Vaccine 32 (2014) 3909–3916

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Oral immunization with *Lactococcus lactis*-expressing EspB induces protective immune responses against *Escherichia coli* O157:H7 in a murine model of colonization

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ARTICLE INFO

Article history:
Received 27 February 2014
Received in revised form 1 May 2014
Accepted 15 May 2014
Available online 28 May 2014

Keywords:
Lactococcus lactis
Enterohemorrhagic *Escherichia coli*
Hemolytic uremic syndrome
EspB
Oral vaccination

ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) have been responsible for several outbreaks of hemolytic–uremic syndrome (HUS) worldwide. HUS is the most common cause of acute renal failure in children and results in fatalities as high as 50% in the elderly. Currently, neither a specific treatment nor a vaccine is available for EHEC. *Lactococcus lactis* is a generally regarded as safe "GRAS" bacterium that offers a valuable platform for oral vaccine delivery. Toward the development of an oral vaccine against EHEC, we have previously constructed a recombinant *L. lactis* strain expressing the EHEC antigen, EspB in the cytoplasmic compartment. However, oral immunization of mice with this strain induced weak priming of the immune system. This outcome was attributed to the rather low levels of EspB expressed by this recombinant strain. Therefore, in the present study we optimized the expression of EspB in *L. lactis* by secreting the antigen either under constitutive or nisin-inducible control. Indeed, oral immunization of mice with the EspB-secreting strains successfully induced specific mucosal and systemic antibody responses. These responses were associated with mixed Th1/Th2 cell responses in Peyer's Patches and mesenteric lymph nodes. Moreover, immunized mice exhibited significant protection against *E. coli* O157:H7 colonization, as indicated by the reduced amount and/or duration of the bacterial fecal shedding. Our results demonstrate the protective potential of EspB as an oral vaccine against EHEC infection. Additionally, the study demonstrates the efficient delivery of recombinant EspB by the "GRAS" bacterium, *L. lactis*. The safety profile of *L. lactis* as a vaccine vehicle can particularly be beneficial to children and elderly as high-risk groups for HUS incidence.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is one group of colonizing enteropathogens that has been responsible for frequent outbreaks of diarrhea and hemorrhagic colitis (HC) worldwide [1]. HC occasionally progresses to hemolytic–uremic syndrome (HUS), which is the most common cause of acute renal failure in children [2] and results in fatality rates as high as 50% in the elderly [3]. HC and HUS are caused by shiga-like toxins (Stxs), which are released by colonizing EHEC into the systemic circulation and induce endothelial damage in intestinal and renal vasculatures [4]. Several studies have linked antibiotic therapy to higher rates of HUS development and prolonged duration of the symptomatic disease, probably due to the excessive release of Stxs upon bacterial lysis [3,5–7]. Therefore, current disease intervention strategies are rather focusing on vaccination. However, no vaccine is currently available for EHEC infections.

Since the Stxs-mediated EHEC diseases develop subsequent to the bacterial intestinal colonization, the latter is considered a key determinant of EHEC pathogenicity and represents a potential target for vaccine design. EHEC colonizes the intestine with the aid of a type III secretion system (T3SS) which enables the bacteria to establish a tight adherence to enterocytes and to modify their cytoskeletal proteins, leading to the characteristic attaching and effacing lesion [8]. EspB is a T3SS protein that plays a central role in mediating the EHEC enterocyte adherence [8–12]. Moreover, EspB is highly immunogenic in human patients [13–15] and in infected or vaccinated animals [13,16]. Therefore, EspB is a potential candidate antigen for vaccination against EHEC.

Oral vaccination can be a particularly efficient approach to interfere with EHEC intestinal colonization, as it can effectively induce local immune responses at the intestinal mucosa, and

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http://dx.doi.org/10.1016/j.vaccine.2014.05.054
0264-410X/

concurrently elicit systemic immune responses [17]. Nevertheless, oral vaccines are at the risk of being degraded by the harsh gastrointestinal conditions. Thus, vaccine delivery systems represent a useful strategy to ensure the efficient oral delivery of antigens [18,19]. One of the interesting oral antigen delivery systems is based on genetically modified lactic acid bacteria (LAB) that simultaneously express and deliver antigens to the intestinal mucosa [20,21]. LAB have been traditionally used in food industry and are generally regarded as safe "GRAS" for human consumption. Therefore, they particularly offer a safe tool for the oral delivery of vaccines. *Lactococcus lactis* is a model LAB that has been extensively studied for oral vaccine delivery. Numerous bacterial, viral and parasitic antigens were expressed in *L. lactis* and the resultant recombinant strains were capable of inducing specific mucosal and systemic immune responses in mice upon oral administration (for a recent review, [22]). We have recently reported the constitutive cytoplasmic expression of EspB in *L. lactis* [23]. However, oral immunization of mice using this recombinant strain induced weak priming of the immune system. This outcome was attributed to the low levels of EspB expressed by this strain [23].

In the present study, we describe the optimized expression of EspB in *L. lactis*. Additionally, we describe the specific immune responses elicited in mice upon oral immunization with the recombinant strains and the protection conferred against a challenge infection with *E. coli* O157:H7.

