immunization with LacVax® OmpA

The schematic representation of study design is depicted in Fig. 1. Briefly, animals were divided in three groups. Each group of twelve mice was orally immunized with either 10^{10} CFU of control strain *L. lactis* NZ9000 (OW) or recombinant vaccine strain; *LacVax®* OmpA (OR) or phosphate buffered saline (PBS) (OS). Bacterial strains were grown as mentioned in Section 2.1 and induced with 10 ng/mL of nisin for 1 h. Cells were harvested by centrifugation at 6000*g* for 10 min and resuspended in 0.5 mL of PBS to obtain 10^8 CFU. Blood samples were collected 0th, 21st, 42nd and 63rd day and fecal samples were collected on 0th, 48th, and 63rd day (Table 2). On day 63, each group was further randomly sub-divided into two sub-groups (n = 6, each group), of which, one sub-group was challenged with *S. flexneri* 2a ATCC 12022 and the other group was sacrificed for splenocytes isolation and other pathophysiological parameters.

2.4. OmpA specific serum IgG and fecal IgA antibodies

OmpA specific serum IgG and fecal IgA antibodies were detected by Enzyme Linked Immunosorbent Assay (ELISA) as described by Yagnik et al. [10]. The ELISA results are expressed as the Optical Density (OD) values measured at 405 nm with a Multiskan GO microplate spectrophotometer (Thermo Scientific, USA) for dilutions of 1:1000 for serum and 1:2 for fecal extracts.

2.5. Cytokine profiling

Six mice from each immunized sub-group were sacrificed on the 14th day of the last booster and spleens were removed. Spleno-

Table 1
Bacterial strains and plasmids used in the study.

Strains or plasmid	Characteristics	Reference
Strains		
Shigella flexneri 2a (ATCC 12022)	Pathogenic strain responsible for shigellosis	Lab Source NCBI Accession No.: KX826786
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® OmpA (r- *L. lactis*::OmpA)	Cm^r, NZ9000 harbouring pSEC:OmpA	[10]
Plasmid		
pSEC:OmpA	Cm^r, *usp45* secretory signal sequence, *ompA* gene under the P_{NisA} promoter, *E. coli*-LAB shuttle vector	[8]

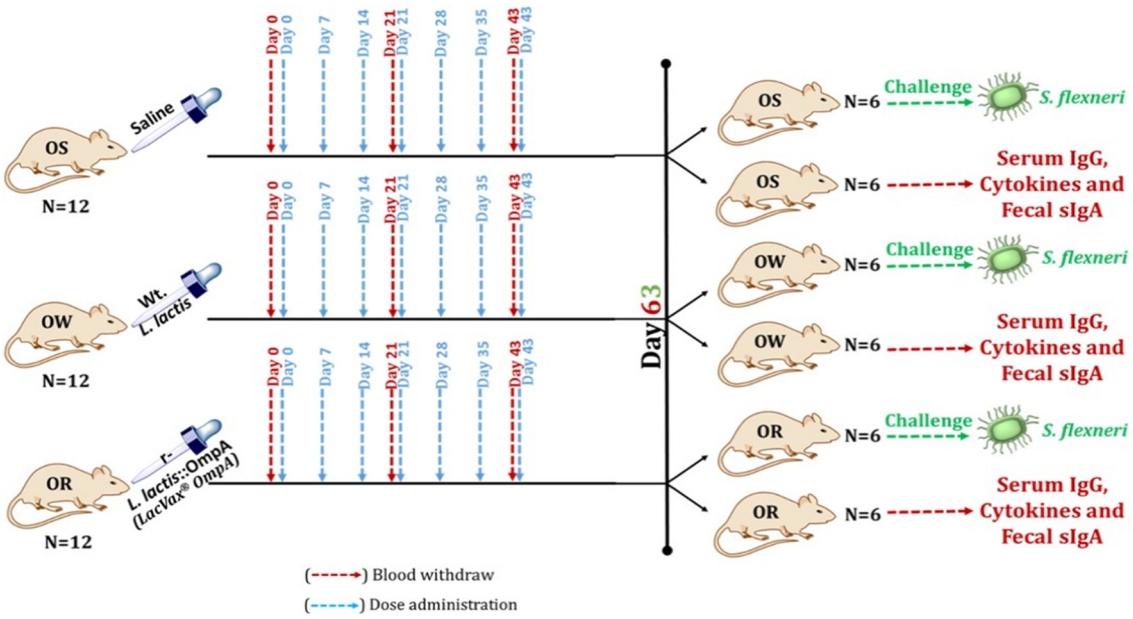

Fig. 1. Schematic representation of the study design.

Table 2
Animal immunization regimen and dose description.

