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Research paper

A systemic vaccine based on *Escherichia coli* O157:H7 bacterial ghosts (BGs) reduces the excretion of *E. coli* O157:H7 in calves

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ABSTRACT

Cattle are the main reservoir of enterohemorrhagic *Escherichia coli* O157:H7, a bacterium that, in humans, causes hemorrhagic colitis and hemolytic uremic syndrome (HUS), a life-threatening disease, especially in children and older people. Therefore, the development of vaccines preventing colonization of cattle by *E. coli* O157:H7 could be a main tool for an HUS control program. In the present study, we evaluated bacterial ghosts (BGs) of *E. coli* O157:H7 as an experimental vaccine against this pathogen. BGs are empty envelopes of Gram-negative bacteria, which retain the morphological surface make-up of their living counterparts and are produced by controlled expression of the cloned protein E, which causes loss of all the cytoplasm content. In this work, *E. coli* O157:H7 BGs were used for subcutaneous immunization of calves. The vaccinated animals elicited significant levels of BG-specific IgG but not IgA antibodies in serum. Low levels of IgA and IgG antibodies against BGs were detected in saliva from vaccinated animals. Following oral challenge with *E. coli* O157:H7, a significant reduction in both the duration and total bacterial shedding was observed in vaccinated calves compared to the nonimmunized group. We demonstrated that systemic vaccination with *E. coli* O157 BGs provides protection in a bovine experimental model. Further research is needed to reach a higher mucosal immune response leading to an optimal vaccine.

1. Introduction

Shiga toxin-producing *Escherichia coli* O157:H7 is a major etiologic agent of disease in humans, and its clinical spectrum includes diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS), a life-threatening disease, especially in children and older people, which is the leading cause of chronic renal failure in children in Argentina and several other countries (Karch et al., 2005; Repetto, 2005). This bacterium produces Shiga toxin (Stx) types 1 and 2 (Jackson et al., 1987; Karpman et al., 1997; Tesh and O'Brien, 1991), which are responsible for systemic damage. The current treatment for this disease is largely limited to supportive care, as no specific regimen against an *E. coli* O157:H7 infection exists. In addition, the use of antibiotics is not recommended because following the antibiotic treatment the bacterium releases Stx, which can worsen the clinical course (Tarr et al., 1998).

The main reservoir for *E. coli* O157:H7 is cattle, which harbor this organism in their intestinal tract (Hussein and

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Bollinger, 2005; Meichtri et al., 2004), especially in the lymphoid follicle-dense mucosa at the terminal rectum (Naylor et al., 2003). The bacteria are usually isolated from healthy animals, although an initial episode of diarrhea can occur in younger animals. Fecal contaminated meats, horticulture products and water supplies are some of the main ways in which this organism can enter the human food chain (Hussein and Bollinger, 2005; Olsen et al., 2002). Vaccination would be a pre-slaughter intervention measure to reduce the carriage of *E. coli* O157:H7 in cattle and thus prevent the risk of human infection.

Many virulence factors of *E. coli* O157:H7 induce an immune response during the course of natural or experimental infections in cattle. Oral inoculation of calves and steers with *E. coli* O157:H7 promotes an increase in serum antibody titers against O157 lipopolysaccharide (LPS) and in neutralizing antibodies to Shiga toxins (Johnson et al., 1996). However, in a similar assay, were only found anti-LPS antibodies (Hoffman et al., 2006). On the other hand, animals develop an immune response against proteins encoded by a large chromosomal locus called LEE (locus of enterocyte effacement) (Elliott et al., 1998). LEE codes for a type III secretion system (McDaniel et al., 1995), an adhesin called intimin, which is required for intimate attachment to host cells, and the secreted proteins EspA, EspD and EspB, which are required for signal transduction events leading to the formation of attachment and effacing lesions (A/E) (Kaper et al., 2004; Kenny et al., 1997). Recently Bretschneider et al. (2007) demonstrated that cattle respond serologically to intimin and EspB during the course of an experimental infection with *E. coli* O157:H7.