2. Materials and methods

2.1. Bacteria, plasmids and culture conditions

A summary of the bacterial strains and plasmids used in this study is shown in Table 1. *L. lactis* was grown at 30 °C in liquid M17 medium (Difco) supplemented with 0.5% glucose. Erythromycin or chloramphenicol was added at concentrations of 5 and 10 μg/ml, respectively. *E. coli* NCTC12900 was grown at 37 °C in Luria–Bertani broth (Difco) supplemented with 80, 15 and 100 μg/ml of novobiocin, naladixic acid and streptomycin, respectively.

2.2. Constructions of the EspB-expressing L. lactis strains

Plasmids pT:SEC-EspB and pNZ:SEC-EspB were constructed for constitutive or nisin-inducible secretion of EspB, respectively (Table 1 and Fig. 1). For construction of plasmid pT:SEC-EspB, the *espB* gene was PCR amplified from plasmid pT:CYT-EspB [23] using sequence-specific primer pairs (forward: 5′-GGGGGATCCAACACTATCGATAACACTCAAG-3′; reverse: 5′-GGGACTAGTTTAACCAGCAA GACG-3′). pT:CYT-EspB carries a codon optimized *espB* gene of *E. coli* O157:H7 (Genbank Accession no: NC_002655), adapted to the preferential codon use of *L. lactis* [23]. The amplified *espB* gene was inserted downstream of the secretion signal peptide SP_{Usp45} at the BamHI/SpeI ends of pTREX1 (Fig. 1). For the construction of plasmid pNZ:SEC-EspB, *espB* along with SP_{Usp45} (SP_{Usp45}–*espB*) was PCR amplified from pT:SEC-EspB using sequence-specific primer pairs (forward: 5′-ATGAAAAAAAAGATTATCTCAGC-3′; reverse: 5′-GGGACTAGTTTAACCAGCAAGACG-3′) and was inserted at the ScaI/SpeI ends of plasmid pNZ8150 (Fig. 1). Plasmids pT:SEC-EspB and pNZ:SEC-EspB were used to transform electrocompetent *L. lactis* MG1363 and *L. lactis* NZ9000 [24] to obtain the recombinant strains LL-pT:SEC-EspB and LL-pNZ:SEC-EspB, respectively (Table 1).

2.3. Induction and quantification of EspB expression in L. lactis

Overnight cultures of the inducible LL-pNZ:SEC:EspB strain or the constitutive, LL-pT:CYT:EspB and LL-pT:SEC:EspB strains were inoculated in GM17 broth at dilutions of 1:25 and 1:100, respectively. All strains were grown to an optical density at 600 nm of 0.4, at which, nisin (10 ng/ml) was added to the culture medium of LL-pNZ:SEC:EspB. LL-pT:CYT:EspB and LL-pT:SEC:EspB cells were harvested by centrifugation and resuspended in buffered-GM9 (BGM9) medium [25]. After a further 4 h incubation, cells or supernatant of equal culture volumes were collected for each strain. *L. lactis* cell lysates were enzymatically obtained as previously described [23]. EspB was detected and quantified in cell lysates and supernatants of the *L. lactis* cultures using a specific sandwich ELISA, as previously described [23].

2.4. Oral immunization of mice with the EspB-expressing L. lactis strains

Six-week-old female BALB/c mice were used in the animal experiments. All experimental and animal management procedures were approved by the animal care and ethics committee of Ghent University, Belgium. The lactococcal strains were grown and induced as described above. Cells of the inducible strains were washed twice with sterile PBS to remove the traces of nisin. Groups of nine mice were immunized orally with LL-pT:SEC:EspB, LL-pNZ:SEC:EspB, purified EspB, LL-pTREX1, LL-pNZ8150 or the inoculation medium BGM9 [25]. As a primary immunization mice received 100 μl of BGM9 containing 2×10^9 CFU of the proper lactococcal strain or 5 μg of purified EspB for three consecutive days. A booster immunization was performed three weeks later using the same regime.

2.5. EspB-specific antibodies

Total serum EspB-specific immunoglobulins (total-Ig) and faecal IgA were measured 10 days after the booster immunization. Preparation of serum and fecal extracts as well as the ELISA procedures were performed as previously described [23]. The ELISA results are expressed as the OD values measured at 405 nm for dilutions of 1:10 for serum and 1:5 for fecal extracts.

2.6. Cytokine ELISA's

Peyer's patches (PP) and mesenteric lymph node (MLN) lymphocytes were isolated from immunized mice ($n = 3$), 10 days after the booster immunization. Cells were resuspended at a concentration of 1.5×10^6 cells/ml in complete RPMI-1640 medium [26] and cultured in 96-well plates. Cells were restimulated with EspB (final concentration: 10 μg/ml) for 48 h. Thereafter, supernatants were collected and assayed for murine INF-γ, IL-4 and IL-10 by quantitative ELISA using the mouse Th1/Th2 ELISA Ready-SET-Go® kit (eBioscience), according to the manufacturer's instructions.

