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Efficacy of a recombinant Intimin, EspB and Shiga toxin 2B vaccine in calves experimentally challenged with *Escherichia coli* O157:H7

Luisina Martorelli [a], Nicolás Garimano [b], Gabriela A. Fiorentino [c], Daniel A. Vilte [a], Sergio G. Garbaccio [a], Stefanie A. Barth [d], Christian Menge [d], Cristina Ibarra [b], Marina S. Palermo [c], Angel Cataldi [e,*]

[a] *Instituto de Patobiología, Centro de Investigación en Ciencias Veterinarias y Agronómicas, Instituto Nacional de Tecnología Agropecuaria (INTA), Hurlingham, Argentina*
[b] *Laboratorio de Fisiopatogenia, Departamento de Fisiología, Instituto de Fisiología y Biofísica Bernardo Houssay (IFIBIO Houssay-CONICET), Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina*
[c] *Laboratorio de Patogénesis e Inmunología de Procesos Infecciosos, Instituto de Medicina Experimental, (IMEX), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)-Academia Nacional de Medicina, Buenos Aires, Argentina*
[d] *Friedrich-Loeffler-Institut/Federal Research Institute for Animal Health, Institute of Molecular Pathogenesis, Jena, Germany*
[e] *Instituto de Biotecnología, Centro de Investigación en Ciencias Veterinarias y Agronómicas, Instituto Nacional de Tecnología Agropecuaria, Hurlingham, Argentina*

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ABSTRACT

Escherichia coli O157:H7 is a zoonotic pathogen of global importance and the serotype of Shiga toxin-producing *E. coli* (STEC) most frequently associated with Hemolytic Uremic Syndrome (HUS) in humans. The main STEC reservoir is cattle. Vaccination of calves with the carboxy-terminal fraction of Intimin γ (IntC280) and EspB can reduce *E. coli* O157:H7 fecal shedding after experimental challenge. Shiga toxin (Stx) exerts local immunosuppressive effects in the bovine intestine and Stx2B fused to *Brucella* lumazine synthase (BLS-Stx2B) induces Stx2-neutralizing antibodies. To determine if an immune response against Stx could improve a vaccine's effect on fecal shedding, groups of calves were immunized with EspB + IntC280, with EspB + IntC280 + BLS-Stx2B, or kept as controls. At 24 days post vaccination calves were challenged with *E. coli* O157:H7. Shedding of *E. coli* O157:H7 was assessed in recto-anal mucosal swabs by direct plating and enrichment followed by immunomagnetic separation and multiplex PCR. Calves were euthanized 15 days after the challenge and intestinal segments were obtained to assess mucosal antibodies. Vaccination induced a significant increase of IntC280 and EspB specific antibodies in serum and intestinal mucosa in both vaccinated groups. Antibodies against Stx2B were detected in serum and intestinal mucosa of animals vaccinated with 3 antigens. Sera and intestinal homogenates were able to neutralize Stx2 verocytotoxicity compared to the control and the 2-antigens vaccinated group. Both vaccines reduced *E. coli* O157:H7 shedding compared to the control group. The addition of Stx2B to the vaccine formulation did not result in a superior level of protection compared to the one conferred by IntC280 and EspB alone. It remains to be determined if the inclusion of Stx2B in the vaccine alters *E. coli* O157:H7 shedding patterns in the long term and after recurrent low dose exposure as occurring in cattle herds.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC), a subset of Shiga toxin-producing *E. coli* (STEC), is a globally important zoonotic pathogen capable of causing Hemorrhagic Colitis (HC) and Hemolytic Uremic Syndrome (HUS) in humans. HUS is often described as an epidemic disease of low incidence in industrialized countries such as USA, Canada and Japan (1–3 cases per 100,000 children under 5 years) [1]. However, Argentina is the country with the highest incidence per year in the world, with 12 to 15 cases per 100,000 children under 5 years old. HUS is the leading cause of acute renal failure in Argentinian children and the second cause of chronic renal failure, and is also responsible for 20% of kidney transplants in children and adolescents [2,3]. Human infection usually results from the consumption of fecal-contaminated foods containing EHEC. The main sources of infection are insufficiently cooked meat, unpasteurized milk, dairy products manufactured with unpasteurized milk, horticultural products contaminated by irrigation or fertilizers, and contaminated water. It can also be transmitted from person to person [1].

The main reservoir of STEC are ruminants, mainly bovine. EHEC O157:H7 preferentially colonizethe lymphoid follicle-dense

* Corresponding author at: Instituto de Biotecnología, INTA, Los Reseros y Nicolás Repetto, CP 1686 Hurlingham, Argentina.
E-mail address: Cataldi.angeladrian@inta.gob.ar (A. Cataldi).

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mucosa at the terminal rectum and the recto-anal junction (RAJ) [4]. The bacterium is shed to the environment intermittently through faecal matter [5], and occurs for longer periods and with a greater number of bacteria shed in young and weaning calves than in adult animals. EHEC O157:H7 has been found in dairy cattle and beef cattle, both grazing and feedlot [5–7].

E. coli O157:H7 is characterized by the possession of a number of virulence traits that confer pathogenicity and colonization properties. Among the first, Shiga toxins (Stx), which can be type 1 or 2, are the principal virulence factors implicated in human disease [8–10]. Despite the fact that cattle do not display any clinical symptoms that relate to EHEC carriage, bovine intestinal epithelial cells as well as peripheral and intraepithelial lymphocytes express functional Stx receptors [11–13]. Stx1 is considered an immunomodulating agent in cattle, blocking the activation and proliferation of bovine PBMC and mucosal lymphocytes *in vitro* [14,15]. Furthermore, an altered cytokine expression pattern has been observed in these cellular populations upon incubation with the toxin [14,16]. Infections with STEC can suppress the development of specific cellular immune responses during the early phase of immune activation in cattle, and this immunosuppression has been linked to Stx2 [17]. Stx2 has also been related to increased intestinal colonization by *E. coli* O157:H7 in mice [18].

The adhesion to intestinal mucosa and the "attaching and effacing" (A/E) lesion relies on a large pathogenicity island called "locus of enterocyte effacement" (LEE), which is a sequence of 35.6 Kb with 5 operons [19–21] that encodes for a type three secretion system (T3SS), i.e., a series of T3SS components, gene regulators and effectors proteins, and the protein responsible for intimate adherence (Intimin) to enterocytes along with its translocated receptor (Tir) [22].

Various *E. coli* O157:H7 virulence factors are capable of inducing an immune response in cattle during natural as well as experimental infections. It has been shown that calves respond serologically to LEE-encoded proteins such as Intimin, EspA and EspB, after an experimental infection with *E. coli* O157:H7 [23]. Antibodies against these proteins have also been found in people infected with EHEC and enteropathogenic *E. coli* (EPEC) [24,25] and in colostrum and milk from naturally infected cows [26].

