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Physiopathological effects of *Escherichia coli* O157:H7 inoculation in weaned calves fed with colostrum containing antibodies to EspB and Intimin

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ABSTRACT

Escherichia coli O157:H7 is responsible for severe intestinal disease and hemolytic uremic syndrome (HUS), a serious systemic complication which particularly affects children. Cattle are the primary reservoir for *E. coli* O157:H7 and the main source of infection for humans. In this study, we evaluated the ability of transferred maternal colostral antibodies against γ-Intimin C_{280} and EspB, to protect young weaned calves from *E. coli* O157:H7 infection. Hyperimmune colostra were obtained by immunization of pregnant cows with a mix of the mentioned antigens. All vaccinated cows mounted a significant IgG response against γ-Intimin C_{280}, and EspB in sera and colostra. Colostrum-fed calves also exhibited high serum IgG titers against γ-Intimin C_{280} and EspB along with a rise in mucosal γ-Intimin C_{280}-specific IgG antibodies at recto-anal junction and ileum. Additionally, 70 day-old calves received a challenge with *E. coli* O157:H7 but no reduction in total bacterial shedding or frequency of *E. coli* O157:H7 excretion from these calves was observed. Most tissue samples showed granulocyte focal infiltrations of the lamina propria and enterocyte erosion. In conclusion, up to the 70th day, the passively acquired γ-Intimin-C_{280} and EspB-IgG antibodies present in sera and recto-anal mucosa reached a titer insufficient to reduce EHEC O157 shedding and damages of experimentally inoculated young calves.

1. Introduction

Escherichia coli O157:H7 is a major etiologic agent of diseases in humans and the clinical spectrum that it causes includes diarrhea, hemorrhagic colitis and haemolytic uremic syndrome (HUS). This particular syndrome is the leading cause of chronic renal failure in children in Argentina and several other countries [1]. This bacterium produces Shiga toxin (Stx) types 1 and 2 [2–4], which are responsible for systemic damage.

The main reservoir of *E. coli* O157:H7 is cattle, which harbor this organism in their intestinal tract [5,6], especially on the lymphoid follicle-dense mucosa at the terminal rectum [7]. The bacteria are usually isolated from healthy animals; however these bacteria can produce an initial episode of diarrhea but only in young animals [5]. Several factors can influence the entry of these bacteria into the human food chain: fecal contamination of meat during slaughter, the use of feces as fertilizer, and the contamination of drinking water [5,8].

In addition to Shiga toxins, *E. coli* O157:H7 is characterized by other virulence-associated traits that enable it to colonize the intestinal mucosa of humans and animals. Infections with *E. coli* O157:H7 cause a histopathological lesion known as "attaching and effacing" (A/E) [9]. A large chromosomal pathogenicity island called locus of enterocyte effacement (LEE) is associated with the A/E activity [10,11]. The LEE encodes a type III secretion system (TTSS) that translocates effector proteins into the host cell, which are responsible for the A/E lesion, into the host cell. The A/E lesion is also characteristic of enteropathogenic *E. coli* (EPEC), another category of *E. coli* strains associated with diarrhea in children [9]. The TTSS forms EspA, a filamentous structure through which

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effector proteins are translocated into the host cell [12]. Additionally, Intimin, a bacterial outer membrane protein, binds to Tir, the bacterial translocated Intimin receptor in the host cell membrane. This binding then leads to the formation of the A/E lesion. EspB is translocated into the host cell and contributes, in turn, to the creation of a pore in the eukaryotic cell membrane [13].

Many virulence factors of *E. coli* O157:H7 induce an immune response during the course of natural or experimental infections in animals and in patients with HUS. Oral inoculation of calves and steers with *E. coli* O157:H7 promotes an increase in serum antibody titers against O157 lipopolysaccharide and neutralizing antibodies to Shiga toxins [14]. On the other hand, mice infected by *Citrobacter rodentium*, a bacterium showing virulence determinants and pathological effects in mice highly similar to those of EPEC in humans, develop an immune response against LEE-encoded proteins and are resistant to bacterial re-infection [15]. Bretschneider et al. [15] demonstrated that cattle respond serologically to Intimin and EspB of *E. coli* O157:H7 during the course of experimental infection [16]. Similar results have been reported in naturally infected bovines [17]. Antibodies against these proteins have also been detected in serum during both human enterohemorrhagic *E. coli* (EHEC) [18] and EPEC infections [19] and in colostra and milk from healthy women [20–23]. According to Steele [24], hyperimmune bovine colostrum (HBC) was used for treating gastrointestinal infections in humans in the past decades and demonstrated the promise of this type of therapeutic for gastrointestinal infectious disease. For instance, this type of therapeutic has been used successfully for treatment or prevention of cryptosporidiosis, shigellosis, rotavirus, enterotoxigenic *E. coli*, and *Clostridium difficile* infection. In general high titers have to be reached to achieve a preventive effect of infectious diseases [25].