Antigen	Group symbol	Route of immunization	Dose description (per dose)	Dosage regimen	Blood withdrawal (on day)	Fecal sample collection (on day)
LacVax® OmpA (r-*L. lactis*::OmpA)	OR	Oral	10^9 CFU of bacteria resuspended in 500 μL PBS	Five doses at the interval of seven days	0th, 20th, 43rd and 49th	49th and 63rd
Wild type *L. lactis* NZ9000	OW	Oral	10^9 CFU of bacteria resuspended in 500 μL PBS			
Saline	OS	Oral	500 μL PBS			

cytes were isolated as previously described by Pore et al. [1]. Briefly, the single cell suspension was prepared in Hanks balanced salt solution medium from isolated spleen using a 70 μm nylon cell strainer (BD Bioscience). Following erythrocytes lysis, cells were resuspended in RPMI 1640 medium containing 10% Fetal Bovine Serum (FBS) at a concentration of 2×10^6 cells per 0.5 mL and cultured in 24 well plate. Cells were incubated at 37 °C and 5% CO_2 for 24 h. Cells were then re-stimulated with purified OmpA for 36 h. Supernatant was then collected and analysed for the quantification of IL-2, IL-4, IL-10 and INF-γ using a mouse Th1/Th2 ELISA Ready-Set-Go® kit (eBioscience) according to the manufacturer's protocol.

2.6. Protective efficacy and pathophysiological observations

In order to assess protective efficacy of *LacVax*® OmpA, six mice from each immunized sub-group were challenged intraperitoneally with 5×10^6 CFU of *S. flexneri* 2a ATCC 12022 as described previously by Sharma et al. [22]. All mice were observed for general physical activity, pathophysiological parameters (body weight, fur ruffling, abdominal swelling, and conjunctivitis), consistency of stool passed and presence of mucus or blood in the feces for 7 days post challenge. Animals were euthanized on day 7 and vital organs such as small intestine, large intestine and spleens were collected. Colon length of all the challenged mice was measured. Collected tissues were subjected to haematoxylin and eosin (H &E) staining.

2.7. Histopathological scoring

Histopathological scores of the stained intestinal sections were blindly scored, ranging from 0 (normal) to 4 (severe). Pathophysiological parameters such as cell death in the crypt, lamina propria, epithelium, muscle layer and epithelium exfoliation were considered to assess the integrity of the whole tissue.

2.8. Defining the correlates of protection

The establishment of *Shigella* infection results in the destruction of host mucosal epithelium and leads to diarrheal condition. Based on this, the cumulative histopathological score (ranging from 0 to 4 and comprised of the individual scores for cell death in the crypt, lamina propria, epithelium, muscle layer and epithelium exfoliation) of intestinal tissue was selected as a quantifiable parameter to define the degree of infection, which is inversely related to the degree of protection. Since the endpoint in the present study is onset of diarrhoea, the integrity of intestinal tissue architecture was used to define the degree of protection and efficacy of the vaccine candidate. The following parameters were evaluated to define the correlates of infection/protection; serum IgG, fecal sIgA, INF-γ, IL-2, IL-10 and IL-4.

2.9. Statistical analyses

All the 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, colon lengths and histopathological scores among different experimental animals. Two-way ANOVA with post-hoc Bonferroni's multiple comparison test was used to determine the statistical significance of serum IgG and fecal sIgA among different experimental animals at different time points.

The linear regression followed by spearman correlation with 95% of confidence interval (CI) was performed to evaluate the correlates of protection.

3. Results

3.1. Humoral response following oral immunization with LacVax® OmpA

We had previously reported the immunogenic potential of orally and nasally administered r-*L. lactis* expressing OmpA (*LacVax*® OmpA) of *S. dysenteriae* type-1 (SD-1) in BALB/c mice [10]. The antibody titer of orally vaccinated animals in the previous study was 1:50, whereas, in the present study, we could achieve 1:1000 anti-OmpA titer by increasing the vaccine dose by 10 fold.

As shown in Fig. 2A, *LacVax*® OmpA immunized mice (OR group) showed a significant increase in anti-OmpA antibodies with the progressive vaccine regimen ($P < 0.001$). The production of antigen specific antibodies was evident immediately after the 3rd dose of *LacVax*® OmpA *i.e.* on 21st day which reached to a maximum titer after 14 days of the last booster (Day 63). However, mice immunized with wt. *L. lactis* (OW) and saline (OS) did not exhibit a significant increase in the antigen specific antibody titers. On day 63rd, OmpA specific IgG levels of OR group were significantly higher than anti-OmpA IgG titers of OW ($P < 0.001$) and OS ($P < 0.0001$) groups (Fig. 2B). The increase in serum anti-OmpA IgG demonstrates the immunogenic potential of the developed vaccine candidate *LacVax*® OmpA.