Various vaccine formulations against *E. coli* O157:H7 infections have been assayed in cattle (Babiuk et al., 2008; Dziva et al., 2007; McNeilly et al., 2008, 2010; Potter et al., 2004; Smith et al., 2008; van Diemen et al., 2007; Vilte et al., 2011) and other animal models (Agin et al., 2005; Cataldi et al., 2008; Dean-Nystrom et al., 2002; Judge et al., 2004; Marcato et al., 2005; Wen et al., 2006) with variable results. Vaccination with bacterial colonization factors has been proposed as a strategy to prevent *E. coli* O157:H7 infection. One new technical approach is the use of BGs as inactivated, whole-cell envelope vaccines. BGs are produced by the controlled expression of the PhiX174 lysis gene *E*. E-mediated lysis of bacteria results in the formation of empty bacterial cell envelopes, which have the same cell surface composition as their living counterparts. They display all surface components in a non-denatured form and are able to induce a strong mucosal immune response (Jalava et al., 2002, 2003). Even highly sensitive and fragile structures like pili are well preserved by this technology, as has been demonstrated for *Vibrio cholerae* BGs expressing the toxin-coregulated pilus (Eko et al., 2000). Furthermore, *Mannheimia* (*Pasteurella*) *haemolytica* BGs, which induce immunity in cattle, exhibit a broader spectrum of protection in rabbits than chemically inactivated *M. haemolytica* (Marchart et al., 2003a, 2003b). Oral immunization of mice with *E. coli* O157:H7 BGs leads to 86.6% protection against a lethal heterologous *E. coli* O157:H7 challenge after one booster immunization (Mayr et al., 2005). In the present work, we evaluated *E. coli* O157:H7 BGs as an experimental systemic vaccine to protect calves from *E. coli* O157:H7 colonization.

2. Materials and methods

2.1. Animals

All animal experiments were performed with the approval of the Instituto Nacional de Tecnología Agropecuaria (INTA) Animal Welfare Committee. Six- to eight-month-old conventionally reared male Holstein-Friesian calves were obtained at a farm from Buenos Aires province, Argentina, and housed at the INTA Experimental Station. Prior to the first immunization and challenge, calves were confirmed twice to be negative for *E. coli* O157:H7 by enrichment of fecal samples followed by immunomagnetic separation as described below. Three days before oral challenge, the calves were allocated in biosafety level 2 facilities and randomly assigned to one of two separated pens according to the immunization group. Calves were fed alfalfa pellets, with free access to hay and water.

2.2. Production of *E. coli* O157:H7 bacterial ghosts

E. coli O157:H7 bacterial ghosts were obtained as previously described (Mayr et al., 2005). EHEC CIP 105282 BGs were produced in a 10-l fermentor (Meredos, Bovenden, Germany) with a stirring rate of 350 rpm and 3.5 l of air per min. No antifoam was added, and pH values were stable in the range between 6.5 and 7.5, necessary for successful E-mediated lysis (Lubitz et al., 1984). To induce nuclease expression, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the *E. coli* cultures at an OD_{600} of 0.3. Protein E-mediated lysis was induced 45 min later by a temperature shift from 28 °C to 42 °C. $MgCl_2$ and $CaCl_2$ were added 90 min after induction of lysis. The number of colony forming units (CFU) within the bacterial samples taken during lysis and nuclease treatment was determined using the spiral plater. Samples were serially diluted in 0.85% NaCl, inoculated onto LB agar plates, and incubated at 28 °C overnight. Preparation of the total DNA of the BG samples and electrophoretic analysis were carried out as recently described (Haidinger et al., 2003). The BGs were collected by centrifugation and washed three times with 0.85% NaCl solution (with 1/3, 1/6, and finally 1/12 of the starting culture volume). The final BG pellet was resuspended in 20 ml distilled water and freeze-dried for 24 h. Ten milligrams of the lyophilized BG preparations was inoculated in LB, incubated for 1 week at 28 °C, and analyzed for living-cell counts by plating on LB agar plates.