Vaccination of cattle with bacterial colonization factors has been suggested as a strategy to reduce STEC colonization of the bovine gastrointestinal tract. Several experimental vaccines have been developed, many of them based on LEE secreted proteins, Tir and Intimin; O157 lipopolysaccharide, siderophores and porin receptors have also been tested with variable results [27–33]. We, along with other groups, have demonstrated that vaccination of calves with T3SS injection apparatus proteins results in reduced excretion of EHEC O157:H7 after experimental challenge [27,30,34,35]. The vaccine tested by our group included the recombinant carboxy-terminal fraction of Intimin γ and EspB with an oily adjuvant and calcitriol. Vaccinated calves showed high titers of serum IgG against both antigens after the first dose and specific IgA increased in saliva, but not in faecal matter. We verified a statistically significant reduction in faecal excretion of EHEC O157:H7 in the vaccinated group compared to the control. This protective effect was observed both at the level and frequency of excretion [27]. These results show that vaccination with T3SS recombinant proteins is a good strategy to reduce faecal shedding. However, vaccine formulations need to be further optimized in order to improve protection.

We have previously observed a specific and neutralizing response against Stx2 upon addition of BLS-Stx2B to the previously mentioned experimental vaccine [36]. The aim of the present study was to assess the ability of the 3-antigen formula to reduce EHEC carriage after experimental challenge. Also, several aspects of the immune response were evaluated.

2. Materials and methods

2.1. Animals

Fifteen 4-month-old conventionally reared Holstein Friesian male calves were obtained from a dairy farm in Buenos Aires Province, Argentina and housed at the Instituto Nacional de Tecnología Agropecuaria (INTA) Research Centre. Animals were selected on the basis of absence of Shiga toxin-producing *E. coli* carriage, assayed by enrichment of recto-anal mucosal swabs streaked onto sorbitol McConkey agar. The confluent growth zone was used as template to perform multiplex PCR to amplify *stx1*, *stx2*, *eae* and $O157_{rbf}$ [37–39]. Furthermore, the selected animals had low levels of serum antibodies against the carboxy-terminal fraction of Intimin γ and EspB (OD < 0.2 under the conditions explained below). Calves were fed alfalfa pellets, with free access to hay and water and treated prophylactically upon arrival with 1% Ivermectin to control intestinal nematodes. All animal experiments were performed with the approval of the Institutional Animal Care and Use of Experimentation Animals Committee (CICUAE) of the INTA in BSL2 containments facilities for large animals. One calf from the 2-antigen vaccinated group died 9 days after the challenge presumably due to pneumonia. It was excluded from all the analysis from that day onwards.

2.1.1. Production of recombinant E. coli O157:H7 proteins

The coding sequences of EspB and IntC280 from the bovine *E. coli* O157:H7 strain 146N were cloned in pRSET-A vector (Invitrogen Corp., Carlsbad, CA) and expressed in *E. coli* BL21 (DE3)/pLysS, as described previously [26]. Briefly, the amino terminal-His-tagged proteins were purified from the lysates by affinity chromatography on Nickel-agarose columns (ProBond nickel-chelating resin; Invitrogen Corp.), eluted under denaturing conditions and dialyzed against PBS at pH7.4.

The B subunit of Stx2 was cloned upstream to the *Brucella* lumazine synthase (BLS) gene and the recombinant protein was expressed as described elsewhere [40]. This antigen was kindly provided by Fernando Goldbaum (Fundación Instituto Leloir, Buenos Aires, Argentina).

Recombinant Stx2B and a small fragment of A subunit were concomitantly expressed as previously described [41]. The A subunit fragment and the B subunit are expressed independently as separate polypeptides. The B subunit is fused to polyhistidine and was purified by Nickel agarose column. This antigen was used in anti-Stx2B ELISA, whole blood re-stimulation and re-stimulation of peripheral blood mononuclear cells (PBMC).

2.2. Immunization protocol

Calves were randomly separated into 3 groups and vaccinated according to the following scheme: non-vaccinated control (n = 5): PBS; Group 3Ag (n = 5): IntC280 + EspB + BLS-Stx2B; Group 2Ag (n = 5): IntC280 + EspB. The immunization scheme consisted on the application of 2 doses, 15 days apart (day 0 and day 15 post vaccination, dpv), with 100 μg of IntC280, 100 μg EspB and 300 μg of BLS-Stx2B by intramuscular route. The antigens were diluted in 1 mL of PBS and emulsified in 1 mL of mineral oil-based adjuvant (Montanide ISA206, Seppic, France). The control group was vaccinated only with PBS emulsified in the adjuvant.

2.3. Challenge

Ten days after the booster, animals were challenged intragastrically with 10^9 CFU of *E. coli* O157:H7 strain 438/99 in 15 mL of sterile PBS. An overnight culture of the challenge strain was diluted

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1/30 in Luria-Bertani broth (LB) with nalidixic acid (20 μg/ml) and grown with aeration until optical density reached 1.1 (5 hours approximately). Animals were challenged with 10 mL of a 1/10 dilution of the previously mentioned culture.

The challenge strain was isolated from a healthy cow and has been used previously in experimental studies [27]. It was selected for spontaneous resistance to nalidixic acid to facilitate recovery from recto-anal mucosal swabs and tissues and possesses the genes for enterohemolysin, Intimin γ, EspA, EspB, Stx2, and the pO157 plasmid.

2.4. Sample collection

Blood samples were collected at different times during the study and recto-anal swabs were collected periodically after the challenge. Samples taken at 0 and 15 dpv were taken prior to challenge. A brief description of the sampling days and the use of the samples are shown in Table 1.

2.5. Immune response assessment

2.5.1. Humoral immune response

All serum samples were analyzed by ELISA to detect specific antibodies against the antigens used in the immunization protocol, as described elsewhere [40,42]. Briefly, 96-well Nunc-Immuno MaxiSorp assay plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 100 μL of either IntC280 or EspB at 1 μg/mL in carbonate buffer pH 9.6 or 100 μL of Stx2B at 5 μg/mL. After three washes with PBS pH 7.4 containing 0.05% Tween 20 (PBST), non-specific binding sites were blocked with 3% skimmed milk in PBS for 1 h at 37 °C. After that, washes were repeated and 100 μL of serum diluted 1/50 was added. Plates were incubated for 2 h at 37 °C. For each plate, two wells were incubated with PBS-T alone (negative control), and a known positive sample was included. Each serum sample was analyzed in duplicate. After washing with PBS-T, wells were incubated for another hour with 100 μL of rabbit anti-bovine IgG1 or IgG2 conjugated with horseradish peroxidase (Bethyl Laboratories, Montgomery, USA), at dilutions of 1:10,000 in PBS-T. Plates were washed three times with PBS-T. Finally, ABTS [2,2-azino-di (3-ethyl-benzthiazoline sulphonic acid)] (Amresco, Solon, USA) in citrate-phosphate buffer pH 4.2 plus 0.01% H_2O_2 (100 μL/well) was added to plates coated with IntC280 or EspB. Reactions were stopped after 10 min with 100 μL/well of 5% SDS and read at 405 nm (OD_{405}) in a BioTekELx808 microplate reader (BioTek Instruments, Winooski, USA). Color reaction in plates coated with Stx2B was developed using OPD (o-phenylenediamine dihydrochloride) and read at 492 nm (OD_{492}). Levels of antibodies are expressed as OD_{405} and OD_{492}, respectively, which represent the mean value of the duplicated minus the OD of the negative sample. Data were analyzed in GraphPad Prism 6.01 by 2-way ANOVA, with Tukey's multiple comparison post-test.