Vaccination with bacterial colonization factors has been proposed as a strategy to prevent *E. coli* O157:H7 infection. Various vaccine formulations have been assayed with variable results in cattle [26–31,31] and in other animal models [32–37]. In this study we evaluated if calves born from cows vaccinated with Esp B and γ-Intimin C_{280} may have a reduced level of colonization after a challenge with *E. coli* O157:H7.

2. Materials and methods

2.1. Animals

The study included 30 Fresian pregnant cows with an average age of 4 years and more than two lactations. Prior to immunization, fecal samples of all animals were confirmed to be negative for *E. coli* O157:H7 by immunomagnetic separation performed as described elsewhere [26]. All deliveries were natural. Calves were allowed to stay with their dams and were also allowed to suckle colostrum for 48 h. Then, feeding of calves was gradually weaned from milk to 25% protein balancer pellet and finally followed by 18% protein balancer pellet. The immunization of pregnant cows was performed at the Estación Experimental Agropecuaria Rafaela, Instituto Nacional de Tecnología Agropecuaria. All the experiments were performed with the ethical approval of the Comité de Bienestar Animal (Animal Welfare Committee) from the Instituto Nacional de Tecnología Agropecuaria.

2.2. Preparation of His-tagged γ-Intimin C_{280} and EspB proteins

Recombinant expression in *E. coli* BL21 (D3) and purification by affinity chromatography from using a column a Nickel-Chelating Resin was performed as previously described [26,30].

2.3. Immunization schema and experimental infection

Fifteen pregnant cows were intramuscularly immunized at approximately 40 and 20 days before the expected delivery date. Vaccines consisted of 100 μg/dose of the two mixed proteins (EspB and γ-Intimin C_{280}) diluted in 1 ml of PBS and mixed with 1 ml of mineral oil-based adjuvant (Montanide ISA206, SEPPIC, France). A control group of 15 animals received only the adjuvant mix with PBS.

After 72 ± 5 days from delivery, six control and six vaccinated calostrum-fed calves were experimentally inoculated with 10^9 CFU of *E. coli* O157:H7 strain 438/99 NalR by the oral route. We have previously tested this dose as effective for a fecal excretion lasting more than 10 days. The challenge strain has the genotype *stx2*, *eae*, and was isolated from a healthy cow in 1999 and selected for spontaneous resistance to nalidixic acid.

2.4. Sample collection

Serum samples were collected from cows at the time of the first immunization and at the delivery as well as from calves at 2, 25, 50, 72, 88 and 93 days of life. All serum samples were stored at −20 °C until further processing (Fig. 1). Colostrum samples were obtained from cows within the first 24 h after parturition for immune assays. These samples were delipidated by centrifugation at 3000 rpm at 4 °C for 45 min and the watery phase was stored at −20 °C until further analysis. After oral inoculation with *E. coli* O157:H7, fecal samples were collected every other day for 3 weeks. At the time of necropsy, samples of ileum, colon and recto-anal junction were removed and processed for physiological and histological studies.

2.5. Quantification of anti-EspB and anti-γ-Intimin C_{280} antibodies

Serum and colostrum samples were analyzed for the presence of IgG antibodies against EspB and γ-Intimin C_{280} by an enzyme-linked immunosorbent assay (ELISA), as described previously [26].

2.6. Quantification of antibodies in tissues

Tissue recto-anal juncture (RAJ), ileum and cecum samples (50–75 mg) were placed in microcentrifugue tubes containing 1 ml of cold sterile PBS and glass beads (acid-washed-Sigma). Then, they were processed in Fast Prep_24 for 10 s at 5.5 m/s. The supernatant was centrifuged for 2 min at 10,000 rpm and stored at −20 °C. For the antibody quantification by ELISA, the supernatant was standardized to a final concentration of 0.5 mg of protein/ml in PBS with 0.5 M NaCl and 0.5% Tween 80.