2.3. Immunization protocol and oral bacterial challenge

Five calves received two doses of vaccine composed of 10 mg of *E. coli* O157:H7 BGs in 5 ml of PBS by the subcutaneous route with an interval of 21 days, whereas other five calves were vaccinated with PBS only (control group). Fourteen days after the second immunization, calves were orally challenged with ca.1×10^{9} CFU of *E. coli* O157:H7

strain 438/99 (stx_2, *eae*-γ) in 15 ml of PBS. The challenge strain was isolated from a healthy cow and selected for spontaneous resistance to nalidixic acid.

The magnitude and duration of fecal excretion of viable *E. coli* O157:H7 were followed daily for the first 3 days post-challenge and every second day until 15 days post-challenge. Two methods were used to increase the probability to detect *E. coli* O157:H7 fecal shedding: (1) bacterial counts were performed by plating serial dilutions of feces taken from the rectum in duplicate onto Sorbitol MacConkey agar (Oxoid, Basingstoke, UK) plates containing 20 μg/ml nalidixic acid (Sigma, St. Louis, USA) and 2.5 μg/ml potassium tellurite (Britania, Argentina) (TN-SMAC); (2) fecal shedding of the microorganism was also monitored by enrichment of swabs obtained from the rectoanal junction, a site of preferencial *E. coli* O157:H7 colonization, in Trypticase soy broth (Oxoid, Basingstoke, UK) containing 20 μg/ml nalidixic acid. After incubation at 37 °C for 18 h, 1 ml of this culture was subjected to *E. coli* O157 immunomagnetic separation, according to the manufacturer's instructions (Dynabeads anti-*E. coli* O157, Invitrogen Dynal AS, Oslo, Norway) and the bead-bacteria mixture was 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 stx_1, stx_2, *eae* and rfb_{O157} genes using the primers described elsewhere (Blanco et al., 2004; Olsvik and Strockbine, 1993; Paton and Paton, 1998). 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% (w/v) agarose. Gels were stained with ethidium bromide and visualized with UV illumination.

In order to monitor antibody responses, serum, saliva and fecal samples were collected before each vaccination, before the oral bacterial challenge, and 2 weeks post-challenge. The calves were euthanized 2 weeks after the bacterial challenge, and samples from the ileum, cecum, colon, and rectoanal junction were cultured to detect *E. coli* O157:H7 after immunomagnetic separation, as described above. Similar intestinal segments were collected for histopathological examination.

2.4. Antibody response

Immediately after their collection, serum samples were stored at −20 °C. Fecal samples were suspended 1:2 (w/v) in sodium acetate buffer (pH 4.5, 10 mM) containing 0.1% (w/v) Protease Inhibitor Cocktail (Sigma–Aldrich Co., Saint Louis, USA). The mixture was centrifuged once at 2500 × *g* for 20 min to sediment larger particles and once at 15,000 × *g* for 30 min; the supernatant was collected and stored at −20 °C until its analysis (Bretschneider et al., 2007). Saliva samples were stored at −20 °C until their analysis, when they were centrifuged at 1000 × *g* for 10 min to remove cells.