2.5.2. Mucosal immune response

Mucosal antibodies from intestinal tissue samples (recto-anal junction, ileum and cecum) taken at necropsy were obtained and assessed by ELISA for the presence of specific antibodies as described in Martorelli et al. [42]. In brief, intestinal tissue samples frozen at −70 °C were thawed and 50–75 mg of mucosa were excised and mixed with 1 mL of ice-cold PBS (pH 7.2). Tissues were processed in a homogenizer (FastPrep®-24, MP Biomedicals) for 10 s at 5.5 m/s using 150–212 μm glass beads (Sigma). The tubes were placed immediately on ice and centrifuged to remove debris. The supernatants protein content was assessed by the Bradford technique and standardized to 0.5 mg/mL in PBS with 0.5% Tween 80 and 0.5 M NaCl, as described by Nart et al. [43]. Data were analyzed in GraphPad Prism 6.01 by 2-way ANOVA, with Tukey's multiple comparison post-test.

2.6. Neutralization of Stx2 cytotoxicity on Vero cells

In order to assess the neutralization ability of sera and intestinal homogenates obtained from vaccinated animals, Stx2 (Phoenix Laboratory, Boston, MA, USA) at a concentration required to kill 50 % of Vero cells (1 CD_{50} = 0.1 ng/ml) was co-incubated with serum (1:1000) or intestinal homogenates (1:50) from the different experimental groups for 1 h at 37 °C with shaking. CD_{50} was determined previously by serially diluting Stx2 on Vero monolayers and performing non-linear regressions to estimate the value. The Stx2 co-incubated samples were then added to monolayers of Vero cells and incubated for 72 h. Vero cells were also incubated under the same conditions with the homogenates without the toxin, in order to assess *per se* cytotoxicity. Cell viability was determined using the neutral red assay adapted from a previously described protocol by Pistone et al. [41]. Results are expressed as percentage of cytotoxic effect neutralization using the following formula:

$$\frac{(H - Stx2)}{(C - Stx2)} \times 100.$$

Table 1
Description of timing, samples and assays performed during the animal study.

Days post immunization (dpv)	Sample	Assay
0 (prior to prime immunization)	Serum	Anti-vaccine antigen ELISAs
		Stx2 neutralization assay
	Heparinized whole blood	IFN-γ release assay (IGRA)
		Cytokine mRNA in stimulated PBMC
15 (prior to boost immunization)	Serum	ELISA/neutralization
24 (prior to challenge infection)	Serum	ELISA
	Heparinized whole blood	IGRA
		Cytokine mRNA
26	Recto-anal swab	*E. coli* O157:H7 detection
28	Recto-anal swab	*E. coli* O157:H7 detection
30	Recto-anal swab	*E. coli* O157:H7 detection
33	Recto-anal swab	*E. coli* O157:H7 detection
36	Serum	ELISA/neutralization
	Heparinized whole blood	IGRA
		Cytokine mRNA
	Recto-anal swab	*E. coli* O157:H7 detection
39–40 (Necropsy)	Intestinal segments	ELISA/neutralization
		E. coli O157:H7 detection

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where H is the absorbance of cells treated with the serum/homogenate and Stx2 mixture, Stx2 is the absorbance of Stx2 treated cells and C is the absorbance of untreated cells. Data were analyzed by non-repeated (intestinal segments) or repeated over time (serum) measures 2-way ANOVA, with Tukey's multiple comparison post-test using GraphPad Prism 6.01 software.

2.7. Interferon gamma (IFN-γ) secretion

IFN-γ secretion was assessed in whole blood stimulated with EspB, IntC280, Stx2B as antigens, and PBS and Pokeweed mitogen (PWM) as negative and positive controls, respectively. Briefly, 200 μL of fresh anticoagulated blood was incubated in 96-well U-bottomed culture plates with 25 μL of antigen solution (36 μg/mL), for 18 h at 37 °C with 5% CO_2. After that, the plates were centrifuged for 30 min at 3500 rpm and the supernatants recovered and stored at −80 °C until use. IFN-γ was assessed using the Bovine Interferon Gamma ELISA Kit (BioRad) following the manufacturer's instructions. Results are expressed as Optical Density Index (ODI), which is the OD obtained for antigen-stimulated blood divided by results from PBS-stimulated cultures. The ODI was calculated for the 3 antigens tested and the 3 immunization groups, at 3 different time points. Data were analyzed in GraphPad Prism 6.01 by 2-way ANOVA, with Tukey's multiple comparison post-test.

2.8. Cytokine mRNA detection in bovine PBMC

Amounts of mRNA molecules for prototypic cytokines were assessed by real-time PCR (RT-PCR) on PBMC stimulated with the antigens used in the vaccination protocol at 0 dpv (pre-immune), 24 dpv (after the booster) and 36 dpv (12 days after the challenge). Briefly, PBMC were separated from heparinized blood by gradient centrifugation over Histopaque 1077 (Sigma-Aldrich) following the manufacturer's protocol. From each animal, 10^7 cells were incubated at 37 °C for 16 h with 21 μg/mL of a mixture of EspB, IntC280 and Stx2B (7 μg/each) in RPMI 1640 complete medium (supplemented with 10% FCS, penicillin, streptomycin and glutamin). A non-stimulated control was included for each animal incubating the cells under identical conditions but with PBS instead of the antigens. After the incubation period, cells were lysed in 1 mL Trizol reagent (Invitrogen) and stored at −80 °C. RNA was extracted following manufacturer instructions which include chloroform extraction. Quality and quantity of total RNA were estimated by UV spectrophotometry (Nanodrop, Wilmington, DE, USA) and electrophoresis on 0.8% agarose gel. DNA-free RNA (1 μg) was mixed with 50 ng of random primers (Invitrogen) in 20 μL of final volume and reverse transcribed to total cDNA with SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's instructions. RT-PCR was performed to detect mRNA encoding the following cytokines: Interleukin (IL)-2, IL-4, IL-8, IL-10, IFN-γ, Tumor Necrosis Factor alpha (TNF-α) and Transforming Growth Factor beta (TGF-β). The housekeeping gene GAPDH was used as a control for constitutive gene expression. The conditions of the real time PCR were performed as described by Sobotta et al. [44]. PCR amplification was performed on an automated fluorometer (ABI PRISM™ 7000 Sequence Detection System, Applied Biosystems) using 96-well optical plates. Each sample was analyzed in duplicates. For PCR, 1.25 μL of cDNA was used in a 25 μL PCR reaction mixture containing 12.5 μL SYBR® Green PCR Master Mix (Life Technologies) and 0.45 μM of each primer. Amplification conditions were the same for all targets assayed: one cycle at 50 °C for 2 min, one cycle at 95 °C for 10 min and 40 cycles at 95 °C for 15 s and at 60 °C for 60 s. Specificity was confirmed analyzing the melting curves and negative samples were given an arbitrary Ct value of 38. REST beta 9 software was used for final calculations and expression variation for each cytokine gene was provided by the software, after normalization referring to the housekeeping gene GAPDH (http://rest.gene-quantification.info).