2.7. Detection of the inoculated strain in tissue samples

Approximately 5 cm^2 of tissue were placed in 5 ml of PBS and vortexed vigorously for 1 min; 500 μl of the previous wash were inoculated into 4.5 ml of LB with 15 μg/ml of nalidixic acid 20 and incubated overnight at 37 °C. Immunomagnetic separation and multiplex PCR were performed as described below.

2.8. E. coli O157:H7 shedding

The magnitude and duration of fecal excretion of viable *E. coli* O157:H7 were followed daily for the first three days post challenge and every other day until day 18th and then day 21st after challenge. Two methods were used to detect *E. coli* O157:H7 fecal shedding: (1) bacterial counts were determined by plating serial dilutions of feces in duplicate onto Sorbitol MacConkey agar (Oxoid, Basingstoke, UK) plates containing 20 μg/ml nalidixic

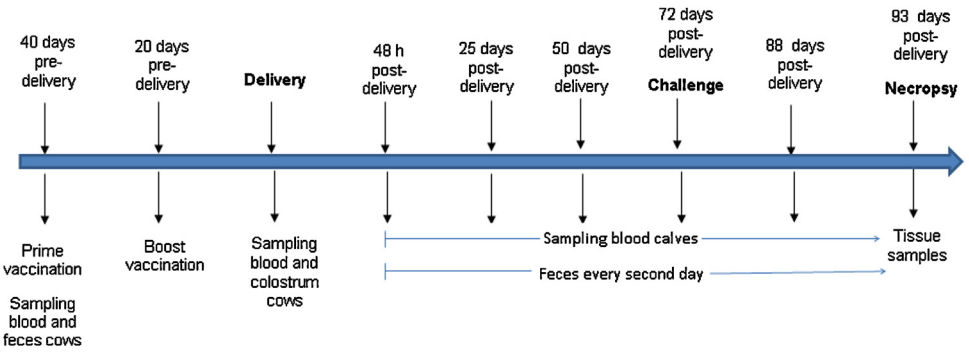

Fig. 1. Vaccination, challenge and sampling schedule. The figure shows the sampling schedule after vaccination of cows and after challenge of calves. Schedule is shown above the time–course thick arrows whereas sampling of blood, excreta or tissues is shown below.

acid (Sigma, St. Louis, USA) and 2.5 μg/ml potassium tellurite (TN-SMAC); (2) fecal shedding of the microorganism was also monitored by enrichment at 37 °C for 18 h of recto-anal junction mucosal swabs in Trypticase soy broth (Oxoid, Basingstoke, UK) containing 20 μg/ml nalidixic acid. Approximately 1 mL of this culture was subjected to *E. coli* O157 immunomagnetic separation performed according to the manufacturer's instructions (Dynabeads anti-*E. coli* O157, Invitrogen Dynal AS, Oslo, Norway) and the bacteria/bead complex was then spread onto TN-SMAC. Non-sorbitol-fermenting colonies were tested for *E. coli* O157 LPS by latex agglutination (Oxoid, Basingstoke, UK). The selected latex-positive colonies were confirmed by a multiplex PCR for the stx1, stx2, eae and rfbO157 genes using the primers described elsewhere [26]. Briefly, PCR assays were carried out in a 25 μl volume containing 2.5 μl of nucleic acid template, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$; 0.6 μM concentrations of each primer, 0.2 mM concentrations of each deoxynucleoside triphosphate, and 2 U of Taq DNA polymerase (Invitrogen Corporation, Carlsbad, USA). Temperature conditions consisted of an initial 94 °C denaturation step for 2 min followed by 30 cycles of 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min. Amplified DNA fragments were resolved by gel electrophoresis using 1% (wt/vol) agarose. Gels were stained with ethidium bromide and visualized with UV illumination.

