Reduced faecal shedding of *Escherichia coli* O157:H7 in cattle following systemic vaccination with γ-intimin C$_{280}$ and EspB proteins

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A R T I C L E I N F O

A B S T R A C T

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 is the most prevalent EHEC serotype that has been recovered from patients with haemolytic uremic syndrome (HUS) worldwide. Vaccination of cattle, the main reservoir of EHEC O157:H7, could be a logical strategy to fight infection in humans. This study evaluated a vaccine based on the carboxyl-terminal fragment of 280 amino acids of γ-intimin (γ-intimin C$_{280}$) and EspB, two key colonization factors of *E. coli* O157:H7. Intramuscular immunization elicited significantly high levels of serum IgG antibodies against both proteins. Antigen-specific IgA and IgG were also induced in saliva, but only the IgA response was significant. Following experimental challenge with *E. coli* O157:H7, a significant reduction in bacterial shedding was observed in vaccinated calves, compared to control group. These promising results suggest that systemic immunization of cattle with intimin and EspB could be a feasible strategy to reduce EHEC O157:H7 faecal shedding in cattle.

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1. Introduction

Enterohaemorragic *Escherichia coli* (EHEC) O157:H7 is a major aetiological agent of diseases in humans, whose clinical spectrum includes diarrhoea, haemorrhagic colitis and haemolytic uremic syndrome (HUS), the leading cause of chronic renal failure in children in Argentina and several other countries [1,2]. This bacterium produces Shiga toxins types 1 and/or 2 [3–5], which are responsible for systemic damage, particularly the vascular endothelium of the kidney and brain, with severe renal and neurological sequelae in children and elder people. Current treatment is largely limited to supportive care, as no specific regimen against an *E. coli* O157:H7 infection exists and the use of antibiotics is not recommended because they can cause the release of Shiga toxins from the bacterium, which can worsen the clinical course [6].

The main reservoir for *E. coli* O157:H7 is cattle, which harbour the bacteria in their intestinal tract [7,8], especially in the lymphoid follicle-dense mucosa at the terminal rectum [9]. The bacteria are usually isolated from healthy animals, although episodes of diarrhoea have been observed in young animals [10,11]. Faecal contamination of meat during slaughter, the use of faeces as fertilizers, and the contamination of drinking water are major ways by which this organism can enter the human food chain [7,12].

Besides producing Shiga toxins, *E. coli* O157:H7 is characterized by other virulence-associated traits, which enable it to colonize the intestinal mucosa of humans and animals with a histopathological lesion known as "attaching and effacing" (A/E) [13]. A large chromosomal pathogenicity island called the locus of enterocyte effacement (LEE) is associated with A/E activity [14,15]. The LEE encodes for a type three secretion system (TTSS) that translocates into the host cell effector proteins responsible for the A/E lesion. The A/E lesion is also characteristic of enteropathogenic *E. coli* (EPEC), another category of *E. coli* strains associated with diarrhoea in children [13]. The TTSS forms EspA, a filamentous structure through which effector proteins are translocated into the host cell [16]. Intimin and its bacterially expressed receptor Tir are translocated by the TTSS in the host cell membrane, leading 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 [17].

Many virulence factors of *E. coli* O157:H7 induce an immune response during the course of natural or experimental infections in animals and patients with HUS. Oral inoculation of calves and steers with *E. coli* O157:H7 promotes an increase in serum antibody titres against the O157 lipopolysaccharide and neutralizing antibodies

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to Shiga toxins [18]. Recently, Bretschneider et al. [19] demonstrated that cattle respond serologically to intimin and EspB of *E. coli* O157:H7 during the course of experimental infection. Antibodies against these proteins have also been detected in serum during both human EHEC [20] and EPEC infections [21] and in colostrum and milk from healthy women [22–25] and cows [26]. In addition, mice infected with *Citrobacter rodentium*, a bacterium that shows virulence determinants and pathological effects in mice highly similar to those of EPEC in humans, develop an immune response against LEE-encoded proteins, which makes them resistant to bacterial re-infection [27].

Vaccination with bacterial colonization factors has been proposed as a strategy to prevent *E. coli* O157:H7 infection. Various vaccine formulations have been assayed, both in cattle [28–33] and in other animal models [34–40], with variable results. In the present study, we evaluated the efficacy of a systemic vaccine composed of γ-intimin C_{280} and EspB, to reduce *E. coli* O157:H7 colonization in cattle.

2. Materials and methods

2.1. Animals

All animal experiments were performed with the ethical approval of the Instituto Nacional de Tecnología Agropecuaria (INTA) Animal Welfare Committee. Eight 6–8-month-old conventionally reared male Holstein-Friesian calves were obtained from a farm in 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 faecal samples followed by immunomagnetic separation as described below. Three days before oral challenge, the calves were allocated in biosafety level 2 rooms and housed in separated pens according to the immunization group. Calves were fed alfalfa pellets, with free access to hay and water.