Serum samples were analyzed for the presence of IgG and IgA antibodies against *E. coli* O157:H7 BGs, and for IgG antibodies to O157 lipopolysaccharide (O157 LPS) by an enzyme-linked immunosorbent assay (ELISA) described previously (Mayr et al., 2005). Briefly, 96-well Nunc-Immuno MaxiSorp assay plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 100 μl of either *E. coli* O157:H7 CIP 105282 ghosts, or O157 LPS (List Biological Laboratories, Inc., Campbell, USA) at 10 μg/ml in 0.05 M carbonate–bicarbonate buffer pH 9.6. Non-specific binding sites were blocked with 3% skim milk in PBS for 1 h at room temperature. Then, plates were incubated for 2 h at room temperature with serial two-fold dilutions of serum. For each plate, two wells were incubated with PBS-T alone (negative control), and a known positive sample was included. Each sample was analyzed in duplicate. Plates were incubated for another hour with 100 μl of sheep anti-bovine IgG or IgA conjugated with horseradish peroxidase (Bethyl Laboratories, Montgomery, USA), at dilutions of 1:8000 for IgG and 1:3000 for IgA, in PBS-T. Peroxidase reactivity was revealed by ABTS [2,2′-azino-di (3-ethyl-benzthiazoline sulfonic acid)] (Amresco, Solon, USA) substrate. Reactions were stopped after 10 min with 5% SDS and read at an OD_{405} in a microplate reader (BioTek Instruments, Winooski, USA). The antibody titer was expressed as the reciprocal of the end-point dilution resulting in an OD_{405} above the cut-off value. The cut-off value was determined by calculating the average plus two times the standard deviation of the optical densities of the samples measured on day 0. For comparison purposes, the serum IgG responses against O157 LPS and O157 BGs were expressed as OD_{405}.

The amounts of *E. coli* O157:H7 BG-specific IgA and IgG present in saliva and feces were determined by a capture ELISA (Cataldi et al., 2008) using sheep anti-bovine IgA or IgG (Bethyl Laboratories) as capture antibody. The plates were further incubated with serial dilutions of either samples or bovine reference serum (Bethyl Laboratories). As secondary antibody, HRP-conjugated sheep anti-bovine IgA or IgG was used. Plates were developed as described above. To compensate for variations in the efficiency of recovery of IgA secretory antibodies between animals, the results were normalized and expressed as end-point titer of antigen-specific IgA with respect to 1 μg of total IgA present in the sample.

In addition, pools of saliva samples of both vaccinated and control groups were examined for anti-*E. coli* O157 BGs IgA and IgG antibodies by Western blot. The *E. coli* O157:H7 BGs were separated by SDS-PAGE under reducing conditions (Laemmli, 1970) loading 2.5 μg BGs per lane, transferred to a nitrocellulose membrane (Amersham Pharmacia, Germany) (Towbin et al., 1979) and blocked with 5% nonfat dry milk. Then these were incubated with 1:2 dilution of saliva pool preparations from immunized and control groups, respectively. Finally, the membranes were incubated with HRP-conjugated rabbit anti-bovine IgG (Bio-yeda, Rehovot, Israel). The blots

were revealed with 4-Cl-1-naphthol (Pierce, Rockford, USA).

2.5. Histological studies

Tissues were fixed in neutral buffered 10% formalin for 24–48 h, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for routine histology.

2.6. Statistical analysis

Data on fecal shedding were log-transformed to attain normality. The resulting variable, log(CFU/g), was analyzed using a linear mixed model for repeated measures (Littell et al., 1998). The model included fixed effects of treatment (two levels: control and vaccinated), and linear, quadratic and cubic terms for the covariate day of measure interacting with treatments, so as to account for the daily trend within treatment (Fig. 5). Animal effects were considered independent random variables, and the covariance of repeated measures within animals was fitted using an autoregressive process of order one (AR(1)) by means of Proc Mixed of SAS. The level of log (CFU/g) for each treatment at any given day, as well as the difference between treatments, was obtained as an estimable linear contrast (Littell et al., 2002; Searle, 1971). A similar procedure was used to test for the difference between vaccinated and control animals for log (CFU/g) during the entire experiment. All tests of hypotheses had their degrees of freedom corrected by the procedure of Kenward and Roger (1997). ELISA data within each group were compared using an unpaired t test. p values of ≤ 0.05 were considered significant.