3.2. sIgA production, a hallmark of mucosal immunity

In order to evaluate whether *LacVax*® OmpA induced mucosal immunity, levels of sIgA, a hallmark of mucosal immunity, were measured in fecal samples of immunized animals. On day 49th, the sIgA levels were significantly higher in OR group as compared to OW and OS groups of mice ($P < 0.01$, **). Interestingly, the rise in sIgA levels was evident even after the 14th day post last booster dose ($P < 0.001$, ***) indicating the strength of *L. lactis* based antigen delivery platform in eliciting a mucosal immune response. The antigen specific sIgA antibodies were not present in fecal samples of OW and OS group on 49th as well as 63rd day (Fig. 2C).

3.3. Th1 dominant cellular response

In order to assess cellular immune response, cytokines were estimated from the supernatant of re-stimulated splenocytes of all vaccinated animals. As shown in Fig. 2D, vaccination with *LacVax*® OmpA resulted in significantly higher production of INF-γ ($P = 0.0093$), a principal Th1 cytokine, IL-2 ($P = 0.0072$) and IL-10 ($P = 0.0131$), as compared to OW and OS groups.

To further investigate the type of the dominant T cell response, ratios of representative Th1 (INF-γ) and Th2 (IL-4) cytokine were calculated. As depicted in Fig. 2E, *LacVax*® OmpA immunized animals showed significantly high INF-γ/IL-4 ratio, suggesting an indicative Th1 immune response as compared to the control animals ($P < 0.0001$).