2.9. Physiological and histological studies

For the physiological studies, Ileum, colon and recto-anal junction fragments were removed immediately after sacrifice of the examined calves and transported to the laboratory in oxygenated ice-cold physiological solution (NaCl, 9 g/L) to preserve the transport functions [38]. The mucosa and submucosa layers were then dissected from the underlying tissue (kept at 4 °C) and mounted as a diaphragm in a modified Ussing chamber (1.76 cm^2). Both sides of the tissues were washed and bathed with a standard Ringer solution (in mM): 113 NaCl, 4.5 KCl, 25 $NaHCO_3$, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 1.2 K_2HPO_4, 0.2 KH_2PO_4, 25 glucose, and bubbled with 95% O_2–5% CO_2. The bathing solution was maintained at 37 °C with water-jacketed reservoirs connected to a constant temperature circulation pump. The water absorption across intestinal mucosa was recorded automatically for 60 min connecting the Ussing chamber to a special electro-optical device [39]. For the histological studies, intestinal mucosa was recovered after physiological measurements and fixed for at least 24 h in cold fixative (4 °C) containing 4% formaldehyde in PBS. After fixation, longitudinal 2–4 μm thick sections were cut, dehydrated, and carefully embedded in paraffin to provide sections perpendicular to the mucosa. Sections were then stained using hematoxylin–eosin (H&E). The slides were then examined blindly by light microscopy.

2.10. Statistical analysis

Statistical analyses were performed using Graph Pad Prism Software (San Diego, CA, USA). Serum ELISA data within each group of cows, between control- and hyperimmune colostrum-fed calves and between colostrum from the immunized and the control group of cows were compared using the Student's *t*-test. Physiological results are reported as means ± SEM and the significance of any differences was determined using the Mann–Whitney test. For fecal bacterial counts, specimens containing fewer bacteria than the detection limit (*E. coli* O157:H7 found only by enrichment) were assigned a value of 10. Negative specimens by both methods were assigned a value of 1. Differences in the total number of bacteria isolated from fecal samples between groups were assessed using the Mann–Whitney test. In all cases, *P* values of < 0.05 were considered significant.

3. Results

3.1. Specific IgG activities in sera and colostra of immunized lactating cows

High titers of IgG antibodies against γ-Intimin C_{280} ($p < 0.0001$) and EspB ($p < 0.01$) were observed in the sera of vaccinated cows at delivery (Fig. 2A). The vaccination of dams also resulted in induction of high titers of IgG antibodies against EspB ($p < 0.01$) and γ-Intimin C_{280} ($p < 0.0001$) in colostrum, compared to those in the control group (Fig. 2B).

3.2. Specific IgG serum from neonatal calves fed with hyperimmune colostrum and challenge with E. coli O157:H7

At 48 h, the sera of calves fed with colostrum from their immunized dams exhibited higher titers of IgG antibodies against γ-Intimin C_{280} ($p < 0.0001$) and EspB ($p < 0.01$) than those of calves fed with colostrum from the control cows (Fig. 3). A significant difference in IgG titers anti-γ-Intimin C_{280} ($p < 0.0001$) and anti-EspB ($p < 0.01$) was also measured at the time of challenge (72 ± 5 days). However the titers decreased from 10.14 $\log_2$ (1/1128) to 7.14 $\log_2$ (1/142) and from 9.08 $\log_2$ (1/544) to 7.14 $\log_2$ (1/142), for C_{280} and anti-EspB, respectively (Fig. 3). For the challenge, 6 calves from both groups were experimentally inoculated by the oral route with 10^9 CFU of *E. coli* O157:H7 strain 438/99 NalR. The time of challenge (72 days) was selected because about 70 days is when bovines convert from lactating to ruminant and in Argentina breeding conditions they start to graze. Throughout the trial, anti-γ-Intimin C_{280} and anti-EspB IgG antibody titers from calves of the control cows significantly increased, as expected, due to the inoculation. Conversely, such titers decreased in calves from the