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

Preparation of His-tagged γ-intimin C_{280} and EspB was performed as described previously [26]. Briefly, constructs for the expression of the gene fragment (843 bp) encoding the 280 carboxyl-terminal amino acids of γ-intimin (γ-intimin C_{280}) and the EspB gene were obtained by PCR amplification from the *E. coli* strain 146N (O157:H7). The amplified DNA fragments were cloned into the His-tag expression vector pRSET-A (Invitrogen Corporation, Carlsbad, USA). The resulting constructs were transformed into chemically competent *E. coli* BL21 (D3)/pLysS as described by the manufacturer. Protein expression was then induced by the addition of 1 mM IPTG. The amino-terminal His-tagged proteins were purified from the lysates by affinity chromatography on nickel-agarose columns (ProBond Nickel-Chelating Resin, Invitrogen Corporation), eluted under denaturing conditions and dialyzed in PBS pH 7.4.

2.3. Immunization protocol and oral bacterial challenge

Four calves received two doses of a vaccine composed of the recombinant proteins EspB (100 μg) and γ-intimin C_{280} (100 μg) diluted in 1 ml of PBS and mixed with 1 ml of mineral oil-based adjuvant (Montanide ISA206; Seppic, France) and calcitriol [1α,25(OH)2D3] (2 μg) by the intramuscular route with an interval of 21 days. A control group of four calves that were vaccinated with PBS plus the adjuvant and calcitriol were included. Fourteen days after the second immunization, calves were orally challenged with ca. 1×10^9 colony forming units (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 faecal excretion of viable *E. coli* O157:H7 were followed every other day until 19 days post-challenge. Two methods were used to increase the probability to detect *E. coli* O157:H7 faecal shedding: (1) bacterial counts were performed by plating serial dilutions of faeces in duplicate onto cefixime-tellurite Sorbitol MacConkey agar (Oxoid, Basingstoke, UK) plates containing 20 μg/ml nalidixic acid (Sigma, St. Louis, USA) (CT-SMAC); (2) faecal shedding of the microorganism was also monitored by enrichment at 37 °C for 18 h of rectoanal junction mucosal swabs in Trypticase soy broth (Oxoid) containing 20 μg/ml nalidixic acid. About 1 ml of this culture was subjected to *E. coli* O157 immunomagnetic separation (IMS) with O157 Dynabeads according to the manufacturer's instructions (Invitrogen Dynal AS, Oslo, Norway) and the bead-bacteria mixture was spread onto CT-SMAC. Non-sorbitol-fermenting colonies were tested for *E. coli* O157 LPS by latex agglutination (Oxoid). 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 [41–43]. 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 Corp.). 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 faecal samples were collected before each vaccination, before the oral bacterial challenge, and 19 days post-challenge, when the calves were euthanized. Samples of the ileum, cecum, colon, and rectoanal junction were examined for *E. coli* O157:H7 by direct plating and IMS 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. Faecal 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 $2500 \times g$ for 20 min to sediment larger particles and once at $15,000 \times g$ for 30 min; the supernatant was collected and stored at −20 °C until its analysis [19]. Saliva samples were stored at −20 °C until their analysis, when they were centrifuged at $1000 \times g$ for 10 min. Serum samples were analyzed for the presence of IgG and IgA antibody responses against γ-intimin C_{280} and EspB by an enzyme-linked immunosorbent assay (ELISA) described previously [40]. Briefly, 96-well Nunc-Immuno MaxiSorp assay plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 100 μl of either γ-Intimin C_{280} or EspB at 10 μg/ml dissolved in PBS pH 7.4. After washing with PBS pH 7.4 containing 0.05% Tween 20 (PBS-T), non-specific binding sites were blocked with PBS-T containing 3% skim milk for 1 h. Serial two-fold dilutions of sera in PBS-T were added (100 μl/well), and plates were incubated for 2 h. 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. After washing in PBS-T, wells 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. Plates were washed four times with PBS-T. Then, ABTS

[2,2′-azino-di (3-ethyl-benzthiazoline sulphonic acid)] (Amresco, Solon, USA) substrate in citrate-phosphate buffer pH 4.2 plus 0.01% H_2O_2 (100 µl/well) was added. Reactions were stopped after 10 min with 100 µl/well of 5% SDS and read at 405 nm (OD_{405}) in a BioTek ELx808 microplate reader (BioTek Instruments, Winooski, USA). The antibody titre 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 calculated as the average plus two times the standard deviation of the optical densities of the samples measured on day 0.