2.7. Challenge with E. coli O157:H7

Groups of seven mice were immunized as described above. Ten days after the booster immunization, mice of each group were randomly divided into two subgroups, of which, one subgroup ($n = 4$) received streptomycin (5 g/l) in drinking water 24 h before infection and till the end of the experiment, while the other subgroup ($n = 3$) was not treated with streptomycin. These subgroups represent two models of varying colonization intensities, since streptomycin treatment enhances EHEC colonization in mice by clearing the competing intestinal flora [27]. Mice were challenged by intragastric inoculation of 10^{10} CFU of *E. coli* NCTC12900 in 100 μl of PBS containing 20% sucrose. Faecal excretion of *E. coli* NCTC12900 was monitored at 2–3 days interval, by both conventional bacterial plating and immuno-magnetic separation, as previously described [28].

Table 1
Bacterial strains and plasmid vectors.

Plasmid or strain	Description	Reference
L. lactis subsp. *cremoris* MG1363	A derivative of the dairy starter strain NCDO712, which was cured of all resident plasmids	[61]
L. lactis NZ9000	*L. lactis* subspecies cremoris MG1363 carrying nisR and nisK genes on the chromosome	[62]
LL-pTREX	*L. lactis* subspecies cremoris MG1363 carrying plasmid pTREX	[23]
LL-pNZ8150	*L. lactis* NZ9000 carrying plasmid pNZ8150	This study
LL-pT:SEC:EspB	*L. lactis* subspecies cremoris MG1363 carrying plasmid pT:SEC:EspB	This study
LL-pNZ:SEC:EspB	*L. lactis* NZ9000 carrying plasmid pNZ:SEC:EspB	This study
E. coli NCTC12900	Shiga toxin-negative mutant of *E. coli* O157:H7, Nov^R, Nal^R and Str^R	[63]
pTREX1	*L. lactis* expression plasmid containing P1 promoter with downstream start codon and secretion signal (SP_{Usp45}), Em^R	[46]
pNZ8150	*L. lactis* expression plasmid containing PnisA promoter with downstream start codon, Cm^R	[64]
pT:CYT:EspB	Modified pTREX1 containing P_1 promoter with downstream *espB* gene, Em^R	[23]
pT:SEC:EspB	Modified pTREX1 containing P_1 promoter with downstream secretion signal (SP_{Usp45}) and *espB*, Em^R	This study
pNZ:SEC:EspB	Modified pNZ8150 containing PnisA promoter with downstream secretion signal (SP_{Usp45}) and *espB*, Cm^R	This study

Nal^R, Str^R, Cm^R, Em^R is resistance against naladixic acid, streptomycin, chloramphenicol and erythromycin, respectively.

2.8. Statistical analyses

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc.). One-way analysis of variance (ANOVA) with Bonferroni post-hoc test was used to analyze the differences in EspB expression levels among the strains as well as the differences in antibody titers, cytokine ratios and duration of fecal shedding among the groups. Pair-wise comparison of individual cytokine levels among immunized and control groups were analyzed using Student's *t*-test. Two-way ANOVA with Bonferroni