3. Results

3.1. Humoral immune response elicited by E. coli O157:H7 bacterial ghosts

Following the second immunization, calves elicited a significant increase in serum *E. coli* O157:H7 BG-specific IgG antibodies (Fig. 1) but not in IgA antibodies (data not shown). IgG antibody titers reached a peak level after booster immunization but did not change after oral challenge with *E. coli* O157:H7. No increase in serum anti-O157 LPS IgG was detected during the immunization period, while a slight increase resulted after the bacterial challenge in both immunized and control groups (Fig. 2).

The mucosal immune response was measured in saliva and fecal samples. Salivary IgA response against *E. coli* O157:H7 BGs showed a non-significant increase following the first immunization which decreased after the challenge in both groups of calves (Fig. 3). No salivary IgG antibodies were detected using ELISA, although a slight response was detected by Western blotting (Fig. 4). All the fecal samples of vaccinated and control calves were negative for anti-*E. coli* O157:H7 BGs IgA and IgG antibodies by ELISA and Western blotting (data not shown).

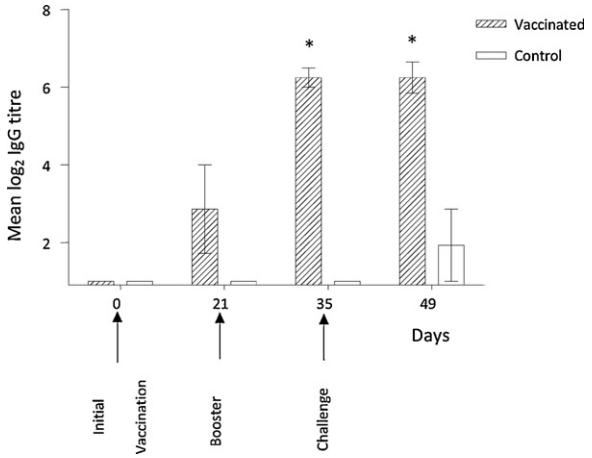

Fig. 1. *E. coli* O157:H7 BGs-specific IgG antibodies in serum, measured by ELISA, from calves immunized with two doses of 10 mg of *E. coli* O157:H7 BGs or placebo; with an interval of 21 days. Results are presented as mean $\log_2$ of IgG titers and SEM is indicated by vertical lines. Significant differences are indicated *($p < 0.001$).

3.2. Challenge and excretion of E. coli O157:H7

Two weeks after the second vaccination, calves were orally challenged with 10^9 CFU of *E. coli* O157:H7. Bacterial shedding post-challenge was calculated quantitatively by direct plating as well as by enrichment culture followed by immunomagnetic separation during the 15 days post-challenge. Specimens containing less than the detection limit (*E. coli* O157:H7 found only by enrichment) were assigned a value of 10. Specimens negative by both methods were assigned a value of 1. No calves experienced diarrhea and no blood was observed in any of the fecal samples collected after inoculation. Bacterial shedding was monitored in feces and rectoanal mucosal swabs by the methods described above. Fig. 5 displays the trends in the estimates of the level of log count (CFU/g) for control and vaccinated animals at any day. All the differences between treatments after day 9 were significantly different ($p < 0.05$). Moreover, the average estimate of the total

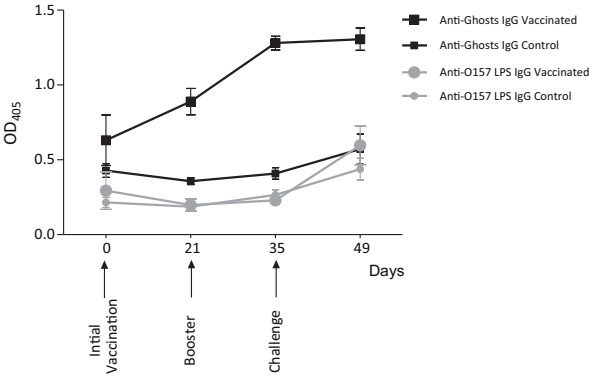

Fig. 2. Serum IgG antibody responses against O157 LPS and *E. coli* O157:H7 BGs, measured by ELISA, from calves immunized with two doses of 10 mg of *E. coli* O157:H7 BGs or placebo; with an interval of 21 days. Results are presented as mean OD_{405} obtained with a serum dilution of 1:400 and SEM is indicated by vertical lines.