The amount of EspB- or γ-intimin C_{280}-specific IgA or IgG present in saliva and faeces was determined by a capture ELISA as previously described [40]. To compensate for variations in the efficiency of recovery of secretory antibodies between animals, the results were expressed as mean OD_{405} of undiluted samples normalized to 1 µg of total IgA or IgG present in the sample, respectively. For this, plates coated with sheep anti-bovine IgA or IgG (Bethyl Laboratories) as capture antibody were further incubated with serial dilutions of the samples or a bovine reference serum (Bethyl Laboratories). As secondary antibody, HRP-conjugated sheep anti-bovine IgA or IgG was used, respectively. Plates were developed as described above.

In addition, pools of serum and saliva samples of both vaccinated and control groups were examined by Western blot to confirm the specificity of the antibody response measured by ELISA. One dimension SDS-PAGE was carried out in a 12.0% polyacrylamide gel under reducing conditions [44], loading 2.5 µg γ-intimin C_{280} or EspB proteins per lane, respectively. Proteins were electrophoretically transferred from the gel onto 0.45 µm nitrocellulose sheets (Amersham-Pharmacia, Germany) for immunoblotting as described by Towbin et al. [45]. Nitrocellulose strips were blocked with 5% nonfat dry milk in PBS pH 7.4, for 2 h under agitation, washed three times with PBS-T, and incubated for 2 h with dilutions of serum (1:4000) or saliva (1:4) from immunized and control groups, respectively. After three washes, the membranes were incubated for 2 h with HRP-conjugated rabbit anti-bovine IgG or IgA (Bio-yeda, Rehovot, Israel) diluted 1:1000 in PBS-T. The blots were revealed with 4-Cl-1-naphthol (Pierce, Rockford, USA). Western blotting was performed on pre-immunization and post-immunization samples, from each immunization group to confirm the specificity of the antibody response.

2.5. Histological studies

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

2.6. Statistical analyses

Faecal shedding counting data were $\log_{10}$ transformed and analyzed as a repeated measures design [46] using Proc Mixed of SAS (SAS v.9.1; SAS Institute Inc., Cary NC, USA). Fixed effects were treatment, and linear, quadratic, and cubic terms for time of measure interacting with treatment. Animals were considered independent random variables. The covariance structure for the repeated measures within animals that gave the best fit displayed heterogeneity of variance across times. The values of each treatment and the difference between treatments at any given day were calculated as estimable linear contrasts [47]. A similar procedure was used to test the difference between vaccinated and control animals for the logarithm of shedding during the complete experiment, as originally proposed by Bono et al. [48]. The procedure of Kenward and Roger [49] was used to correct the degrees of freedom of all linear hypotheses. ELISA data within each immunization group and
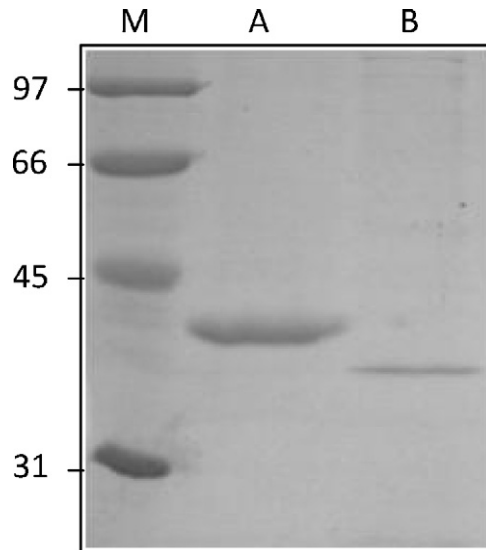
Fig. 1. Coomassie blue-stained polyacrylamide gel of purified γ-intimin C_{280} (A) and EspB (B) antigens used in immunizations.

between groups were performed with the Student's *t*-test. In all cases P values of <0.05% were considered significant.

3. Results

3.1. Purification of recombinant proteins

EspB and γ-intimin C_{280} proteins were analyzed by SDS-PAGE and Coomassie blue staining, and single bands of the predicted molecular weight were observed (Fig. 1).

3.2. Immune response elicited after intramuscular immunization with EspB and γ-intimin C_{280}

The systemic immunization of 6–8-month-old calves with two doses of EspB and γ-intimin C_{280} antigens formulated with ISA206-D3 induced a strong and significant increase in serum IgG titres against both proteins after the first immunization ($P < 0.05$) but not IgA antibodies (data not shown). IgG-antibody titres showed a 13-fold increase on day 21 and remained at the same level throughout the experiment (Fig. 2). A small but not significant increase in EspB and γ-intimin C_{280}-specific IgG titres was observed in the control group after *E. coli* O157:H7 oral administration.