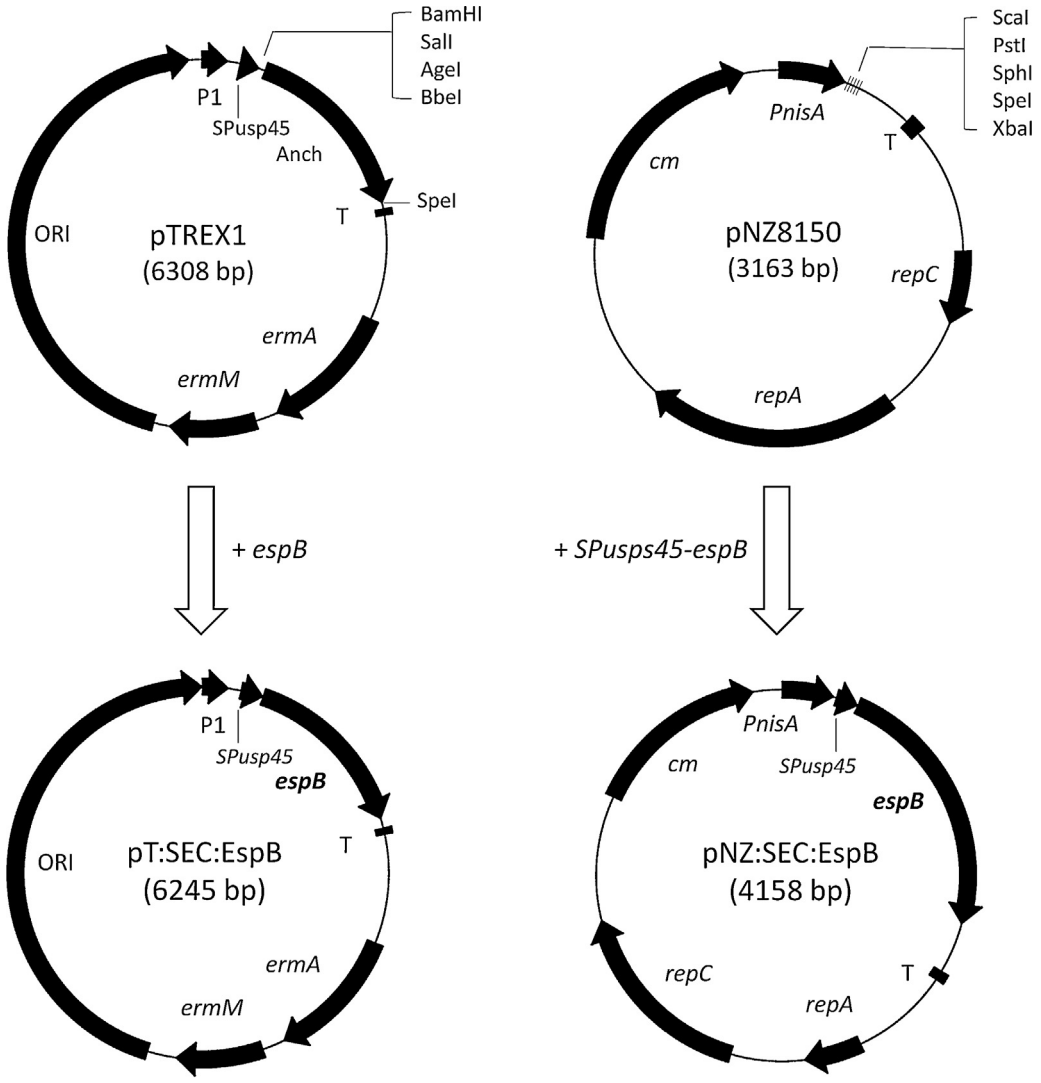

Fig. 1. *Strategies for espB gene cloning in L. lactis*. (A) The expression plasmid pTREX1 containing the constitutive promoter P1 and the secretion peptide signal SP-Usps45 was used for the insertion of the *espB* coding sequence between BamHI and SpeI restriction sites to form the plasmid pT:SEC:EspB. (B) The expression plasmid pNZ8150 containing the nisin-inducible promoter PnisA was modified by inserting a fragment combining the *espB* gene to an upstream secretion signal (SP_{Usp45}-*espB*) between ScaI and SpeI restriction sites to form the plasmid pNZ:SEC:EspB.

post-hoc test was used to analyze the differences in fecal loads of *E. coli* NCTC12900 over time among the groups. Differences with a $P < 0.05$ were considered statistically significant.

3. Results

3.1. ELISA quantification of EspB expression by the recombinant L. lactis strains

EspB was detected only in the cell lysate of LL-pT:CYT:EspB, while it was detected in both cell lysates and supernatants of LL-pNZ:SEC:EspB and LL-pT:SEC:EspB (Table 2). The detection of EspB in cell lysates of the EspB-secreting strains corresponds to the presence of the cytoplasmic precursor of the mature (secreted) protein. The total amount of EspB expressed by the secreting strains LL-pNZ:SEC:EspB and LL-pT:SEC:EspB (both cytoplasmic and secreted) was, respectively, 56- and 25-fold higher than that expressed by the cytoplasmic expression strain LL-pT:CYT:EspB ($P < 0.05$). The nisin-inducible LL-pNZ:SEC:EspB strain showed a 2.2-fold higher total EspB expression than the constitutive LL-pT:SEC:EspB strain ($P < 0.05$) (Table 2). Both LL-pNZ:SEC:EspB and LL-pT:SEC:EspB were used for the immunization experiments.

3.2. EspB-secreting L. lactis strains induce specific antibody responses after oral administration to mice

Mice orally immunized with either LL-pNZ:SEC:EspB or LL-pT:SEC:EspB showed significantly higher levels of EspB-specific serum total-Ig ($P < 0.0001$) and faecal IgA ($P < 0.0001$) compared to those immunized with the corresponding control strains, LL-pNZ8150 and LL-pTREX1, respectively (Fig. 2). The highest antibody responses were elicited by the nisin-inducible strain LL-pNZ:SEC:EspB compared to the constitutive strain LL-pT:SEC:EspB ($P < 0.0001$). Mice immunized with purified EspB showed no significant difference in total serum Ig and faecal IgA compared to those receiving the BGM9 medium.

3.3. Oral immunization with L. lactis-secreting EspB induces mixed Th1/Th2 immune response

To characterize the cellular immune responses induced by the oral immunization with the EspB-secreting *L. lactis* strains, the production of INF-γ (Th1 cytokine), IL-4 (Th2 cytokine) [29] and IL-10 (a broadly expressed anti-inflammatory cytokine) [29,30] was measured in PP (Fig. 3A) and MLN (Fig. 3C) lymphocytes after in vitro restimulation with EspB. Lymphocytes of both LL-pT:SEC:EspB and LL-pNZ:SEC:EspB immunized mice showed a significant higher production of all cytokines compared to those immunized with LL-pTREX1 and LL-pNZ8150, respectively ($P < 0.0001$). This finding indicates a mixed Th1/Th2 cell response. Mice immunized with purified EspB showed no significant increase in cytokine production compared to those received the BGM9 medium.

To determine the Th1/Th2 cell-type dominance, the IL-4/INF-γ ratio was further analyzed for PP (Fig. 3B) and MLN (Fig. 3D) lymphocytes. The ratio in both LL-pNZ:SEC:EspB and LL-pT:SEC:EspB immunized mice was greater than one and was significantly higher than the baseline ratios shown by control mice immunized with the respective empty vector strain or with the BGM9 medium. This finding indicates a dominance of the Th2 response.