2.9. Shedding

Shedding of viable *E. coli* O157:H7 was followed in recto-anal mucosal swabs during a 15-day period after challenge, as previously described [45]. Briefly, bacterial CFU/swab were determined by vortexing the swabs in Trypticase soy broth (TSB, Oxoid, Basingstoke, UK), plating serial dilutions on Sorbitol MacConkey agar (Oxoid, Basingstoke, UK) containing 20 μg/mL nalidixic acid (Sigma, St. Louis, USA), 2.5 μg/mL potassium tellurite and 0.05 μg/mL cefixime (CT-SMAC-NAL). When direct cultures were negative, swabs were enriched at 37 °C for 18 h and 1 mL of this culture was subjected to *E. coli* O157 immunomagnetic separation (IMS) performed according to the manufacturer's instructions (Dynabeads anti-*E. coli* O157, Invitrogen Dynal AS, Oslo, Norway). The bead-bacterium mixture was plated on CT-SMAC-NAL. Samples that resulted culture-positive by IMS were considered positive (and assigned a value of 10 CFU), while samples culture-negative by IMS were deemed negative (and assigned a value of 1 CFU). Non-sorbitol-fermenting colonies were tested for *E. coli* O157 LPS by latex agglutination (Oxoid, Basingstoke, UK) and confirmed by a multiplex PCR for the *stx1, stx2, eae* and rbf_{O157} genes, as described previously [37–39].

Recto-anal junction segments obtained at necropsy were enriched overnight in 3 mL of TSB and subjected to IMS for the detection of *E. coli* O157:H7.

The analysis of shedding required the data to be transformed to normality by means of the Cox-Box procedure, as discussed by Peltier et al. [46]. Transformed data was analyzed with a model including fixed effects of treatment and linear and quadratic effects of the covariate time (in days) nested within treatment. The covariance structure of error terms accounted for heterogeneity of variance due to animal, where the range of heteroscedasticity was almost 34 times larger (0.27–9.12). The model was fitted with PROC MIXED of SAS (SAS v.9.2; SAS Institute Inc., Cary NC, USA). Linear contrasts were formed to test for the linear functions of interest with degrees of freedom corrected by the procedure of Kenward and Roger [47]. The hypothesis of interest was whether the estimated area under the curve (AUC) differed between the three immunization groups.

3. Results

3.1. Humoral immune response

Specific serum antibodies were detected in vaccinated animals against the respective antigens, while unvaccinated control animals did not increase the level of antibodies against IntC280, EspB or Stx2 even after challenge (Fig. 1).

3.1.1. IntC280 and EspB antibodies

IgG1 antibodies against IntC280 and EspB were significantly higher in immunized calves after just one immunization dose, while the second dose increased further more IgG1 antibodies against both antigens. Vaccinated animals also developed IgG2 antibodies against these 2 antigens, although at lower levels compared to IgG1 (Fig. 1).

3.1.2. Stx2B antibodies

IgG1 antibodies against the B subunit of Stx2 were only detected in animals immunized with the 3-antigen vaccine and raised at day 24, after the second immunization dose. Booster was necessary to significantly increase Stx2B antibodies above

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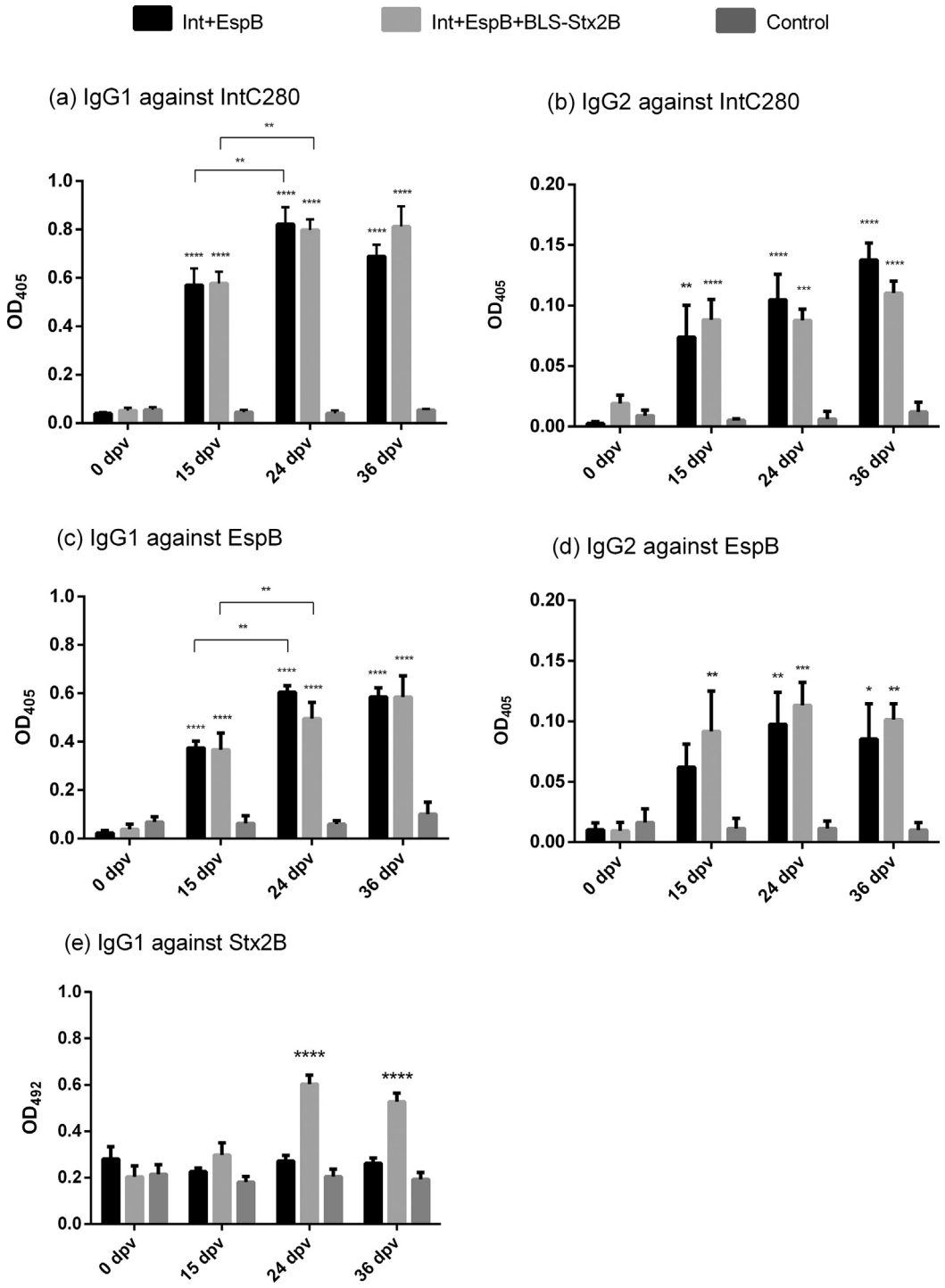