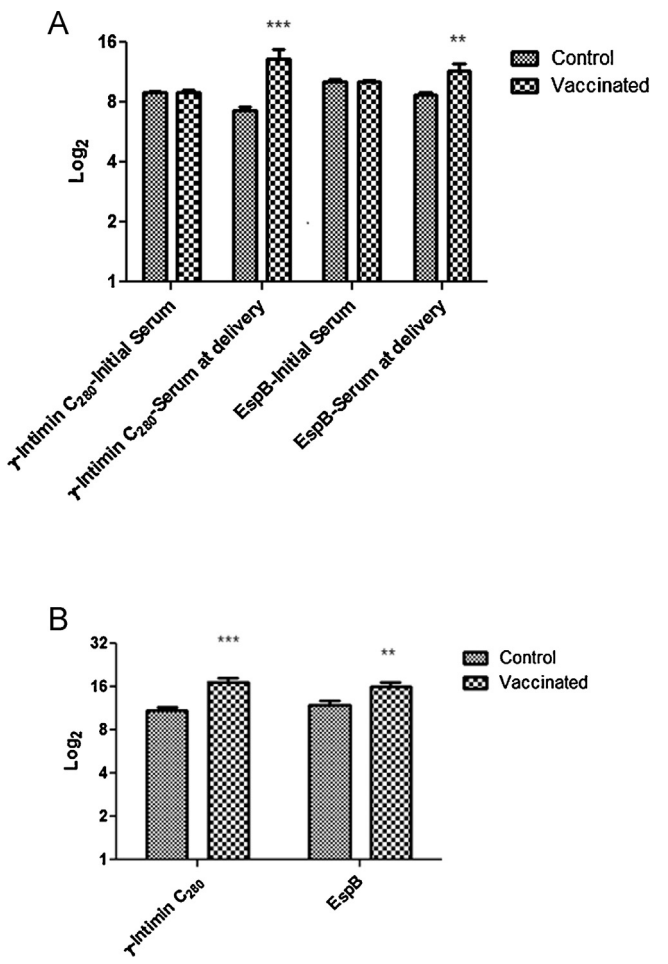

Fig. 2. (A) IgG responses in sera from vaccinated cows (dotted bars) with γ-Intimin C_{280} and EspB measured by ELISA. Control cows are shown in gray bars. Data are shown as mean $OD_{405} \pm SEM$. A significant increase in serum IgG against the γ-Intimin C_{280} (***$p < 0.0001$) and EspB was observed at delivery (**$p < 0.01$) compared to the initial (pre-vaccination) specific IgG. (B) IgG responses in calostra from vaccinated cows (dotted bars) with γ-Intimin C_{280} and EspB measured by ELISA. Control cows are shown in gray bars. Data are shown as mean OD405 ± SEM. The sera of calves fed with colostrum from their immunized dams exhibited higher titers of IgG antibodies against γ-Intimin C_{280} (***$p < 0.0001$) and EspB (**$p < 0.01$) than those of calves fed with colostrum from the control cows.

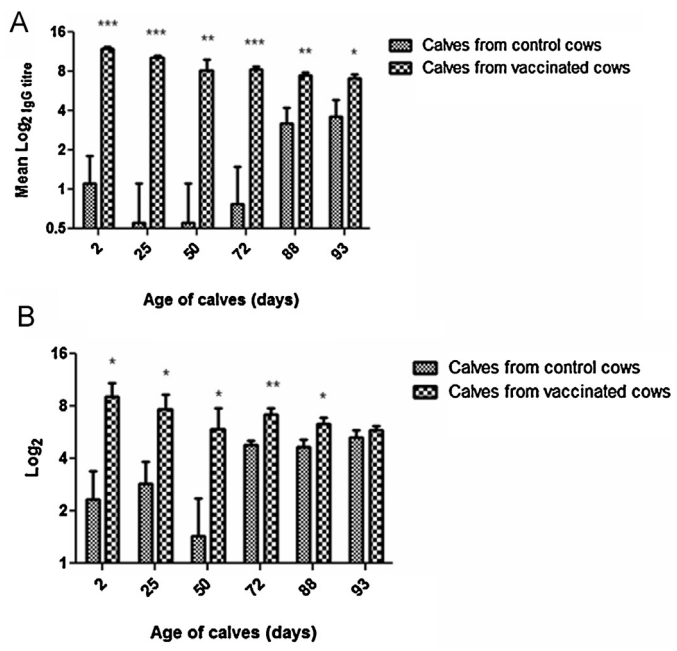

Fig. 3. Progression of IgG responses in sera from calves. Sera from calves were sampled at 2, 25, 50, 72, 88 and 93 days. IgG responses to γ-Intimin C_{280} (A) and EspB (B) in sera from calves fed with hyperimmune colostra (from vaccinated cows) (dotted bars) and in those fed with control colostra (gray bars) were measured by ELISA. Statistical differences are shown between sera from calves fed with hyperimmune colostra and those fed with control calostra at every time.