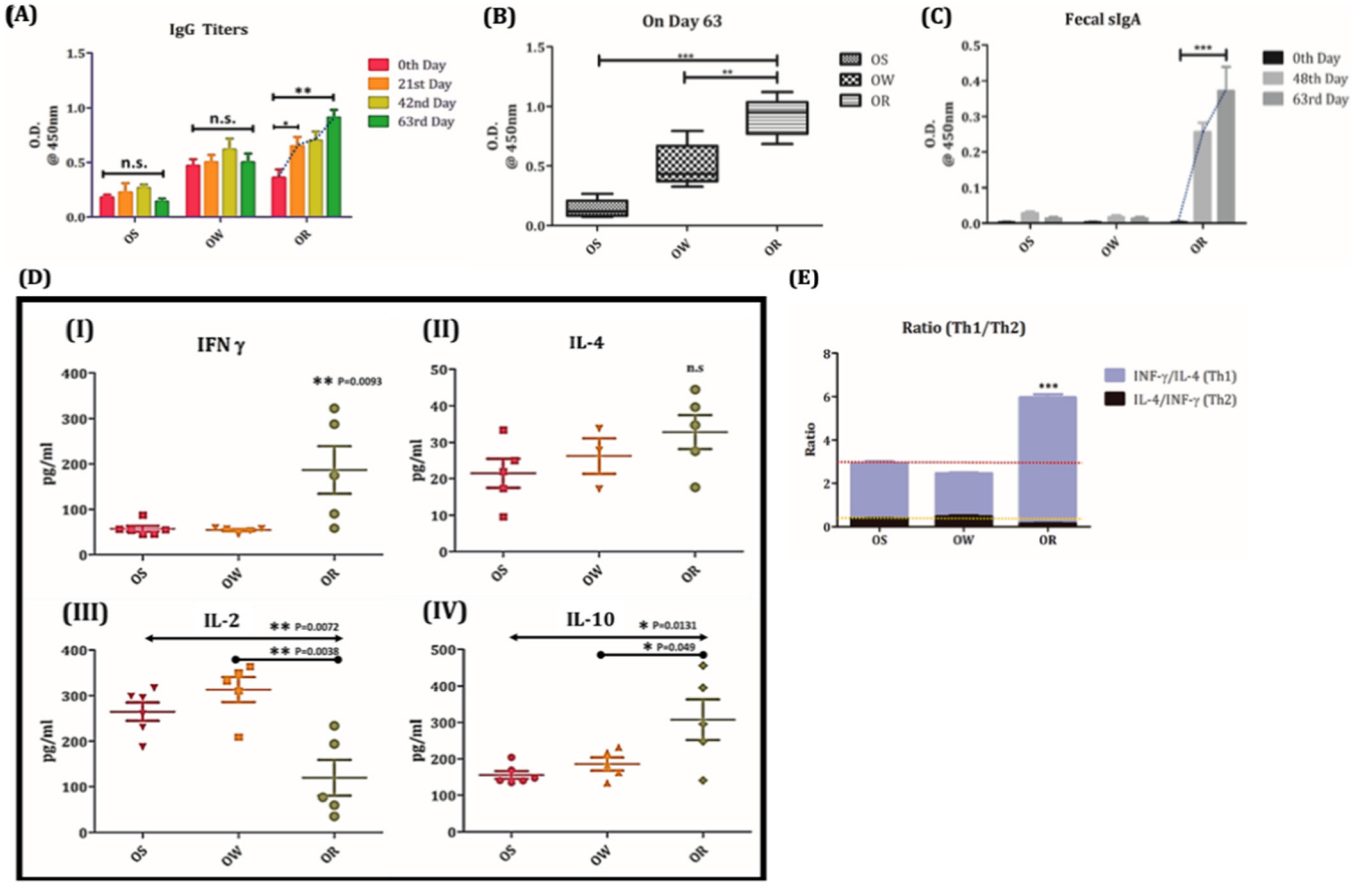

Fig. 2. Humoral and cellular immune response following *LacVax*® OmpA administration in immunized animals. (A) Levels of anti-OmpA IgG antibodies at different time points of vaccination (**$P < 0.001$; n.s. = non-significant). (B) Anti-OmpA IgG antibody levels on the day 63 (**$P < 0.001$; ***$P < 0.0001$). (C) OmpA specific fecal sIgA levels on different time points of vaccination (***$P < 0.001$). (D) Levels of representative Th1 (INF-γ and IL-2) and Th2 (IL-4 and IL-10) cytokines in immunized animals on the day 63. (E) INF-γ/IL-4 cytokine ratio (an indicative of Th1/Th2 ration) of all immunized animals.

3.4. Pathophysiological responses

3.4.1. Diarrhoea

In order to assess the protective efficacy of vaccine candidates, we have previously developed human like shigellosis murine model and established disease parameters to be observed following intraperitoneal *Shigella* infection. Induction of diarrhoea in BALB/c mice is one of the salient features which mimics human shigellosis. In the present study, we did not observe any signs of diarrhoea in *LacVax*® OmpA immunized mice following challenge with *S. flexneri* 2a ATCC 12022 (Fig. 3A). In contrast, the OS and OW group exhibited diarrhoea, similar to that of human shigellosis (Fig. 3A). Interestingly, we also observed a significant difference in the diarrheal symptoms between OW and OS groups. The wild-type *L. lactis* NZ9000 receiving OW group exhibited semi-solid diarrheal episodes, in contrast to saline receiving OS group which exhibited frequent liquid diarrhoea (Fig. 3A). This observation indicates the protective properties of food grade organism *L. lactis* NZ9000 in maintaining the integrity of intestinal tissue during *Shigella* infection.

3.4.2. Conjunctivitis, an indicator of systemic infection

We have earlier reported the development of conjunctivitis as a consequence of the systemic spread of *Shigella* infection in BALB/c mice [20]. In the present study, we observed conjunctivitis in saline receiving BALB/c mice (OS) following *Shigella* infection, which was not the case in OW and OR groups. This observation not only supports the protective efficacy of the vaccine candidate, *LacVax*® OmpA but also strengthens the protective nature of vaccine carrier *L. lactis* in limiting *Shigella* penetration and spread.

3.4.3. Colon length

Reduction in the colon length is one of the key indicator of inflammation and tissue destruction [22]. In the present study, we have measured colon length of all the vaccinated animals before *Shigella* challenge, 24 h post-*Shigella* challenge and 7 days