Fig. 1. Serum antibodies at different times after vaccination: (a) IgG1 against IntC280, (b) IgG1 against EspB, and (c) and IgG1 against StxB. n = 5 animals per group; samples of each animal/time point were tested in duplicate; mean values of duplicate determinations were used for further statistical analysis. Statistically significant differences are marked for each time point in vaccinated groups when compared to the non-vaccinated controls and between sampling days between the same immunization group (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

the control level (Fig. 1). IgG2 against Stx2B was not significantly detected in neither of the groups (data not shown). The response against the carrier protein BLS was also measured. At 1/500 dilution of sera, the OD492 was around 0.8 (data not shown) suggesting that the level of these antibodies is higher than that observed for Stx2B antibodies.

3.1.3. Stx2 neutralization in serum

In order to determine the reactivity of antibodies against Stx2 present in sera, their cytotoxicity neutralization capacity on Vero cells was analyzed. Sera samples taken at 0, 15, 24 and 36 dpv were tested at a 1:1000 dilution. Sera from animals vaccinated with 3 antigens exhibited a significant neutralization of Stx2 cytotoxicity

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on Vero cells compared to calves vaccinated with 2 antigens and the non-vaccinated group (Fig. 2).

Significantly higher cytotoxic neutralization capacity for the 3-antigen vaccinated group was observed at day 24 and 36 dpv compared to 15 dpv. No neutralization capacity was observed at 0 dpv in neither of the 3 groups of calves (Fig. 2).

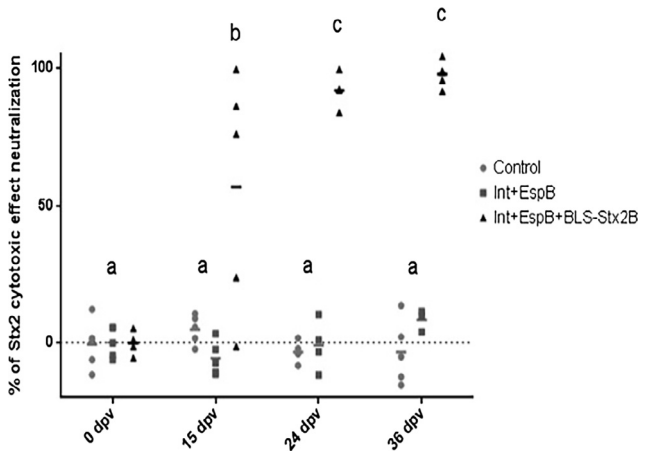

Fig. 2. Percentage of Stx2 cytotoxic effect neutralization in sera (1:1000 dilution) from vaccinated and control animals evaluated at different times post vaccination. Each point represents an individual, and horizontal lines represent the group media. Two-way, matched values ANOVA with Tukey's post-test was used ($p < 0.01$). Treatments displaying the same letter showed no significant differences between them, whereas treatments displaying different letters showed statistically significant differences among them.

3.2. Mucosal immune response

Antibodies in the mucosa of ileum, cecum and recto-anal junction were assessed in intestinal segments obtained at necropsy from all the animals involved in the study, except the one calf that died before the necropsy was performed.

3.2.1. IntC280 and EspB antibodies

Mucosal IgG1 antibodies against IntC280 were significantly higher in ileum, recto-anal junction and cecum of animals vaccinated with 2 as well as 3 antigens, when compared to non-vaccinated calves. As regards anti-EspB antibodies, a significant difference was observed in RAJ of the 2 groups immunized with this antigen (Fig. 3). No differences were observed between the three intestinal segments within groups.

IgG2 against these antigens was detected at very low levels and no significant differences were found (data not shown).

3.2.2. Stx2B antibodies

Ileum was the segment that rendered higher OD values of anti Stx2B antibodies. The level of antibodies in this tissue from animals immunized with 3 antigens was higher than in ileum from the animals vaccinated with 2 antigens or controls. No significant differences were found in recto-anal junction and cecum between the 3 groups of calves (Fig. 3).