Mucosal anti-EspB and anti-γ-intimin C_{280} IgA and IgG antibodies were quantified in saliva and faecal samples. Specific salivary IgA antibodies against both proteins showed a significant 4–7-fold increase after the first immunization but were undetectable on day 19 post-challenge ($P < 0.05$) (Fig. 3). Salivary anti-EspB and anti -γ-intimin C_{280} IgG antibodies were also detected but the increase after vaccination was not significant (Fig. 4). The bacterial challenge did not result in any detectable increase in salivary antigen-specific IgG or IgA antibody responses. The specificity of the immune response was confirmed by western blot analysis of pools of antisera and saliva from vaccinated and control groups (Fig. 5). Faecal IgG or IgA response to both antigens was not detected (data not shown).

### 3.3. Effect of systemic immunization on E. coli O157:H7 shedding

Two weeks after the second immunization, animals were orally challenged with 10^9 CFU of *E. coli* O157:H7. Bacterial shedding post-challenge was calculated quantitatively by direct plating of serial
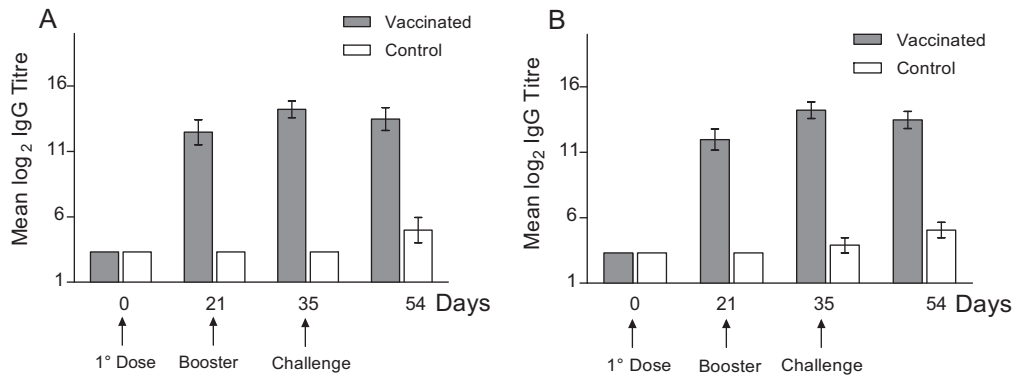
Fig. 2. Serum IgG responses in calves vaccinated with γ-intimin C_{280} and EspB measured by ELISA. (A) Antibody titres against γ-intimin C_{280}. (B) Antibody titres against EspB. Results are presented as mean $\log_2$ of IgG titres and SEM is indicated by vertical lines. A significant increase in serum IgG against the two antigens was observed at the moment of the second immunization ($P < 0.05$).
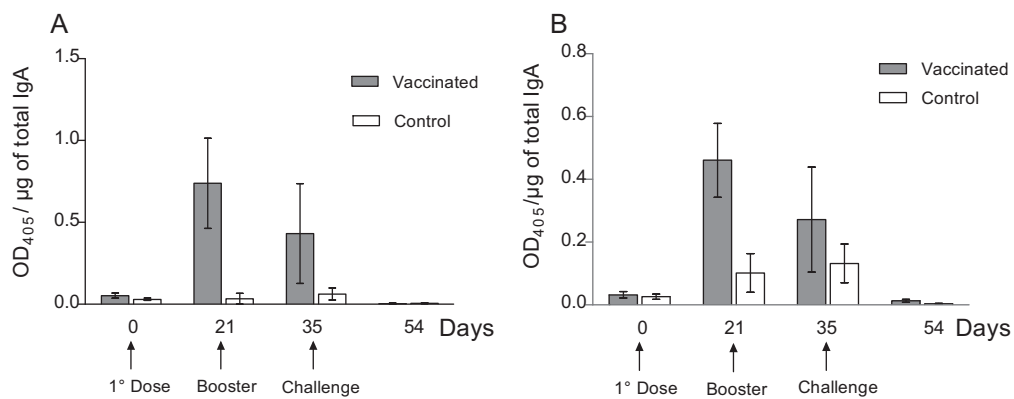