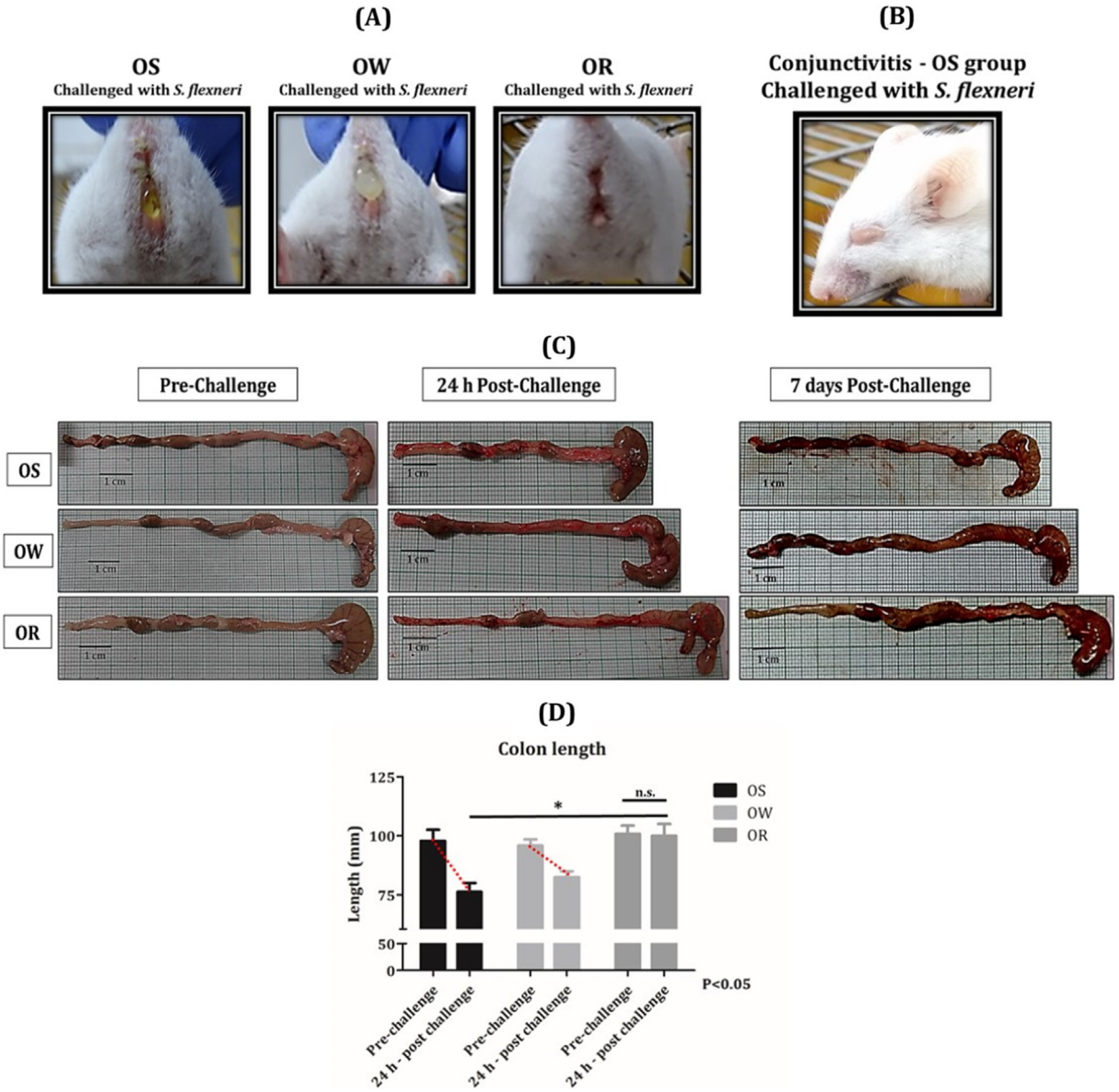

Fig. 3. Pathophysiological parameters of immunized animals following intraperitoneal (i.p.) administration of *S. flexneri* 2a. (A) Photographs of anal region of representative animal from each group following *S. flexneri* 2a infection. The mucus secretion in the OS and OW group of mice indicate bacterial dysenteriae. (B) Photograph of bacterial conjunctivitis in OS group of mice, a hallmark of systemic infection of *S. flexneri* 2a in BALB/c mice. (C) Representative photographs of the colon of immunize animals before and after *S. flexneri* 2a infection. (D) Comparison of the colon lengths (in mm) of all immunized animals before and 24 h after *S. flexneri* 2a infection ($^{*}P < 0.05$).

post *Shigella* challenge. Of note, colon lengths of control animals; OW (P = 0.05) and OS (P = 0.05) were significantly reduced 24-h post *Shigella* infection. Interestingly, there was no change in the colon length of *LacVax*® OmpA immunized mice. After 7 days of *Shigella* infection, the colon length of all the challenged animals was restored to the normal average length, indicating the clearance of *Shigella* infection and associated inflammation.

3.4.4. Histopathological scores of H & E stained intestinal tissues of Shigella challenged animals

The induction of mucosal and systemic immunity prevents *Shigella* penetration, infection and thereby inhibiting the intestinal tissue destruction. Twenty four hours post *Shigella* challenge, the tissue integrity of *LacVax*® OmpA immunized animals did not exhibit any prominent changes as evident by the cumulative histopathological score assigned by sample-blind pathologist, whereas control groups of animals showed severe tissue destruction as marked by cell death in the crypt (P < 0.0001), lamina propria (P < 0.0001), epithelium (P = 0.0017), muscle layer (P = 0.0004) and disturbed whole tissue integrity (P < 0.0001) (Fig. 4).