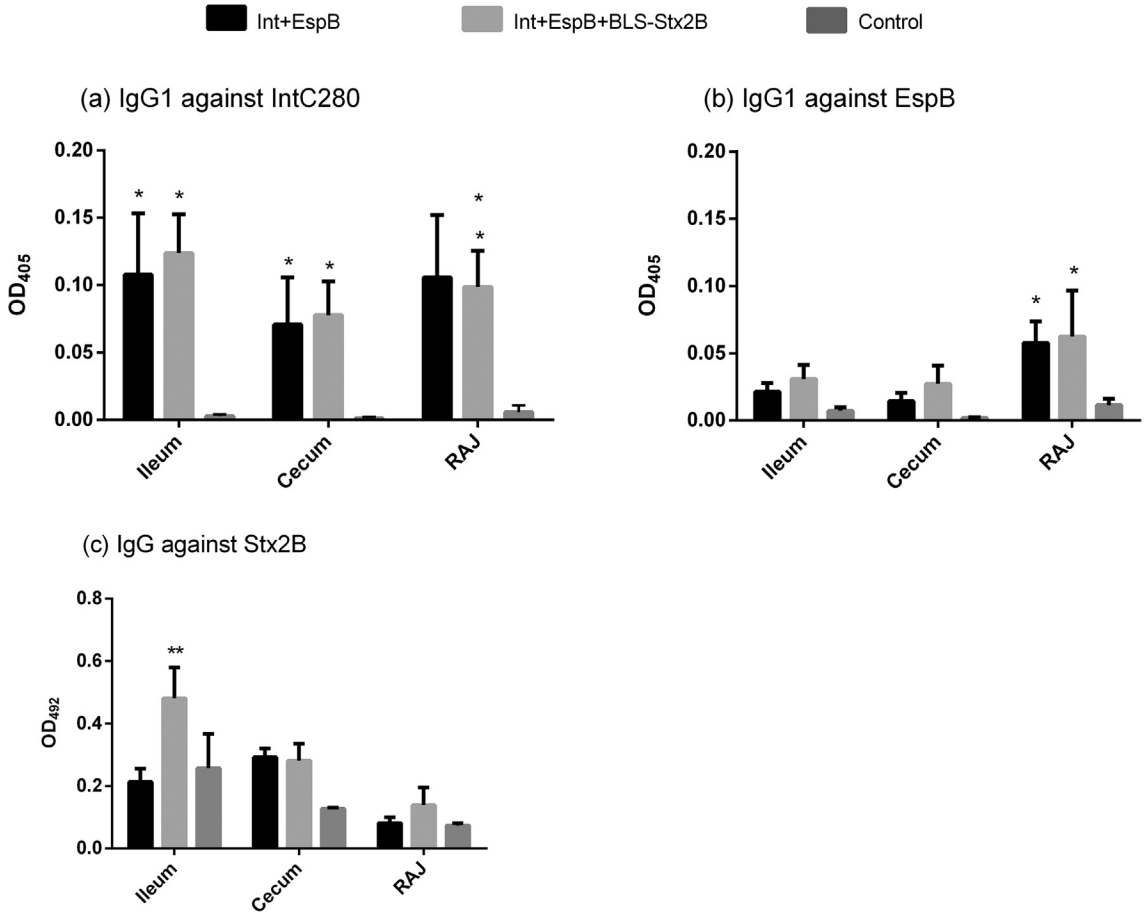

Fig. 3. IgG1 mucosal antibodies in different intestinal tissues taken at necropsy: (a) IgG1 against IntC280, (b) IgG1 against EspB, (c). IgG against Stx2B, in ileum, cecum and recto-anal junction of calves vaccinates with 2 as well as 3 antigens and control non-vaccinated animals. Statistically significant differences (*, $p < 0.05$) between intestinal segments of vaccinated animals compared to the same segment from non-vaccinated animals are shown.

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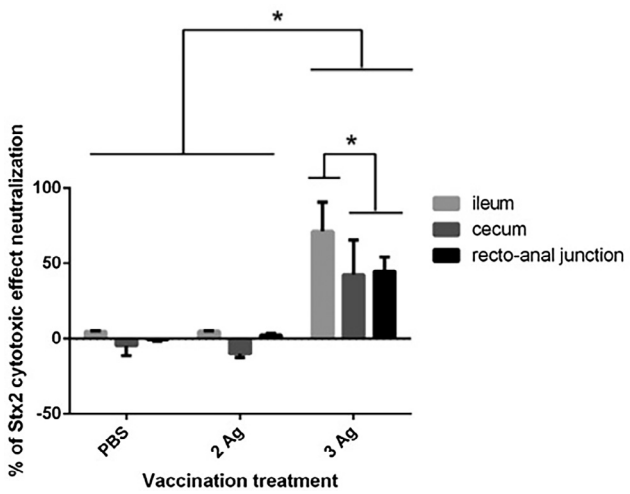

Fig. 4. Stx2 cytotoxic effect neutralization at 1:50 dilution of the intestinal homogenates of vaccinated and control animals. PBS, control; 2Ag, Int + EspB; 3Ag, Int + EspB + BLS-Stx2B. Significant differences (*, $p < 0.05$) were found for the 3 intestinal segments between animals vaccinated with 3 antigens when compared to the other 2 groups. A significant increase in neutralization was also found in ileum of the calves vaccinated with Int + EspB + BLS-Stx2B when compared to cecum and recto-anal junction.

3.2.3. Stx2 neutralization activity in mucosa

Intestinal tissue homogenates (1:50) were tested, using Stx2 1 CD_{50} (0.1 ng/ml, as indicated previously) to assess their ability to neutralize Stx2 cytotoxicity. A significant difference ($p < 0.05$) was observed between animals vaccinated with 3 antigens when compared to the 2-antigen vaccinated calves and the control group for the 3 intestinal segments. Also, the ileum homogenate of the 3-antigen vaccinated group showed a significantly higher neutralization ability compared to cecum and recto-anal junction (Fig. 4).

3.3. Cytokine mRNA detection in PBMC

To assess the cytokine's expression pattern after vaccination and challenge, mRNA was determined in stimulated (mix of EspB, IntC280, and Stx2B) and unstimulated PBMC from the 2 vaccinated and control groups before immunization (0 dpv), after the booster (24 dpv) and 12 days after challenge (36 dpv). To measure if the vaccination itself influences immune cell responsiveness, we compared cytokine expression in unstimulated PBMC of the different groups after vaccination or challenge (samples at 24 and 36 dpv) with their unstimulated pre-immune condition and found no significant differences (data not shown). Similarly, before vaccination all animals exhibited a comparable cellular immune status as no significant differences where found in relative cytokine expression after stimulation in the vaccinated groups (2Ag and 3Ag) when compared to the control at 0 dpv (see supplemental table). After immunization (24 dpv) the cytokine IL-4 was the only parameter significantly upregulated in re-stimulated PBMC (on average) in the group of calves that received either the two or three-antigen immunization, when compared to control animals. At day 36, i.e., after challenge, no significant differences in cytokine expression were found between none of the vaccinated calves when compared to the non-vaccinated control. However, IL-4 was upregulated in the group immunized with BLS-Stx2B when compared to the group that received the 2-antigen vaccine (Fig. 5).

3.4. Interferon-γ release assay (IGRA) in stimulated whole blood

IGRA showed a mild secretion of IFN-γ after stimulation with IntC280 as well as EspB of blood samples from calves vaccinated with 2 and 3 antigens at 24 and 36 dpv. However, these results are not statistically significant different. Stimulation with Stx2B resulted in a statistically significant secretion in the group immunized with 2 antigens at day 24 (supplementary material).

3.5. Shedding

E. coli O157:H7 shedding was followed by recto-anal junction swabs for 15 days after challenge and the areas under the shedding curves were estimated according to the model fitted with PROC MIXED of SAS. Vaccinated animals excreted significantly less than the non-vaccinated group. In turn, there were no significant differences between the groups vaccinated with three or two antigens (Fig. 6). Without taking into account the magnitude of shedding and considering the individual animal excretion as a dicotomic value (y/n), each group was sampled 35 times (5 calves/group in 7 sampling points). The 2-antigen vaccinated group was positive 19 times, the group vaccinated with 3 antigens, 22 times and the non-vaccinated group 27 times. If only direct plating and colony counting are considered the values are 16, 22 and 25, respectively. *E. coli* O157:H7 was not detected after IMS of cultured recto-anal junction segments in neither of the animals involved in the protocol.

4. Discussion

Type-three secretion system proteins have been considered vaccine candidates to mitigate fecal STEC shedding by cattle and comprehensively tested in animal challenge studies or under field conditions of natural exposure with partial success [27,28,30,35,48]. Although the basis of protection is not fully understood, we previously demonstrated that antibodies directed against these virulence factors inhibit *E. coli* O157:H7 adherence to epithelial cell lines and neutralize red blood cell lysis provoked by the T3SS [36]. Stx has been suggested to have a local immunosuppressive effect in the bovine gut. Given that infections with STEC can suppress the development of specific cellular immune responses in cattle promoting intestinal colonization of the bacteria, it may be pertinent to include Stx in a vaccine formulation. The elicitation of specific and neutralizing antibodies may abrogate its immunosuppressive action and thus, a stronger anti-STEC response may be obtained. Several studies have used Stx1 or Stx2, either the A or B subunit, mixed or fused as a vaccine antigen and assessed its protective efficacy in different animal models mostly to prevent Stx-mediated disease [49]. Kerner et al. prepared mutated toxoids of Stx1 and Stx2, which are devoid of immunosuppressive effects in bovine immune cell cultures and, when applied to cattle, led to an induction of neutralizing antibodies [50]. Very recently, first evidence was presented that immunization with these toxoids enables calves to actively mount a more rapid and effective immune response after natural exposure to STEC strains circulating in the respective cohort which results in a reduction of STEC shedding under field-like conditions [51]. However, such antigens have not yet been tested in cattle upon experimental challenge with STEC. In order to assess Stx as candidate vaccines, their intrinsic low antigenicity should be taken into account. To circumvent this problem we used Stx2B fused to *Brucella* lumazine synthase (BLS) that has the ability to enhance the antigenicity of proteins fused to it [52–55]. Particularly, the Stx2B subunit fused to BLS induced a prominent antibody response capable of neutralizing holotoxin and thereby protecting mice [40,56]. No immunization trial with an antigen fused to BLS has been published yet in cattle. In our hands, the humoral response to BLS in calves vaccinated with BLS-Stx2B was strong (data not shown). Morover, BLS was immunodominant in cattle infected by or vaccinated against *Brucella* spp. [57].