Fig. 3. Saliva IgA response in calves vaccinated with γ-intimin C_{280} and EspB measured by ELISA. (A) Antibody titres against γ-intimin C_{280}. (B) Antibody titres against EspB. Results are presented as mean OD_{405} of undiluted samples normalized to 1 μg of total IgA. SEM is indicated by vertical lines. A significant increase in IgA antibodies against the two antigens was observed at the moment of the second immunization ($P < 0.05$).
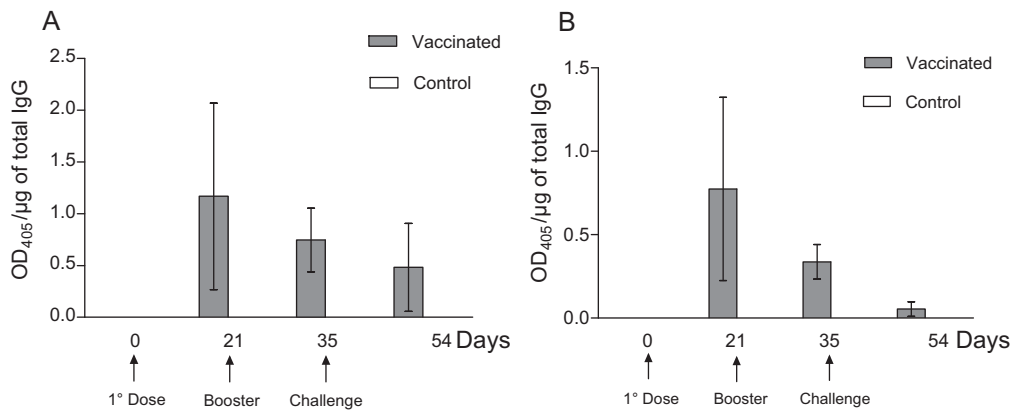
Fig. 4. Saliva IgG response in calves vaccinated with γ-intimin C_{280} and EspB measured by ELISA. (A) Antibody titres against γ-intimin C_{280}. (B) Antibody titres against EspB. Results are presented as mean OD_{405} of undiluted samples normalized to 1 μg of total IgG. SEM is indicated by vertical lines. The response to γ-intimin C_{280} and EspB increased on day 21 in vaccinated animals as compared with control calves.

dilutions of faeces as well as by broth enrichment of rectoanal mucosal swabs followed by IMS during the 19-day post-challenge period. Specimens containing less 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. No calf had diarrhoea after inoculation. Calves of the placebo group were successfully colonized by *E. coli* O157:H7. This is supported by the observation that bacterial shedding was detectable at least until day 11 following oral inoculation in all the animals. Fig. 6B shows the trend in the estimates of the level of $\log_{10}$ (CFU/g) for con-

trol and vaccinated animals on any day. The animals of the control group displayed a larger value of bacterial shedding. The differences between the animals in the control and vaccinated groups were significant ($P < 0.05$) up to day 13. Later, the difference became smaller and not significant. Total bacterial shedding, expressed as the area under the curve, was estimated to be 22.2 for the vaccinated animals and 35.7 for the control group, showing a significant difference ($P < 0.05$). At necropsy, *E. coli* O157:H7 was not recovered from any intestinal sample from animals of both groups. The individual values of bacterial excretion can be seen in Fig. 6A. In
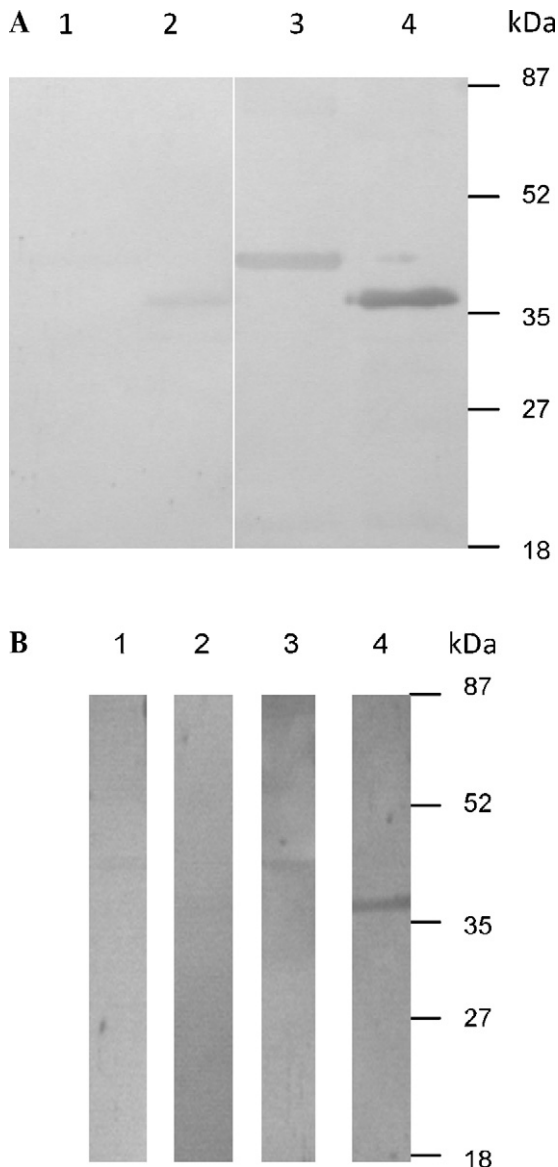
Fig. 5. Western blotting of EspB and γ-intimin C_{280} with pools of serum (A) or saliva (B) samples, demonstrating the specificity of the immune response measured by ELISA. (A) Serum IgG; (B) saliva IgA. Lanes 1 and 2, pre-immunization samples reacting with EspB and γ-intimin C_{280}, respectively; lanes 3 and 4, post-immunization samples reacting with EspB and γ-intimin C_{280}, respectively. Serum and saliva samples were diluted 1:4000 and 1:4, respectively.
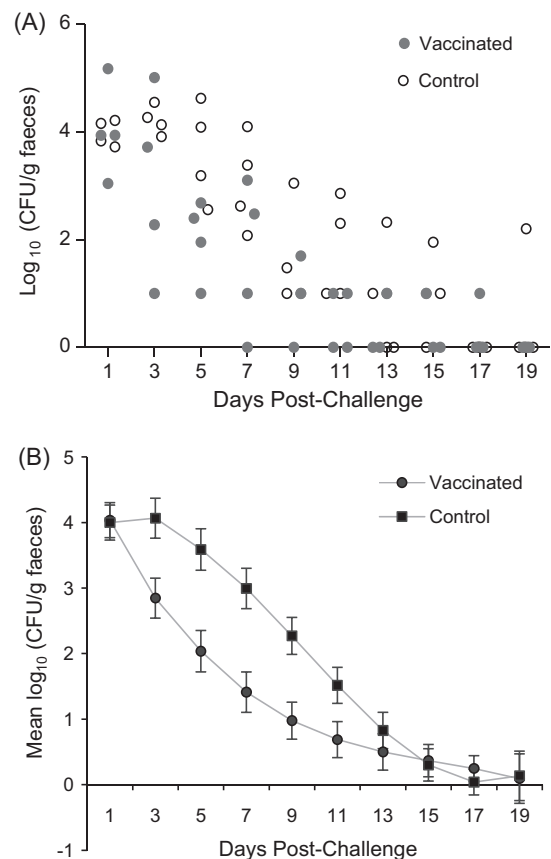
most animals of both groups, no bacteria were detected after day 17 post-challenge.