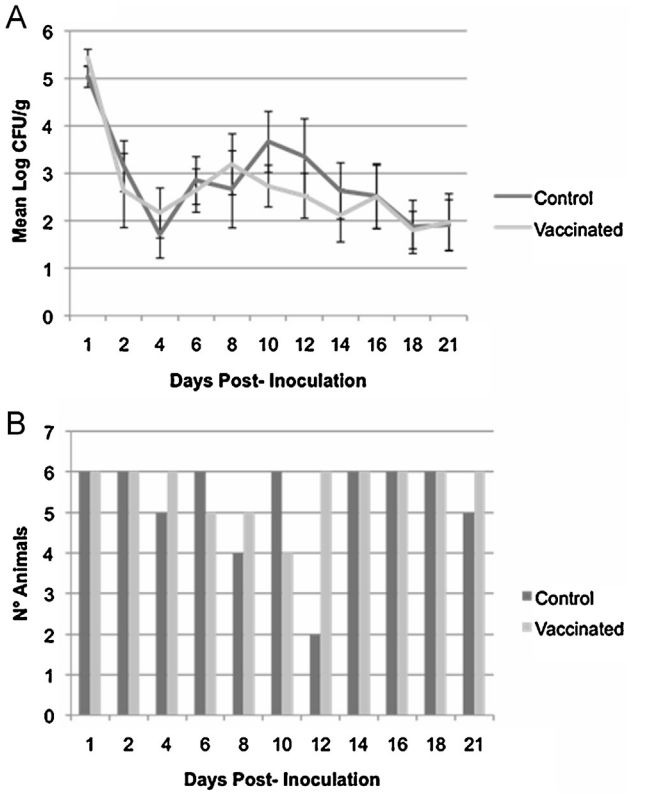

Fig. 4. Fecal shedding of *E. coli* O157:H7 of calves fed with hyperimmune colostra and controls. Fecal excretion of *E. coli* O157:H7 (log10 CFU/g ± SEM) at different times after inoculation were measured by counting colonies on Sorbitol MacConkey agar (A). Fecal shedding of the microorganism was also monitored by enrichment of recto-anal junction mucosal swabs in Trypticase soy broth followed by immunomagnetic separation and the bacteria/bead complex was then spread onto TN-SMAC. Non-sorbitol-fermenting colonies were tested for *E. coli* O157 LPS by latex agglutination and confirmed by a multiplex PCR for the stx1, stx2, eae and rfbO157. Results are expressed as the number of animal positive (B).

vaccinated cows (Fig. 3). However, in the case of the anti-γ-Intimin C_{280} titers, these titers remained higher in calves fed with hyperimmune colostra (from vaccinated cows) than in those fed with control colostra (from cows vaccinated with the adjuvant mix with PBS).

3.3. Fecal shedding

The excretion was followed for 21 days prior to euthanasia. Bacterial shedding in feces and recto-anal mucosal swabs were monitored through direct CFU counting (Fig. 4). Another monitoring was performed through enrichment followed by immunomagnetic separation and subsequent PCR and latex agglutination (Fig. 5). At sacrifice, 92% of the calves excreted *E. coli* O157:H7 and there was no significant decrease in total excretion or excretion rate of passively immunized calves. The excretion level in both groups reached a peak of 1×10^5 CFU/g feces on day 1, then declined and finally reached a new peak on days 8–10 (Fig. 4A). The animals of both groups continued excreting until the sacrifice (90 days) (Fig. 4B). The total number of bacteria isolated from fecal samples was not significantly different