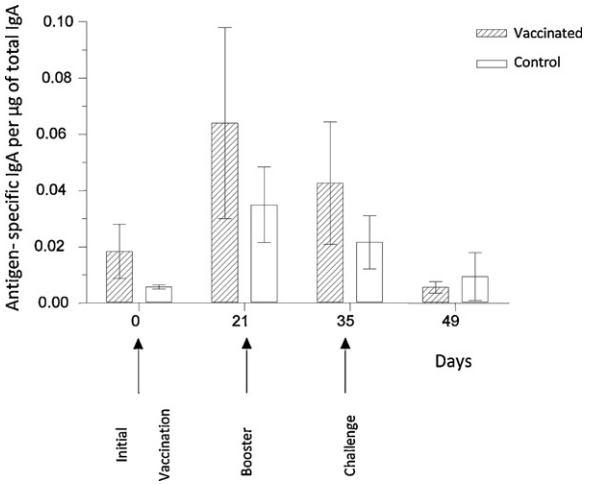

Fig. 3. *E. coli* O157:H7 BGs-specific IgA antibodies in saliva measured by ELISA, from calves immunized with two doses of 10 mg of *E. coli* O157:H7 BGs or placebo; with an interval of 21 days. Results are presented as end-point titer of antigen-specific IgA with respect to 1 µg of total IgA. SEM is indicated by vertical lines.

shedding, expressed as the area under the curve (AUC) of log (CFU/g), for the control during the 15 experimental days was 36.6, whereas for the vaccinated group was 28.7, which was significantly different ($p < 0.05$). In terms of estimated total amount of bacteria shed per group over the entire

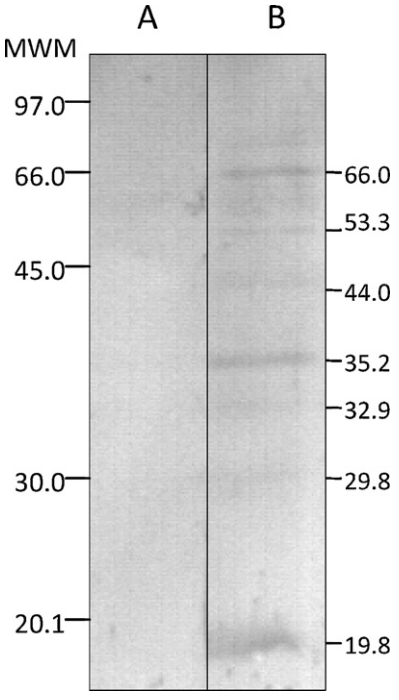

Fig. 4. Western blot of saliva IgG response against *E. coli* O157:H7 BGs. Dilution 1:2 of pool samples obtained from control (A) or immunized calves (B), 2 weeks after the last immunization were analyzed by loading 2.5 µg *E. coli* O157:H7 BGs per lane The pool sample from immunized cows showed reactivity against proteins of different sizes of *E. coli* O157:H7 BGs. MWM, molecular weight marker. All molecular weights are expressed as kDa.