3.4. Histopathology

No histological changes consistent with attaching and effacing lesions or adherent bacterial layers were seen in intestinal sections of either group of animals at necropsy on day 19 post-challenge.

4. Discussion

Many efforts have been directed to the development of vaccines against enteric pathogens that are able to induce strong mucosal immune responses capable of preventing intestinal colonization. In this sense, immunization of cattle to reduce *E. coli* O157:H7 shedding should be aimed to block the adhesion and colonization process, thereby reducing the risk of microbial transmission

Fig. 6. Faecal shedding of *E. coli* O157:H7 of calves immunized with EspB and γ-intimin C_{280}. (A) Dot plot of $\log_{10}$ CFU/g data for each calf over the entire trial period. In most animals of both groups, no bacteria were detected after day 17 post-challenge. (B) The modeled faecal excretion of *E. coli* O157:H7 shows the trend in the estimates of the level of $\log_{10}$ (CFU/g) ± SEM for control and vaccinated animals at any day of the experiment. The animals of the control group displayed a larger value of bacterial shedding. The differences between the animals in the control and vaccinated groups were significant ($P < 0.05$) up to day 13. Later, the difference became smaller and not significant. One animal in the control group had a random pattern of measures, thus increasing the heterogeneity of the variance. This also explains why curves cross each other on day 15. Total bacterial shedding, expressed as the area under the curve, was estimated to be 22.2 for the vaccinated animals and 35.7 for the control group, showing a significant difference ($P < 0.05$).

to other susceptible hosts. As reported, intimin is a major antigen of bacteria producing attaching and effacing lesions and a key virulence factor that allows strong binding of *E. coli* O157:H7 to the rectal mucosa of calves [53–55]. Thus, intimin and other LEE encoded type III secreted proteins, including Tir, have been tested as vaccine antigens in cattle, with variable results [31–33]. With respect to EspB, although its central role in *E. coli* O157:H7 colonization of the intestinal epithelium has been demonstrated [56,57], this protein has not yet been evaluated as antigen in *E. coli* O157:H7 vaccines for cattle.

In this study, we demonstrated that two doses of a systemic vaccine containing the carboxyl-terminal domain of γ intimin and EspB reduces bacterial colonization and shedding following challenge with *E. coli* O157:H7 in cattle. The immunization induced high serum IgG antibody titres and a mucosal response consisting of both IgA and IgG antibodies against the two antigens. These results are consistent with previous observations that antibodies against intimin are capable of blocking *E. coli* O157:H7 adherence to cultured cells [58,59]. In other studies, systemic vaccines that elicit seric and mucosal IgG antibodies against TTSS antigens have demonstrated the ability to protect streptomycin-treated mice or cattle against *E. coli* O157:H7 colonization [31,34]. The possible explanation for the reduced bacterial shedding observed in the

current study could then be the presence of IgG and IgA antibodies in the intestinal mucosa. Although copro-antibodies were undetectable despite the appropriate processing of faecal samples, we hypothesize that the detection of anti-EspB and anti-γ-intimin C_{280} IgA and IgG in saliva would be related to the presence of specific antibodies also in intestinal mucosa. Vaccination studies where samples were taken by rectal swabbing for antibody determination have shown that systemic immunization of cattle produced similar antibody responses in different mucosal secretions [39].