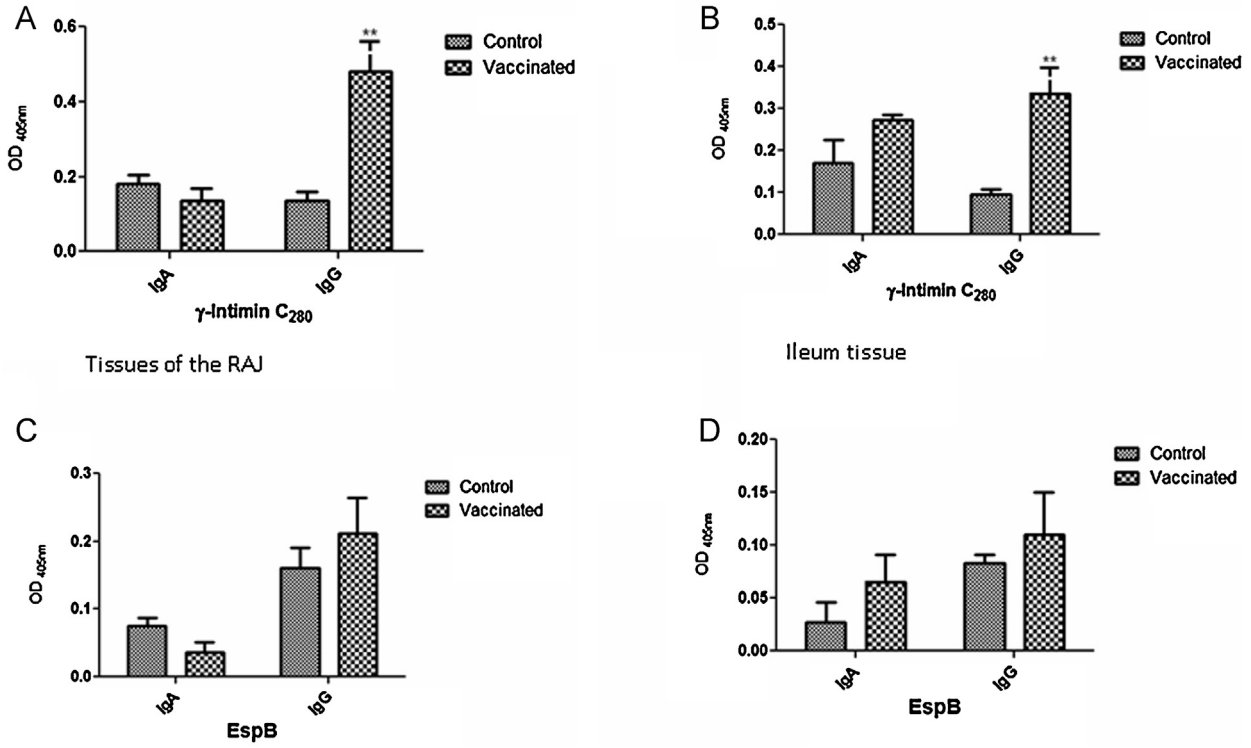

Fig. 5. Presence of specific IgG and IgA in intestinal tissues. IgA and IgG responses to γ-Intimin C_{280} and EspB in sera from calves fed with hyperimmune colostra (dotted bars) and in those fed with control colostra (gray bars) were measured by ELISA. (A) anti-γ-Intimin C_{280} antibodies extracted from RAJ; (B) anti-γ-Intimin C_{280} antibodies extracted from the Ileum; (C) anti-EspB antibodies extracted from RAJ; and (D) anti-EspB antibodies extracted from the ileum.

among the individuals of the group of hyperimmune colostrum-fed calves as compared to the colostrum-fed group (control group) ($p = 0.8$).

As expected, the titer of anti-EspB and anti-γ-Intimin C_{280} antibodies in the non-vaccinated group further increased after challenge. Anti-EspB, but not anti-γ-Intimin C_{280} titer, was raised earlier in time, probably due to an unnoticed exposure to an attaching and effacing *Escherichia coli* (AEEC).

3.4. Detection of mucosal antibody responses by ELISA

After calf necropsy, IgG and IgA antibodies against γ-Intimin C_{280} and EspB were analyzed in RAJ and ileum. In both intestinal tissues, an increase in IgG titers against γ-Intimin C_{280} was observed in calves fed with hyperimmune colostrum compared to colostrum-fed calves (Fig. 5). By contrast, no difference was observed in the response against EspB. Similar IgA titers against γ-Intimin C_{280} and EspB were also detected in both groups.

3.5. Detection of O157:H7 in tissues

The presence of *E. coli* O157:H7 was analyzed in RAJ, ileum and cecum tissues. The bacteria were found by culture or PCR or through both techniques in 2 colostrum-fed animals and 6 out of 6 in hyperimmune colostrum-fed animals. Individual tissue analyses showed colonization of control animals by the challenge strain in the cecum and RAJ of one animal and in the RAJ only of another animal. The challenge strain was observed the RAJ and ileum of three of the animals feed with colostrum from vaccinated cows. In consequence, the RAJ was the anatomical site of the intestinal segments where *E. coli* O157:H7 was most frequently found.