3.4.5. Correlates of protection

As shown in Fig. 5, the antigen specific serum IgG (P = 0.0001), fecal sIgA (P = 0.0019), INF-γ (P = 0.02) and IL-10 (P = 0.02) levels

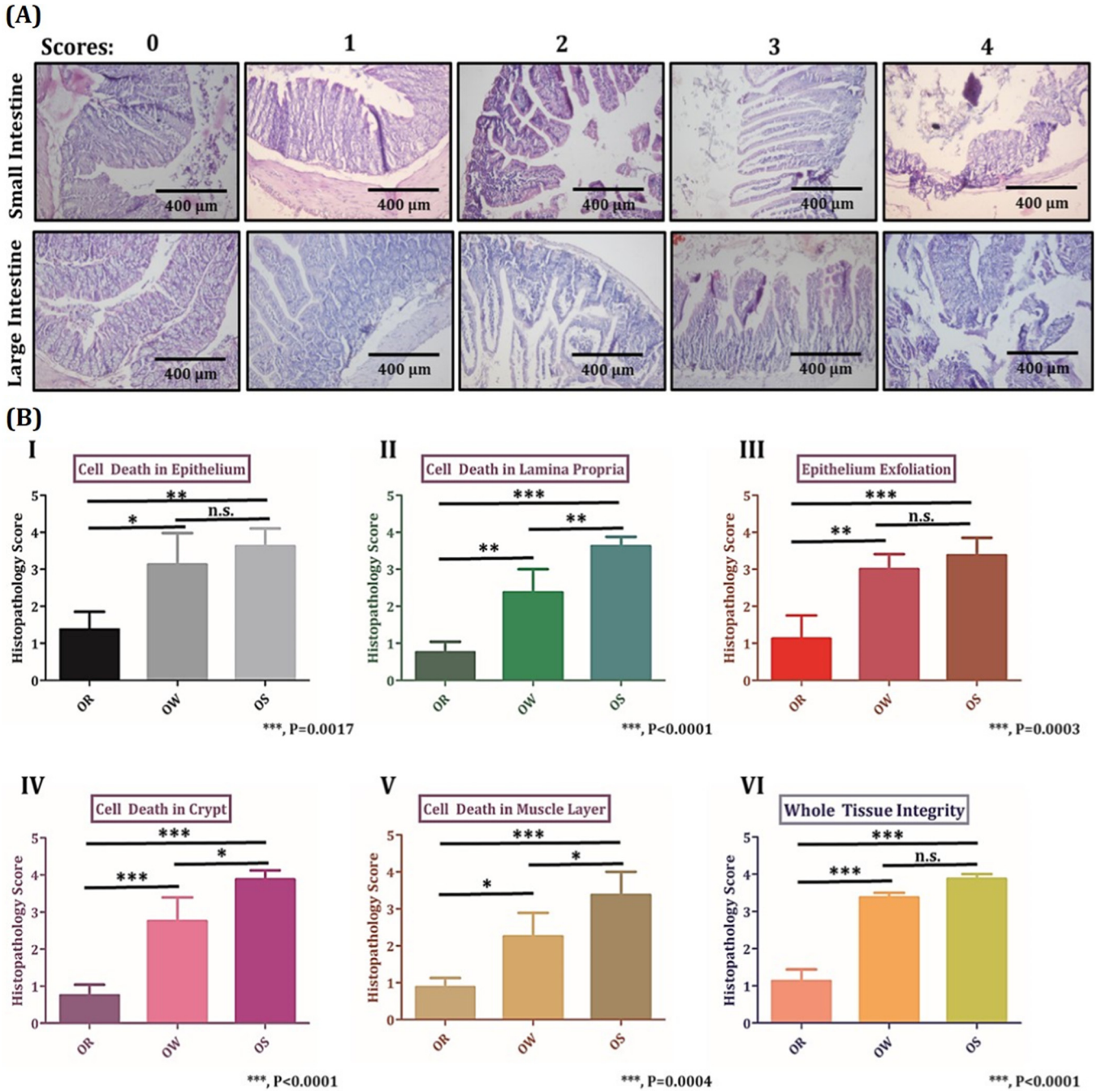

Fig. 4. Histopathological changes in the small and large intestine of immunized animals following intraperitoneal (i.p.) administration of *S. flexneri* 2a. (A) Blind scoring of H & E stained samples of small and large intestine. Scoring is based on overall tissue structure and integrity. The score ranges from 0 to 4 indicating the severity of disease characteristics. (B) The tissue sections of all the animals were further assessed for particular parameters; (I) Cell death in epithelium, (II) Cell death in lamina propria, (III) Epithelium exfoliation, (IV) Cell death in crypt, (V) Cell death in muscle layer, (VI) Whole tissue integrity and blindly scored from 0 to 4 where 0 indicates minimal change and 4 indicated maximum disruption.