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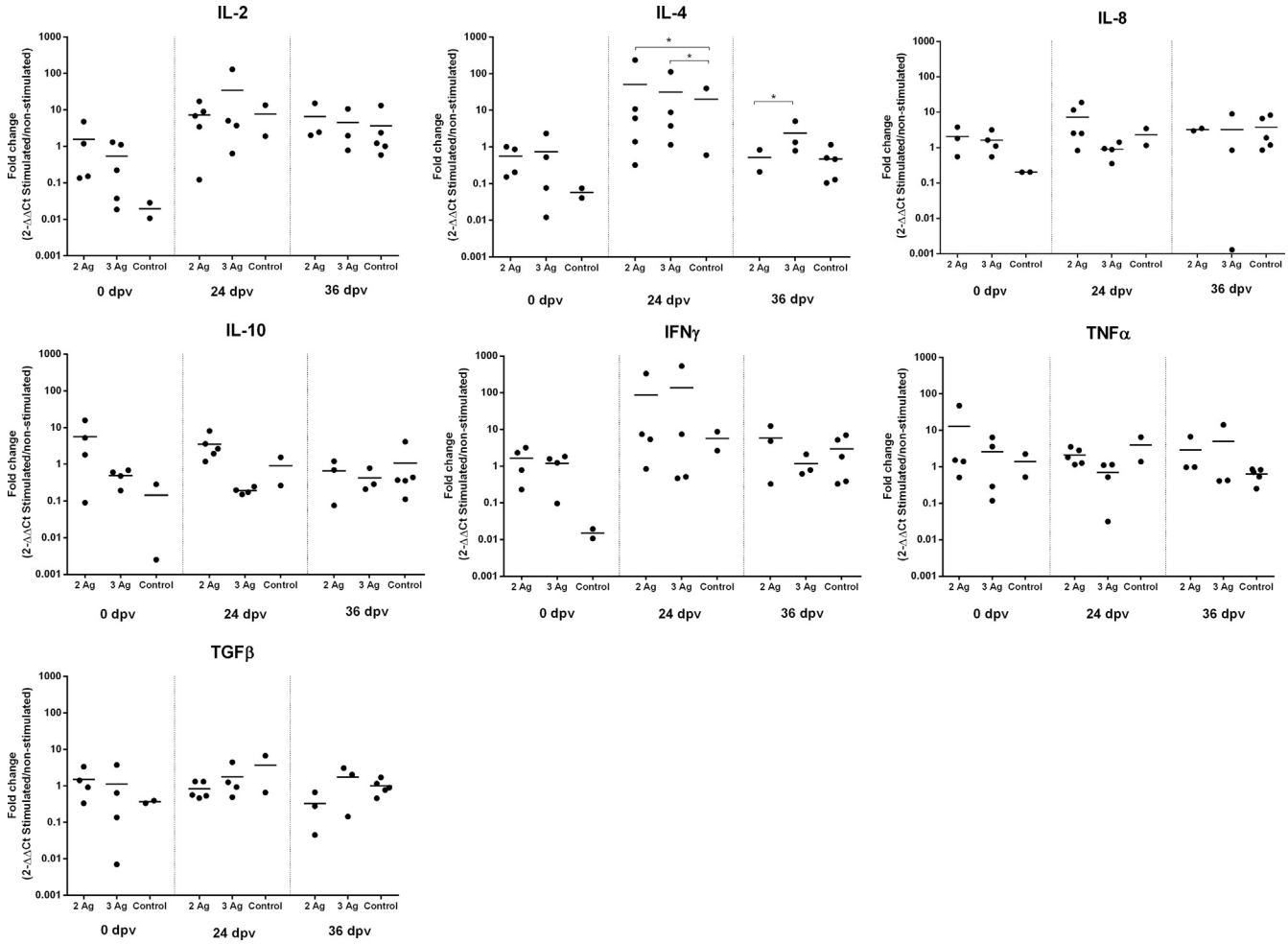

Fig. 5. Cytokine's relative expression in the two vaccinated and control groups at 0, 24 and 36 days post vaccination. From each animal, 10^7 cells were incubated at 37 °C for 16 h with 21 μg/mL of a mixture of EspB, IntC280 and Stx2B (7 μg/each) or in the absence of antigens (unstimulated controls). Results are shows as fold change ($2^{-\Delta\Delta Ct}$) between stimulated and unstimulated samples for each time point. Dots represent the values obtained for each individual animal of which samples were available for the respective time point, while horizontal lines represent the group media. Statistical analysis was performed using REST beta 9 software and significant differences are shown (*, $p < 0.05$).

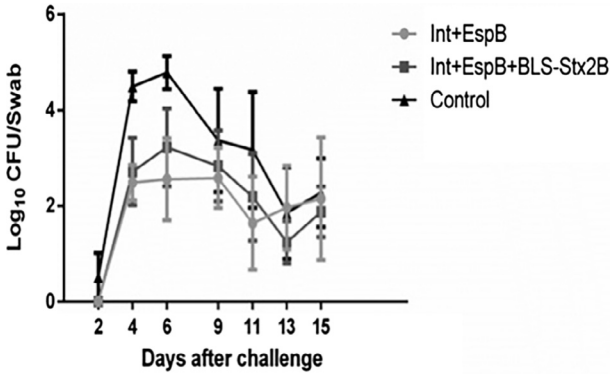

Fig. 6. *E. coli* O157:H7 shedding in vaccinated and control animals during 15 days after challenge. For statistical analysis, areas under the shedding curves (AUC) are compared (IntC280 + EspB vs IntC280 + EspB + BLS-Stx2B p = 0.662; IntC280 + EspB vs control p = 0.0024; IntC280 + EspB+BLS-Stx2B vs control p = 0.0051).