3.4. Oral immunization with L. lactis-secreting EspB protects mice against E. coli O157:H7 colonization

Comparisons of *E. coli O157:H7* fecal counts among the immunization groups within either streptomycin-treated (Fig. 4A) or non-streptomycin-treated (Fig. 4B) challenge groups show significantly reduced counts in LL-pNZ:SEC:EspB and LL-pT:SEC:EspB groups in comparison to LL-pNZ8150 and LL-pTREX1 groups, respectively, or to the BGM9 group, at most of the time points ($P < 0.05$). This reduction was significantly higher in the LL-pNZ:SEC:EspB immunization group compared to the LL-pT:SEC:EspB group ($P < 0.05$) (Fig. 4A and B).

Additionally, comparisons of the durations of *E. coli O157:H7* fecal shedding among the streptomycin-treated immunization groups show a significantly shorter duration in the LL-pNZ:SEC:EspB group (11.52 ± 2.87 days) compared to LL-pNZ8150 and BGM9 groups (30.5 ± 1.73 and 29.75 ± 1.5 days, respectively) ($P < 0.05$). However, the duration of fecal shedding shown by the LL-pT:SEC:EspB group was not significantly shorter than that of the LL-pTREX1 or the BGM9 group (26.75 ± 1.5 days versus 29.75 ± 1.5 days). Within the non-streptomycin-treated immunization groups, the duration of fecal shedding shown by the LL-pNZ:SEC:EspB group (11.52 ± 2.87 days) and the LL-pT:SEC:EspB group (16 ± 1.73 days) was significantly shorter than that of the LL-pNZ8150 group

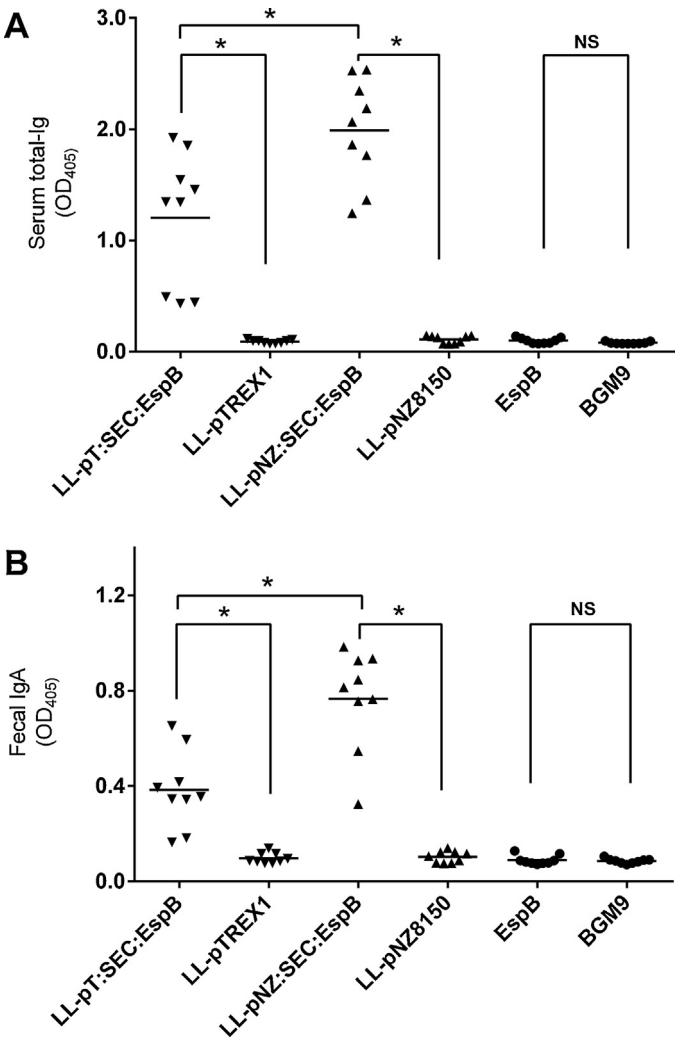

Fig. 2. *Antibody responses in mice immunized with EspB-secreting lactococci.* Groups of mice ($n = 9$) were immunized using the constitutive strain LL-pT:SEC:EspB, the inducible strain LL-pNZ:SEC:EspB or purified EspB. Corresponding control groups were immunized using LL-pTREX1, LL-pNZ8150 or the oral inoculation medium, BGM9. Ten days after the booster immunization serum and fecal samples were assayed by ELISA for the presence of EspB-specific total-Ig (A) or IgA (B), respectively. Data present individual values (triangles) and means (horizontal line) of nine mice per group. Statistical differences were determined using one-way ANOVA with Bonferroni post-hoc test (* $P < 0.0001$; NS = non significant difference).

Table 2
ELISA quantification of EspB production by the recombinant *L. lactis* strains.