The published efficacy of systemic vaccines to elicit a mucosal IgA response is variable [31–34]. Although the IgA response after intramuscular immunization is induced mainly in systemic lymphoid tissues, an increased homing of antigen-specific IgA-secreting cells (ASC) to the gut-associated lymphoid tissue has been observed when the antigen is supplemented with $1\alpha,25(OH)2D3$ [60]. It has been shown that this steroid hormone can enhance the antigen-specific IgA response in serum as well as in mucosal secretions after intramuscular immunization. This molecule is the active metabolite of vitamin D, a lipophilic steroid hormone classified as a Th2-immunomodulating adjuvant [61]. In our study, the addition of $1\alpha,25(OH)2D3$ to the vaccine formulation could shift the immune response to EspB and γ-intimin C_{280}, favouring an antibody response and stimulating mucosal immunity. We also speculate that calves could be mucosally primed to both proteins. It has been shown that prior to mucosal priming it is required to generate an intestinal antigen-specific IgA response to systemic immunization [62,63]. In a previous study, we found that 77% of colostrum samples from cows from farms in Buenos Aires contained IgG antibodies against EspB and γ-intimin C_{280} [26]. These data indicate a high exposure of cattle to either *E. coli* O157:H7 or other *E. coli* strains encoding highly homologous EspB and γ-intimin proteins.

We observed the highest circulating antibody response at challenge. However, although the seric IgG antibodies remained high through the whole experiment, mucosal antibodies apparently decreased after the second immunization, until it became almost undetectable on day 54. The time between the second immunization and the next sampling may have been too long to detect a possible increase in mucosal antibodies.

The time-course of the excretion showed an initial increase followed by a sharp drop in bacterial shedding. A similar pattern of excretion has been observed in older calves or adult cattle [50–52] but not in very young (1–2 months) calves, which shed *E. coli* O157:H7 for longer periods after experimental inoculation [50]. The absence of histopathological lesions in the intestinal samples was expected if it is considered that the inoculum bacteria were recovered on day 19 post-challenge only from one animal of the control group, indicating low intestinal levels of *E. coli* O157:H7, probably insufficient to observe A/E lesions.

5. Conclusions

Cattle vaccination would be a feasible pre-slaughter intervention key to reduce *E. coli* O157:H7 faecal shedding and a good strategy to lower the risk of contamination for humans. Our results in a cattle infection model suggest that systemic immunization with two doses of a vaccine containing recombinant intimin and EspB could reduce *E. coli* O157:H7 colonization and shedding in cattle. Further field trials are needed to evaluate the efficacy of the vaccine to reduce bovine *E. coli* O157:H7 carriage under natural conditions.