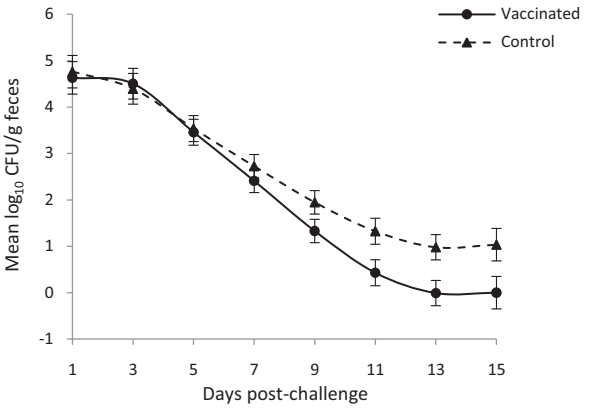

Fig. 5. Fecal shedding of *E. coli* O157:H7 until 15 days post-challenge of calves. Two weeks after the second vaccination, calves were orally challenged with 10^9 CFU of *E. coli* O157:H7. Data represents estimates of mean daily fecal excretion of *E. coli* O157:H7 per gram of feces ($\log_{10} \pm$ SEM). Significant differences were found on days 11 ($p = 0.0315$), 13 ($p = 0.0106$) and 15 ($p = 0.0427$). The estimate of the total shedding, the AUC, for the control group during the 15 experimental days was 36.6, whereas that for the vaccinated group was 28.7, being the difference significant ($p < 0.05$).

15-day period, the vaccinated group showed a reduction of 52.3%. At necropsy, *E. coli* O157:H7 was recovered from the rectoanal junction and ileum samples from one animal belonging to the control group. No histological changes consistent with attaching-and-effacing lesions or adherent bacterial layers were seen in intestinal sections of either group of animals. However, all animals showed scanty neutrophils in the *lamina propria* from different sections of intestine.

4. Discussion

Reduced colonization or shedding of *E. coli* O157:H7 in cattle by the administration of systemic vaccines has been previously observed by us and others (McNeilly et al., 2008, 2010; Potter et al., 2004; Thornton et al., 2009; Vilte et al., 2011). Some of these studies have found evidence of systemic and mucosal antibody responses following immunization with *E. coli* O157:H7 colonization antigens (McNeilly et al., 2008, 2010; Potter et al., 2004; Vilte et al., 2011). In this study, we demonstrated that subcutaneous vaccination of cattle with an *E. coli* O157 bacterial ghost formulation effectively reduced the length of *E. coli* O157:H7 excretion as also the total bacterial shedding in the vaccinated group compared to the control group. The lack of protection at short times post-challenge was likely due to a low overall quantity of anti-*E. coli* O157:H7 antibodies in the intestinal mucosa and led to an overall reduction 50% in the total amount of bacteria excreted by the vaccinated animals.

Our results showed that the *E. coli* O157:H7 inoculum dose used in the experimental infection (ca. 1×10^9 CFU) was sufficient to induce colonization, as evidenced by the duration of fecal shedding in the non-vaccinated calves, which continued at least until day 11 post-challenge, whereas that four of the animals were still shedding at day 15, when the study ended. In contrast, none of the vaccinated calves were excreting a detectable level of *E. coli*

O157:H7 at day 11 post-challenge. The time-course of the *E. coli* O157:H7 excretion initially showed a high level of shedding followed by a sharp decrease through the two-week sampling period. In previous works, weaned calves (Brown et al., 1997; Dean-Nystrom et al., 2008; Hoffman et al., 2006) and adult cattle (Cray and Moon, 1995; Snider et al., 2006), which were experimentally infected with Stx+ *E. coli* O157:H7 strains, showed a similar shedding pattern, while pre-weaning calves had a greater and more prolonged excretion (Cray and Moon, 1995; Dziva et al., 2007). These responses to an experimental challenge are consistent with farm surveys in which rates of isolation of *E. coli* O157:H7 from immature animals exceed those from adults (Wells et al., 1991).

In the present study, we have chosen to utilize conventional reared calves, which in spite of being negative for *E. coli* O157:H7 might have had prior exposure to the organism. The reason for this choice over the conventional gnotobiotic calves is that we are currently seeking to produce a vaccine that decreases the excretion of *E. coli* O157:H7 in naturally infected animals.