Strain	Expression features	EspB expression in fractions of cultures (μg/ml)[†]		
		Cell lysate	Supernatant	Total
LL-pNZ:CYT:EspB	Nisin-inducible cytoplasmic	0.082 ± 0.02	ND[*]	0.082 ± 0.02[a]
LL-pNZ:SEC:EspB	Nisin-inducible secretion	0.83 ± 0.19	3.8 ± 1.1	4.63 ± 1.29[a]
LL-pT:SEC:EspB	Constitutive secretion	0.15 ± 0.05	1.9 ± 0.4	2.05 ± 0.45[a]

[†] Culture density approximately equal to 8×10^8 CFU/ml. Data represent means ± standard deviation of three independent measurements.
[a] Significant differences among the strains using one way-ANOVA with Bonferroni post-hoc test ($P < 0.05$).
[*] ND: non detectable.

(19.3 ± 2.31 days) and the LL-pTREX1 group (22 days), respectively or than that of the BGM9 group (22 days) ($P < 0.05$). The shorter duration of fecal shedding shown by the LL-pNZ:SEC:EspB group in comparison to the LL-pT:SEC:EspB group was significant ($P < 0.05$).

4. Discussion

We have recently reported the constitutive cytoplasmic expression of EspB in *L. lactis* (LL-pT:CYT:EspB) [23]. However, oral vaccination of BALB/c mice with this strain induced a weak priming the immune system [23]. This outcome was attributed to the low amount of EspB expressed by LL-pT:CYT:EspB, presumably due to the antigen degradation by the lactococcal cytoplasmic proteases [31–33]. Therefore, in the present study we hypothesized that secretion could allow EspB to escape cytoplasmic proteolysis and thus would result in a higher expression yield. Additionally, we compared a nisin-inducible and a constitutive expression system for the highest protein expression. In accordance with