We have previously tested the immunogenicity of a 100 μg-dose of Stx2B-BLS alone or applied in combination with EspB and IntC280 in calves and observed a moderate response with antibody levels much lower than those obtained against IntC280 or EspB, but still able to neutralize Stx2 cytotoxicity *in vitro* [36]. In order to evaluate the ability of these antibodies to neutralize Stx2 *in vivo*, the BLS-Stx2B dose was increased from 100 to 300 μg so as to augment the level of specific antibodies against this antigen. At this dose, the inclusion of BLS-Stx2B did not have a significant immunostimulatory effect on the humoral response against IntC280 nor EspB, as we had previously observed [36].

In this work, vaccination with BLS-Stx2B induced antibodies in sera which were efficiently transferred to the mucosa of the large and small intestine, as well as to the recto-anal junction. Neutralizing capacity of sera at 15 dpv showed a dichotomous response, as some sera samples exhibited a maximum neutralizing response, while others showed no response at all. This is probably due to the fact that a 2nd booster vaccination was necessary to elicit an antibody response in some calves, as all sera samples corresponding to 24 and 36 dpv showed a maximum neutralizing response. A more pronounced neutralization of Stx2 cytotoxicity was observed with ileum homogenates as in the other two intestinal segments. This effect could be probably due to the more abundant Peyer's patches present in the ileum, which make this intestinal segment particularly suited to evoke a local immune response.

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Cultivation of PBMC obtained from all calf groups in the presence of a mixture of all antigens deployed in the study (EspB, IntC280 and Stx2B) led to an increase in the abundance of IL-4-specific mRNA from 0 to 24 dpv independent of vaccination status, with minor but significant differences between groups. It appears that all animals have experienced a phase of a Th2-biased immune response subsequent to the application of the adjuvant which rendered the PBMC prone to non-specifically respond to exposure to bacterial products. Amounts of IL-4 mRNA returned to levels similar to those determined at the beginning of the study after challenge infection. Of note, re-stimulated PBMC of 3-Ag vaccinated animals exhibited highest values. Elevated IL-4 mRNA levels were also observed in bovine ileal intraepithelial lymphocytes after incubation with Stx1 [16]. Interestingly, this *in vitro* effect was neither observed in circulating lymphocytes nor upon incubation with the recombinant Stx1 and Stx2 toxoids [50], suggesting that a functional toxin is required to stimulate *IL-4* transcription. Taking into account that samples were not available for analysis from any calf deployed in the study at every time point and the groups differed in their PBMC's cytokine profile at the beginning of the study already, it remains to be proven that StxB2 immunization preserves or even promotes a Th2-biased immune response in cattle following contact with shigatoxigenic *E. coli* O157:H7.

Although the immunization induced a significant and neutralizing IgG response against Stx2B in sera as well as intestinal tissues, the addition of BLS-Stx2B conferred no further protection from shedding in the observation period compared to IntC280 and EspB alone after experimental *E. coli* O157:H7 challenge. Several hypotheses may explain these results:

(i) Robinson et al. [18] demonstrated that Stx increased the adherence of EHEC to the bovine intestinal epithelium, releasing Stx locally in adherence sites of microcolonies. In this situation it may be possible that anti-Stx specific antibodies cannot penetrate the space between the mucosa and the microcolonies and no neutralization occurs before the resorbed Stx has reached cells of the immune system, in particular after experimental high dose challenge. In a different setting of natural – presumably low dose – exposure to STEC strains in a calf cohort subject to a more comprehensive immunization scheme (transfer of high immune colostrum plus active vaccination with Stx toxoids), vaccinated animals were less frequently positive for STEC shedding during a study period of 55 weeks [51].

(ii) The level of specific antibodies against Stx2B obtained are low and cannot neutralize all the Stx2 produced in cattle intestine. Antibodies obtained in this and in a previous work [36] are lower than those observed for Stx2B and other BLS fused antigens in other species, mainly mice [40,56,58–60]. Despite the fact that the B subunit is fused to BLS in order to increase its antigenicity, the moderate level of antibodies elicited suggests this antigen is tolerated to some extent by the bovine immune system. Indeed, domains resembling the receptor (globotriaosylceramide) binding sites of StxB have been identified in the bovine interferon-α receptor qualifying presumably protective epitopes in StxB as potential autoantigens [61,62].

(iii) Immunosuppression by Stx has been linked to the blockage of early phases of immune activation in naïve calves, rather than suppressing an established immunity [17]. Given that calves become exposed to a plethora of STEC strains early in life [63] and that calves of our study possibly had pre-existing antibodies against the toxin, we cannot rule out the possibility that Stx2 produced in the intestine at the age of the calves and for the duration of the study has had little effect on the pre-shaped immune response in these animals. Hoffman et al. observed that an *E. coli* O157:H7 strain devoid of *stx2* had a significantly reduced shedding when compared to the parental strains and concluded that STEC can suppress the development of specific cellular immune responses in cattle [17]. However, Stx or genes encoded in Stx carrying phages may affect gene expression of other virulence related genes in *E. coli* O157:H7 [64]. In consequence the lack of immunosuppressive effect in Stx-deleted mutants may not be attributed to Stx itself.

(iv) Antibodies against the StxA subunit might be needed for an effective neutralization of Stx2 toxicity [65], since *in silico* analysis of this subunit revealed both B and T cell epitopes [66]. However it has been demonstrated *in vitro* that antibodies directed against the B subunit are able to efficiently neutralize Stx [40,67].

(v) Stx2 neutralization in the bovine intestine impairs the development of a mild but already established immunity against the toxin. A similar effect has been observed after vaccination with H7 flagellin, where systemically-induced H7-specific IgG neutralized TLR5 activation and subsequently reduced vaccine efficacy [48]. As shown in the shedding dynamics figure, the AUC of the group of calves vaccinates with 3 antigens was higher than AUC of animals immunized with 2 antigens, although this difference is far from being significant.

In spite of the fact that no higher protection was observed when Stx2B was added to the vaccine formulation after experimental challenge, it remains to be determined if E. coli O157:H7 shedding patterns are altered in the long term and after recurrent low dose exposure as occurring in cattle herds. We also consider that there is still room to improve the role of Stx2 as an immunogen present in a vaccine to control cattle colonization by EHEC. Several strategies such as increasing the dose of Stx2 antigen, evaluate new adjuvants to improve the mucosal response or include the Stx2A subunit, are to be further explored.

Declarations of interest

None.

Contributors

L M: performed the experiments, conducted statistical analysis, analyzed the results, wrote the manuscript,
N G: performed the experiments, conducted statistical analysis,
G A: performed the experiments, conducted statistical analysis,
D A V: performed the experiments,
S G G: performed the experiments in bovines,
S A B: performed the experiments, conducted statistical analysis, analyzed the results,
C M: analyzed the results, wrote the manuscript,
C I: analyzed the results, wrote the manuscript,
M S P: analyzed the results, wrote the manuscript,
A C: analyzed the results, wrote the manuscript.

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Appendix A. Supplementary material

Interferon-γ released from whole blood stimulated with the antigens employed in the immunization protocol, at different times post vaccination. Results are expressed as ODI (OD antigen-stimulated/OD PBS-stimulated; $^{*}p < 0.05$). Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.05.059.

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