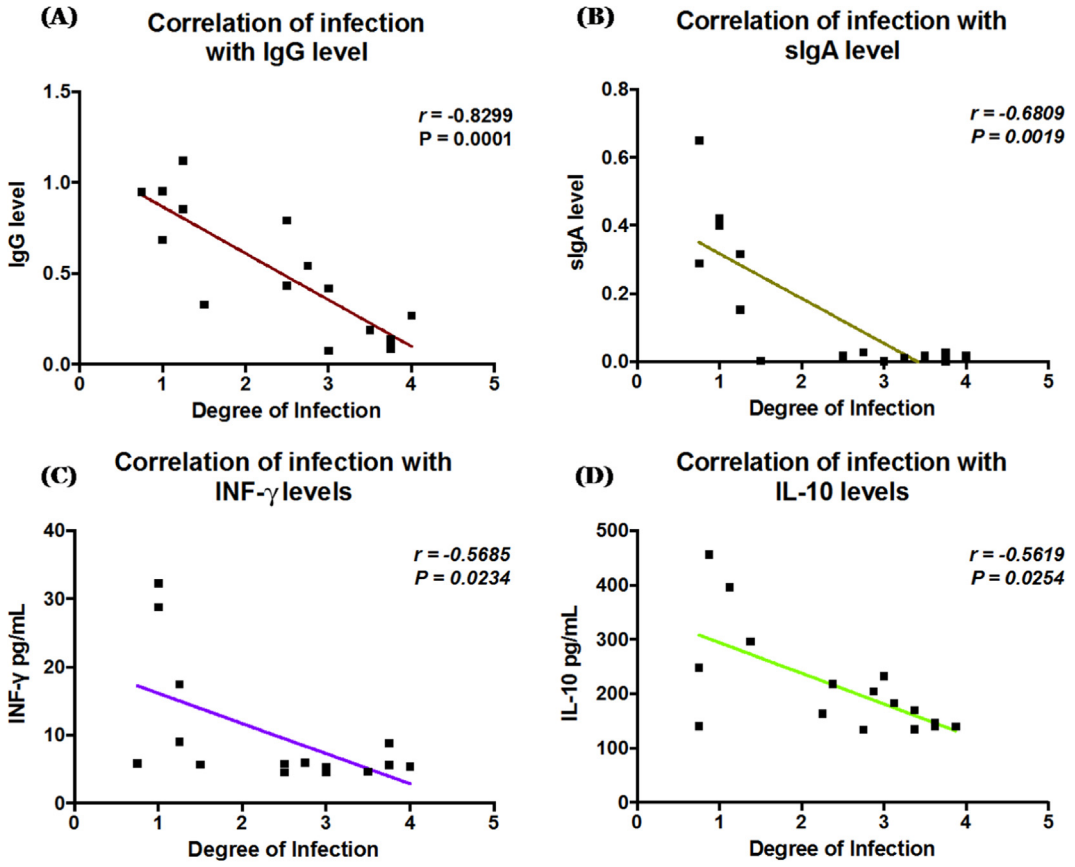

Fig. 5. Correlation of protection. Correlation analysis between the degree of *Shigella* infection (as defined by cumulative histopathological score) and serum IgG (A), fecal sIgA (B), INF-γ levels (C) and IL-10 (D).

were significantly negatively correlated with the degree of infection. Higher levels of antigen specific IgG and sIgA antibodies and cytokine levels of INF-γ and IL-10 in OR group of animals suggest that these immunological parameters protected animals from *Shigella* infection and are strong correlates of protection. We didn't observe significant correlation between degree of infection with any other immunological parameters such as IL-4 (P = 0.85) and IL-2 (P = 0.17) levels (Supplementary Fig. 1).

4. Discussion

Despite of numerous attempts of vaccine development, there is not even a single licensed vaccine available in the market against multi-drug resistant *Shigella*. Considering the potential threat of shigellosis, World Health Organization (WHO) placed the development of a *Shigella* vaccine at the top of its priority list of awaited vaccines against enteric infections [23,24].

Various approaches were used to develop a vaccine against *Shigella* which include the use of genetically attenuated *Shigella*, or killed whole cell vaccines, or sub-cellular vaccines, or O-polysaccharide-protein conjugates [25]. These approaches either suffer from low immunogenicity or adverse side effects. Attenuated pathogens as vaccines successfully activate both the arms of immunity and provide long lasting immunity but poses a threat of reversion to virulent phenotype. On the other side, purified antigen components as subunit vaccines are safer alternatives to attenuated pathogens however they fail to activate cellular immunity [3].

Against this background, *Lactococcus lactis* (*L. lactis*) is considered an attractive and safe alternative to subunit vaccines and attenuated pathogens. Moreover, the immunostimulatory properties of *L. lactis* act as an adjuvant, encouraging its use as an antigen delivery vehicle. We have previously reported that r-*L. lactis* expressing a well conserved, immunodominant *Shigella* antigen, OmpA, induced antigen specific systemic and mucosal immune responses when given orally to BALB/c mice [8,10].