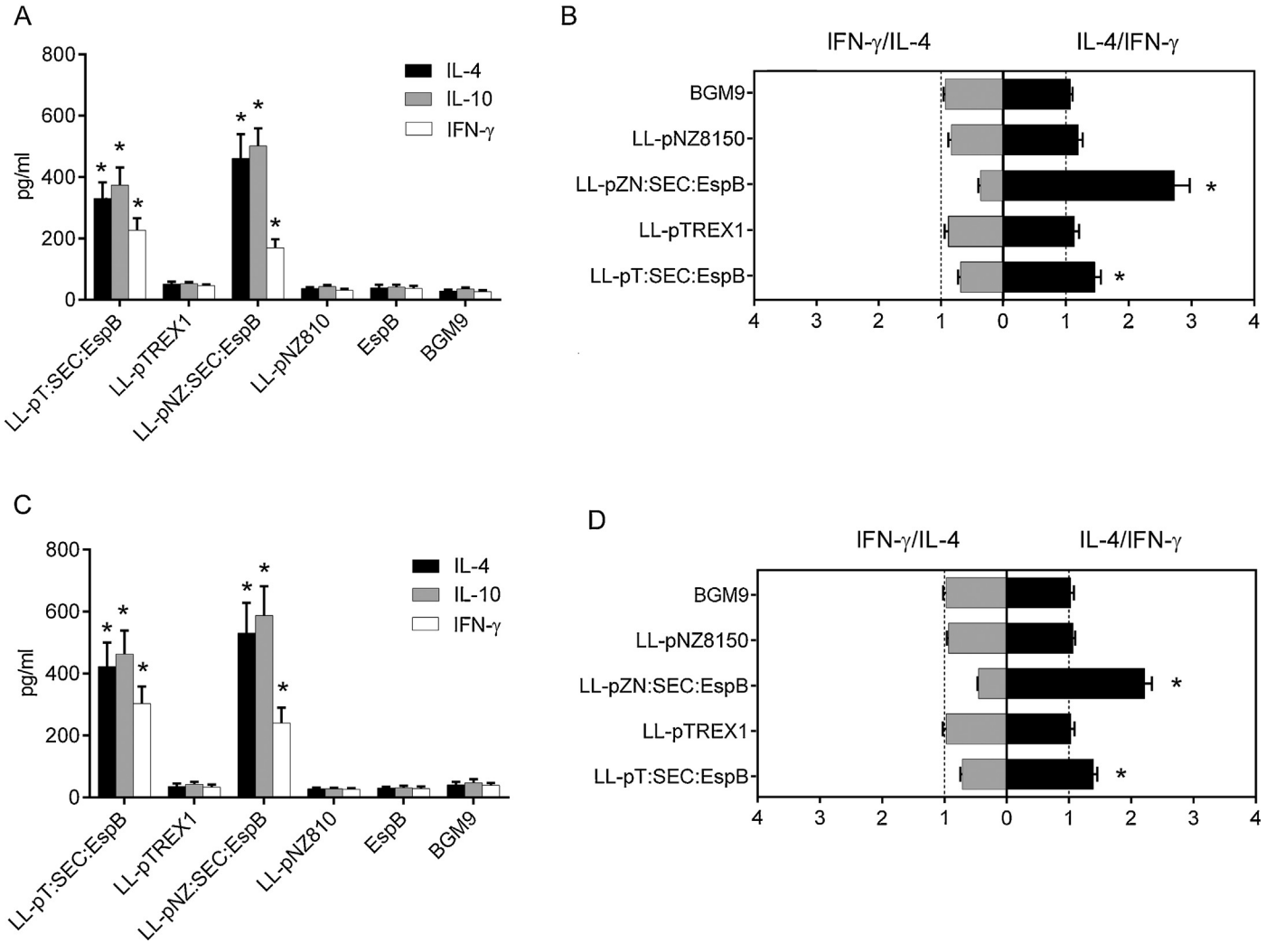

Fig. 3. Cytokine responses of Peyer's patches (PP) (A and B) and mesenteric lymph nodes (MLN) (C) and (D) lymphocytes from mice orally immunized with EspB-secreting *L. lactis*. Ten days after the booster immunization, lymphocytes were isolated from PP and MLN of mice ($n = 3$) of immunized mice; restimulated with EspB, and assayed for production of IFN-γ, IL-4 and IL-10 cytokines. Data present the mean ± SD. Asterisks indicate significant difference between the immunization groups LL-pT:SEC:EspB, LL-pNZ:SEC:EspB and EspB compared to the control groups LL-pTREX1, LL-pNZ8150 and BGM9, respectively ($P < 0.05$). Statistical differences were determined using Student's *t*-test ($P < 0.05$). IL-4/INF-γ ratios were calculated to determine the dominant T-helper response in PP (B) and MLN lymphocytes (D). Data present the mean ± SD. Asterisks indicate significant difference between the immunization groups LL-pT:SEC:EspB and LL-pNZ:SEC:EspB compared to the respective control groups LL-pTREX1 and LL-pNZ8150, or to the BMG9 control group ($P < 0.05$). Statistical differences were determined using one-way ANOVA with Bonferroni post-hoc test ($P < 0.05$).

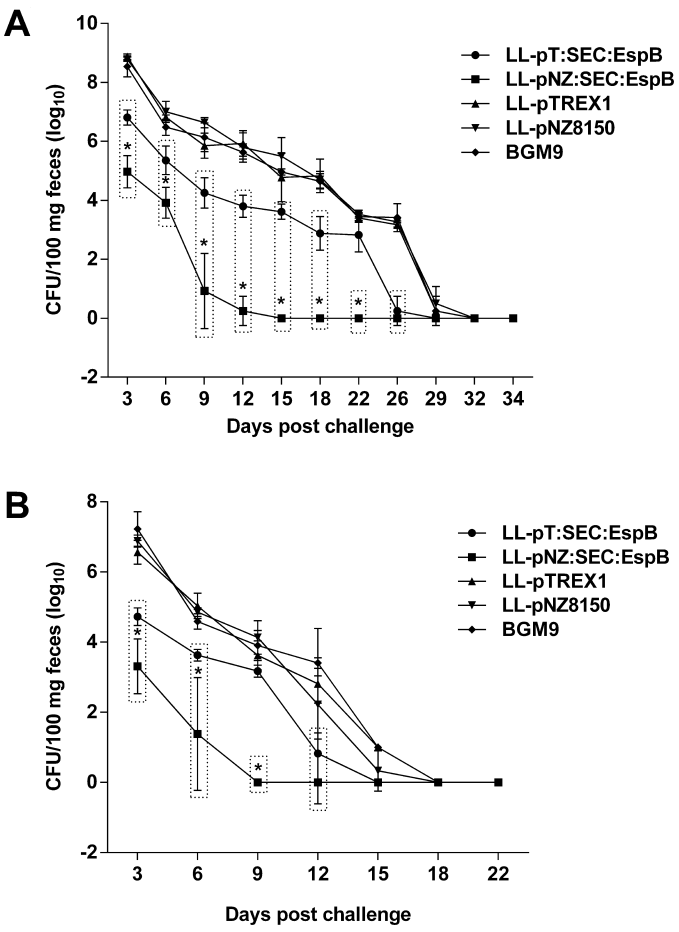

Fig. 4. *Kinetics of fecal shedding of E. coli O157:H7 in mice immunized orally with EspB secreting lactococci.* Ten days after the booster immunization, groups of immunized mice were orally challenged with 10^{10} CFU of *E. coli* NCTC12900 (a non-toxigenic mutant of *E. coli* O157:H7), either under oral streptomycin treatment condition ($n=4$) (A) or without streptomycin treatment ($n=3$) (B). Data present the mean ± SD. Data points in rectangles indicate a significant difference between the LL-pT:SEC:EspB and pNZ:SEC:EspB immunization groups compared to the respective control groups LL-pTREX1 and LL-pNZ8150 or to the BGM9 control group ($P<0.05$). Asterisks indicate statistical differences between the LL-pT:SEC:EspB and LL-pNZ:SEC:EspB immunization groups ($P<0.05$). Data present the mean ± SD. Statistical differences were determined using two-way ANOVA with Bonferroni post-hoc test ($P<0.05$).

our hypothesis, secretion of EspB either under constitutive (LL-pT:SEC:EspB) or inducible (LL-pNZ:SEC:EspB) conditions resulted in a great enhancement of the total antigen yield compared to the cytoplasmic expression by LL-pT:CYT:EspB (56- and 25-fold increase, respectively). A similar enhancement of the total protein yield by secretion in comparison to cytoplasmic expression has been reported for the expression of the staphylococcal nuclease [34] and the *Brucella abortus* ribosomal protein L7/L12 [35] in *L. lactis*.