3.6. Clinical symptoms, physiological and histological findings

Some calves, regardless of having been fed with colostrum from the control or from a vaccinated cow, had a mild or moderate non-bloody diarrhea (Table 1). However, water absorption (in $\mu l/min\,cm^2$) measured across the intestinal mucosa mounted in the Ussing chamber was similar in all calves regardless of the type of feeding: hyperimmune or control colostra (ileum: 0.35 ± 0.02 vs 0.31 ± 0.04; colon: 0.20 ± 0.04 vs 0.24 ± 0.06, RAJ: 0.30 ± 0.02 vs 0.45 ± 0.13, respectively). Light microscopy showed loss of the superficial epithelium, interstitial edema,

Table 1
Score of diarrhea in fecal samples collected after inoculation.

DPC	No. of animals	Diarrhea
1	826 (P)	++
	827 (P)	++
	832 (V)	++
	835 (V)	++
6	830 (P)	++
8	831 (V)	++
	832 (V)	++
	832 (V)	+
10	827 (P)	+
	829 (P)	+
	831 (V)	+
	832 (V)	+
	836 (V)	+
12	830 (P)	+
	831 (V)	++
	832 (V)	+++
	836 (V)	+

DPC, day post challenge; calves fed with control calostrum (P), or hyperimmune calostrum (V), respectively. +, lower diarrhea; ++, mild diarrhea; +++, severe diarrhea.

leukocytes infiltration and rupture of the gland structure in both groups of calves (Fig. 6S).

4. Discussion

In a previous study, we demonstrated that the vaccination of pregnant cows with three doses of recombinant EspB and γ-Intimin C_{280} and inactivated Stx2 proteins caused high titers of IgG against γ-Intimin C_{280}, EspB and Stx2 in colostrum that were efficiently transferred to calves when administered separated [39,40]. In agreement with this previous study, we demonstrated that IgG anti-EspB and anti-γ-Intimin C_{280} from immunized cows were efficiently transferred to the calves from colostrum when mothers were vaccinated with a mix of both antigens. To the best of our knowledge, no previous research on the use of hyperimmune colostrum to prevent cattle from colonization of *E. coli* O157:H7 has been reported. Nevertheless, Funatogawa et al. [41] administered IgG-enriched (non-hyperimmune) colostrum preparation to streptomycin-treated mice and observed a decrease of *E. coli* O157:H7 shedding and attachment to gut tissues. Dean-Nystrom et al. [42] used a model of piglets suckling colostrum from dams vaccinated with Intimin and demonstrated similar results upon EHEC inoculation (although with a Stx negative strain).

In spite of the effective colostrum transferred to calves, the animals of this study were not protected from a challenge with *E. coli* O157:H7. This was evidenced by the excretion rate and the presence of *E. coli* O157:H7 in intestinal segments in both groups. At the time of challenge (67–77 days old), the hyperimmune calostrum-fed calves showed significant differences in IgG-antibody titers anti-EspB and anti-γ-Intimin C_{280} in comparison with calves from the control group. However, calves from both groups suffered a fall of 3.8 and 7.9 times, respectively, compared with the titers obtained in 2-day-old calves. Furthermore, this study showed that calves fed with either colostrum presented intestinal alterations compatible with diarrhea. Yet a higher content of IgG against γ-Intimin C_{280} was observed in RAJ and ileum of calves fed with hyperimmune colostrum.

The lack of protection may be related to a low titer of specific IgG antibody to γ-Intimin C_{280} and EspB in sera. In a previous study with vaccinated calves [26], the detected protection, also with specific IgG antibodies to γ-Intimin C $_{280}$ and EspB, was 250 fold higher than that observed in the present research. Similarly, McNeilly et al. demonstrated protective results with high anti Intimin titers [29]. Besides, Dean-Nystrom et al. [42] demonstrated that young calves (3–5 months of age) have other attachment sites apart from the recto-anal junction, notably the illeo-cecal valve. This suggests that a stronger immune response is needed in young bovines to prevent EHEC colonization.

In conclusion, bovine colostrum with elevated levels of antibodies against EHEC O157:H7 was obtained by systemic immunization of cows and specific antibodies were efficiently transferred to newborn calves by feeding with this hyperimmune colostrum. However, these antibodies passively acquired by the calves were insufficient to protect them from EHEC O157 experimental infection. Further research to found the best protocol to efficiently transfer antibodies to colostrum that may protect calves from EHEC O157 infection is already in progress.

Immune colostrum and milk may be an alternative to protect calves from early colonization by EHEC O157:H7 and may also be a key source of antibodies which could block colonization and toxic activity of that bacterium in the human intestine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.04.073.

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