Vaccinated calves elicited significantly higher BG-specific IgG antibody titers in serum than control cattle, indicating that the BGs inoculated subcutaneously were able to generate an immune response in cattle without the addition of any adjuvant. However, the saliva anti-*E. coli* O157:H7 BGs IgG and IgA antibody response was poor, whereas copro-antibodies against *E. coli* O157:H7 BGs were undetectable, despite the processing with protease inhibitors and freezer storage of fecal samples to minimize proteolysis. Coincidentally, Babiuk et al. (2008) showed that subcutaneous immunization of mice with type III secreted proteins could prevent colonization and shedding of *E. coli* O157:H7 without any detectable antigen-specific IgA in feces. However, the lack of detection of copro-antibodies in our study was possibly due to insufficient antibody titers in fecal samples. A previous *E. coli* O157:H7 vaccine trial (McNeilly et al., 2008), which used rectal swabbing to measure bovine mucosal antibody response, showed that systemic immunization of cattle produced a similar pattern of IgG and IgA antibodies in different mucosal secretions. Thus, we hypothesize that the detection of anti-BG antibodies in saliva could be also correlated with the presence of certain amount of *E. coli* O157:H7 BGs specific antibodies in the intestinal mucosa.

No attaching and effacing lesions were observed in any of the intestinal samples. Dean-Nystrom et al. (2008) observed no A/E O157 bacteria in the distal colon, cecum, ileum, gall bladder or bile of weaned calves experimentally inoculated with STEC O157 that were shedding fewer than 10^5 CFU of inoculum-type bacteria per gram of feces 4 days after inoculation, but observed A/E O157 bacteria in the rectoanal junction or ileocecal valve of some inoculated calves that were shedding $<10^5$ CFU of STEC O157 bacteria per gram of feces. In our study, the inoculum bacteria were recovered only by the enrichment procedure on day 15 post-challenge, indicating low levels of *E. coli* O157:H7 populations in intestinal segments, probably insufficient to observe A/E lesions. The mild inflammatory response represented by scanty neutrophil infiltration of the *lamina propria* in both groups of animals should not be associated to *E. coli* infection, since there were no differences between animals that were positive or negative for *E. coli* O157:H7 at end of study.

A vaccine against *E. coli* O157:H7 should prevent colonization of the intestinal tract of cattle. In order to prevent the pathogen from adhering to the mucosa, a vaccine has to include important cell surface factors. BGs retain even fragile surface structures such as pili, as it has been demonstrated for *V. cholerae* BGs expressing the toxin-coregulated pilus (Eko et al., 2000).

In studies carried out in mice with the same *E. coli* O157:H7 BGs used in the present study, oral immunization with one booster led to 100% protection against the challenge, whereas a single rectal immunization was more effective since no further booster immunization was needed to reach full level of protection (Mayr et al., 2011). The study with *E. coli* O157:H7 BGs in mice indicates that, in order to reach a higher and better level of protection, it is important and more effective to first immunize animals at the site of infection. As the reservoir and area of *E. coli* O157:H7 colonization is the rectum, which is rich in underlying lymphoid tissue, this route of immunization should be used to immunize animals. In addition, oral/sublingual application of *E. coli* O157:H7 BGs could increase the level of sIgA, which might be beneficial to capture *E. coli* O157:H7 bacteria.

At present, the specificity of the BG antibodies is unknown as well as the protective antigen(s) in the BG composition The lack of antibodies against LPS observed in the present study could be dependent on the growth conditions used to enrich the BGs for this component. The latter aspect, as well as a different regime of immunization, such as the application of *E. coli* O157:H7 BGs in drinking water of early age calves followed by repeated boosters, should be considered in further immunization studies, since this may be a useful pre-harvest intervention strategy.

5. Conclusions

The development of vaccines preventing colonization of cattle by *E. coli* O157:H7 could be a main tool to lower the risk of contamination for humans. In the present study, we could reduce *E. coli* O157:H7 colonization and shedding in cattle by subcutaneous immunization with *E. coli* O157:H7 bacterial ghosts. Further studies are needed to increase the mucosal antibody response and therefore the level of protection of intestinal colonization by *E. coli* O157:H7.

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