Here, in order to obtain higher antigen specific IgG and sIgA titers, we have increased the antigen dose by 10-fold and assessed the humoral response. The IgG antibody titers increased by 20-fold by increasing the vaccine dose by 10-fold. The serological IgG levels, following oral inoculation of r-*L. lactis* expressing OmpA, were significantly higher than the control groups (P < 0.01). There was a progressive increase in serum IgG levels with each round of vaccine administration. The highest serum IgG titers were observed after 14th day post last booster. The possible explanation could be the increase in circulating antigen specific memory B cells following the last booster administration [26]. The observations of an increase in antigen specific antibody titers after the last booster dose are in agreement with other researchers reporting, enhanced antibody titers after last vaccine dose of nasally administered r-*L. lactis* expressing pneumococcal protective protein A (PppA) [27].

In the present work, the protective efficacy of the developed vaccine candidate was also evaluated in an active human like shigellosis murine model [22]. Enteric invasive pathogens such as *Shigella* breach the epithelial barrier and establish their infection. The presence of commensal bacteria and antigen specific sIgA resist the host pathogen interaction and invasion of the pathogen [28]. In the present study, immunization with *LacVax*® OmpA resulted in four fold increase in OmpA specific fecal sIgA. The sIgA production also increases after 14 days post vaccination, following the similar trend as that of serum IgG. The presence of sIgA in fecal samples indicate the stimulation of mucosal immunity and supports the protective efficacy of the developed vaccine candidate [29,30].

In order to evaluate the activation of cellular arm of immunity, the cytokine profile of vaccinated animals, before *Shigella* challenge was studied. An indicative Th1 dominant response (high INF-γ/IL-4 ratio) in vaccinated animals advocates the elicitation of a strong cellular response essential to combat intracellular pathogens such as *Shigella*. The surge in INF-γ levels ensures the inhibition of cytosolic replication of *Shigella* and thereby reducing the susceptibility of *Shigella* infection in vaccinated animals [31]. Moreover, the pro-inflammatory cytokine, INF-γ, is also reported to have anti-inflammatory activities along with the interplay of other cytokines [31,32]. The anti-inflammatory properties of INF-γ as well as IL-10 are also observed in the present study wherein the colon lengths of vaccinated animals did not reduce even following *Shigella* infection, strengthening the protective role of INF-γ and IL-10 during *Shigella* infection. The observation of protective IL-10 induction following vaccination in our study are in line with the IL-10 role in preventing bacterial induced inflammation following vaccination as established by other researchers [33,34].

Collectively, the observed protection in *LacVax*® OmpA immunized animals can be attributed to the presence of antigen specific IgG, sIgA and higher levels of INF-γ and IL-10 at pre-infection time point. Our observations are in accordance with the existing literature where the antigen specific IgG and sIgA antibodies and higher levels of INF-γ and IL-10 strongly correlated with the protection from *Shigella* infection following vaccination [33–39], further strengthening our vaccine candidate *LacVax*® OmpA. However, further detailed immunological studies are warranted to understand the underlying mechanism of protection which would further strengthen the candidature of *LacVax*® OmpA.

The colon length reduction and disruption of tissue integrity are the key indicators of severe inflammation following *Shigella* infection [22,40,41]. Intact intestinal tissues and adequate colon lengths of vaccinated animals following *Shigella* infection demonstrate the protective immune response of vaccine candidate.

The potential of *L. lactis* based antigen delivery platform in generating protective immune response is established even for various other infections such as *Listeria monocytogenes* [13], *Escherichia coli* O157:H7 [42], *Pneumococcal* serotypes [27], Rotavirus [43], Human Papillomavirus [44] and severalother diseases [45].

This developed vaccine candidate has several benefits such as low production cost, safe profile and needle-free administration which is convenient for mass immunization [46]. Furthermore, the immunostimulatory adjuvant properties of *L. lactis* helps in counteracting vaccine loss and make up for the lower immunogenicity of non-living vaccines [47–49]. Considering the patient compliance, unsafe usage of needles, cost and rapid increase in the incidence of cross-contamination, the use of *L. lactis* based vaccine platform is a well suited model of a needle-free oral mucosal vaccine for enteric pathogens. Moreover, this platform can also be used to develop protective prophylactics against other enteric pathogens.

Disclosure

(1) All authors concur with the submission.
(2) All funding for the studies in the manuscript, together with the names of the principal funding recipients, are listed in the Acknowledgements.
(3) All persons cited in the manuscript by way of the Acknowledgements, personal communications, unpublished observations/data concur with the citation.
(4) The work has not been published elsewhere, either completely, in part, or in another form.
(5) The manuscript contains experiments using animals. The permission of the national authorities is included.
(6) The manuscript does not contain human studies.
(7) Authors declare no financial/commercial conflicts of interests.

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Conflict of interest

The authors declare no conflict of interest with respect to authorship, funding and publication of this article.

Authors contribution

BY, DS, HP and PD conceptualized and designed the experiments. BY, DS and PD performed the experiments and acquired the data. BY compiled the results and wrote the manuscript. BY, HP, and PD improvised the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.04.053.

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