The immune response induced by the EspB-secreting lactococci is expected to be influenced by EspB expression levels, which are at least two times higher for the nisin-inducible strain, LL-pNZ:SEC:EspB compared to the constitutive strain, LL-pT:SEC:EspB. On the other hand, LL-pT:SEC:EspB is capable of constant secretion of EspB during the intestinal passage, which may compensate for the higher expression levels of LL-pNZ:SEC:EspB, whereas LL-pNZ:SEC:EspB may not continue EspB secretion along the entire duration of its intestinal passage as nisin is absent in vivo. However, the highest immune responses were obtained by the inducible LL-pNZ:SEC:EspB strain. In this regard, previous reports have demonstrated the ability of nisin-inducible expression systems to continue protein expression for at least 10 h after removal of nisin from the culture medium [34]. Additionally, considering that the reported transit time of *L. lactis* throughout the mouse gastrointestinal tract is approximately 12 h [36], it is highly likely that both strains had a comparable persistence of EspB secretion in vivo, while the higher responses induced by LL-pNZ:SEC:EspB are a direct result of its higher capacity for EspB expression.

Unlike the mice immunized with the EspB-expressing *L. lactis* strains, those immunized with purified EspB did not show specific immune responses, even though EspB was given at a dose approximately equal to that present in the inoculum of LL-pT:SEC:EspB (5 μg). This finding could be explained by the ability of *L. lactis* to maintain metabolic activity [37–39] and de novo secretion of recombinant proteins [38,39] during its gastrointestinal passage, leading to the delivery of higher amounts of EspB than those measured in the administered inocula [40]. Additionally, purified EspB could have been influenced by the degrading effects of the gastrointestinal acidity and enzymes, a bottleneck that can be overcome by using *L. lactis* as an antigen delivery vector. Moreover, *L. lactis* exhibits adjuvant effects [41], which can enhance the immune response to recombinant EspB in comparison to the purified protein [42–44]. These arguments support the pharmacological and the immunological value of *L. lactis* as an oral antigen delivery system [45–47].

To characterize the cellular immune responses induced by oral immunization of mice with the EspB-secreting strains, we isolated lymphocytes from PP and MLN, the main inductive sites of the gut mucosal immune system [48] and measured the production of INF-γ (Th1 cytokine) and IL-4 (Th2 cytokine) [29]after stimulating the cells with EspB in vitro. Both cytokines were produced by lymphocytes of immunized mice, indicating the induction of a mixed Th1/Th2 cell response. Previous reports have similarly described a mixed Th1/Th2 cell response to antigens orally delivered by *L. lactis* [46,49,50]. Additionally, analysis of the IL-4:INF-γ ratio's indicated a dominance of the Th2 response, which is a typical characteristic of an intestinal mucosal response [29,46]. The Th2 dominance corresponds well with the induced mucosal IgA responses, since Th2 cells and cytokines are known to contribute to mucosal IgA class switching in PP and MLN [51,52].

Reduced intestinal colonization is considered an important criterion for protection against EHEC virulence [53,54]. Our findings show that oral immunization of mice with the EspB-secreting *L. lactis* strains resulted in a reduction of *E. coli* O157:H7 fecal shedding. Moreover, the reduced bacterial shedding was consistent in two infection models of varying colonization intensities indicating the efficacy and the reproducibility of our vaccination approach. This pattern of protection could be correlated with an intestinal EspB-specific IgA response, which is consistent with the protective role of secretory IgA in mucosal infections [18]. However, previous immunization studies in mice models, particularly those involving T3SS antigens, lack consensus on the necessity of intestinal IgA responses for clearing an EHEC infection (recently reviewed in [55]). For instance, intramuscular immunization with a mixture of T3SS proteins eliminated EHEC shedding, despite the absence of fecal Tir- or EspA-specific IgA responses [56]. On the other hand, a subcutaneous immunization with Tir failed to induce intestinal IgA responses and to protect mice against a challenge [57]. With regard to EspB, previous reports have described the protective effect of parenteral vaccination with formulations containing EspB [58,59]. However, these studies were performed in cattle, a species in which IgG responses contribute largely to intestinal mucosal protection [60]. The present study, however, is the first to describe the protective potential of EspB upon oral vaccination.

In conclusion, oral immunization of mice with recombinant *L. lactis* secreting EspB induces specific mucosal and systemic immune responses and confers protection against an *E. coli*

O157:H7 challenge infection. These findings demonstrate the feasibility of an oral vaccine based on the recombinant expression of EspB in *L. lactis* to control EHEC infection. The safety profile of *L. lactis* as a vaccine delivery vector can particularly be beneficial to children and elderly, the high-risk groups for HUS complications [1].

Author contribution statement

All authors have substantially contributed to the study design, the acquisition, analysis and interpretation of the data, the manuscript drafting and the revision of its intellectual contents. All authors have approved the submitted manuscript.

Acknowledgments

This work was supported by the Special Research Fund (BOF) of Ghent University and the (project G0A4312N) and FOD health, food chain safety and environment. We gratefully acknowledge Dr. Erik Remaut, Department of Biomedical Molecular Biology, Ghent University, for providing the strain *L. lactis* GM1363 as well as the lactococcal expression vector, pTREX1. We also gratefully acknowledge Pieter Almgren, for his technical help.

Conflict of interest statement: The authors declare no conflicts of interest with respect to authorship, funding and publication of this article.

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