Acknowledgements

References

[1] Karch H, Tarr PI, Bielaszewska M. Enterohaemorrhagic *Escherichia coli* in human medicine. Int J Med Microbiol 2005;295:405–18.
[2] Repetto HA. Long-term course and mechanisms of progression of renal disease in hemolytic uremic syndrome. Kidney Int Suppl 2005;97:S102–6.
[3] Tesh VL, O'Brien AD. The pathogenic mechanisms of Shiga toxin and the Shiga-like toxins. Mol Microbiol 1991;5:1817–22.
[4] Jackson MP, Newland JW, Holmes RK, O'Brien AD. Nucleotide sequence analysis of the structural genes for Shiga-like toxin I encoded by bacteriophage 933J from *Escherichia coli*. Microb Pathog 1987;2:147–53.
[5] Karpman D, Connell H, Svensson M, Scheutz F, Alm P, Svanborg C. The role of lipopolysaccharide and Shiga-like toxin in a mouse model of *Escherichia coli* O157:H7 infection. J Infect Dis 1997;175:611–20.
[6] Tarr PI, Neill MA, Christie DL, Anderson DE. *Escherichia coli* O157:H7 hemorrhagic colitis. N Engl J Med 1988;318:1697.
[7] Hussein HS, Bollinger LM. Prevalence of Shiga toxin-producing *Escherichia coli* in beef cattle. J Food Prot 2005;68:2224–41.
[8] Meichtri L, Gioffré A, Miliwebsky E, Chinen I, Chillemi G, Masana M, et al. Prevalence and characterization of Shiga toxin producing *Escherichia coli* in beef cattle from Argentina. J Food Microbiol 2004;96:189–98.
[9] Naylor SW, Low JC, Besser TE, Mahajan A, Gunn GJ, Pearce MC, et al. Lymphoid follicle dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host. Infect Immun 2003;71:1505–12.
[10] Kang SJ, Ryu SJ, Chae JS, Eo SK, Woo GJ, Lee JH. Occurrence and characteristics of enterohemorrhagic *Escherichia coli* O157 in calves associated with diarrhoea. Vet Microbiol 2004;98:323–8.
[11] Ezawa A, Gocho F, Kawata K, Takahashi T, Kikuchi N. High prevalence of enterohemorrhagic *Escherichia coli* (EHEC) O157 from cattle in selected regions of Japan. J Vet Med Sci 2004;66:585–7.
[12] Olsen SJ, Miller G, Breuer T, Kennedy M, Higgins C, Walford J, et al. A waterborne outbreak of *Escherichia coli* O157:H7 infections and hemolytic uremic syndrome: implications for rural water systems. Emerg Infect Dis 2002;8:370–5.
[13] Kaper JB, Nataro JP, Mobley HL. Pathogenic *Escherichia coli*. Nat Rev Microbiol 2004;2:123–40.
[14] Elliott SJ, Wainwright LA, McDaniel TK, Jarvis KG, Deng YK, Lai LC. The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escherichia coli* E2348/69. Mol Microbiol 1998;28:1–4.
[15] McDaniel TK, Jarvis KG, Donnenberg MS, Kaper JB. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc Natl Acad Sci USA 1995;92:1664–8.
[16] Kenny B, DeVinney R, Stein M, Reinscheid DJ, Frey EA, Finlay BB. Enteropathogenic *Escherichia coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. Cell 1997;91:511–20.
[17] Ide T, Laarmann S, Greune L, Schillers H, Oberleithner H, Schmidt MA. Characterization of translocation pores inserted into plasma membranes by type III-secreted Esp proteins of enteropathogenic *Escherichia coli*. Cell Microbiol 2001;3:669–79.
[18] Johnson RP, Cray Jr WC, Johnson ST. Serum antibody responses of cattle following experimental infection with *Escherichia coli* O157:H7. Infect Immun 1996;64:1879–83.
[19] Bretschneider G, Berberov EM, Moxley RA. Isotype-specific antibody responses against *Escherichia coli* O157:H7 locus of enterocyte effacement proteins in adult beef cattle following experimental infection. Vet Immunol Immunopathol 2007;118:229–38.
[20] Li Y, Frey E, Mackenzie AMR, Finlay BB. Human response to *Escherichia coli* O157:H7 infection: antibodies to secreted virulence factors. Infect Immun 2000;68:5090–5.
[21] Martínez MB, Taddei CR, Ruiz-Tagle A, Trabulsi LR, Girón JA. Antibody response of children with enteropathogenic *Escherichia coli* infection to the bundle-forming pilus and locus of enterocyte effacementencoded virulence determinants. J Infect Dis 1999;179:269–74.
[22] Loureiro I, Frankel G, Adu-Bobie J, Dougan G, Trabulsi LR, Carneiro-Sampaio MM. Human colostrum contains IgA antibodies reactive to enteropathogenic *Escherichia coli* virulence-associated proteins: intimin, BfpA, EspA, and EspB. J Pediatr Gastroenterol Nutr 1998;27:166–71.
[23] Manjarrez-Hernandez HA, Gavilanes-Parra S, Chavez-Berrocal E, Navarro-Ocaña A, Cravioto A. Antigen detection in enteropathogenic *Escherichia coli* using secretory immunoglobulin A antibodies isolated from human breast milk. Infect Immun 2000;68:5030–6.
[24] Noguera-Obenza M, Ochoa TJ, Gomez HF, Guerrero ML, Herrera- Insua I, Morrow AL, et al. Human milk secretory antibodies against attaching and effacing *Escherichia coli* antigens. Emerg Infect Dis 2003;9:545–51.
[25] Parissi-Crivelli A, Parissi-Crivelli JM, Girón JA. Recognition of enteropathogenic *Escherichia coli* virulence determinants by human colostrum and serum antibodies. J Clin Microbiol 2000;38:2696–700.
[26] Vilte DA, Larzábal M, Cataldi AA, Mercado EC. Evidence of antibodies against the LEE-encoded proteins γ-Intimin and EspB from attaching and effacing

Escherichia coli strains in non-immune bovine colostrum. Clin Vaccine Immunol 2008;15:1208–13.

[27] Ghaem-Maghami M, Simmons CP, Daniell S, Pizza M, Lewis D, Frankel G, et al. Intimin-specific immune responses prevent bacterial colonization by the attaching-effacing pathogen *Citrobacter rodentium*. Infect Immun 2001;69:5597–605.

[28] Potter AA, Klashinsky S, Li Y, Frey E, Townsend H, Rogan D, et al. Decreased shedding of *Escherichia coli* O157:H7 by cattle following vaccination with type III secreted proteins. Vaccine 2004;22:362–9.

[29] Smith DR, Moxley RA, Peterson RE, Klopfenstein TJ, Erickson GE, Clowser SL. A two-dose regimen of a vaccine against *Escherichia coli* O157:H7 type III secreted proteins reduced environmental transmission of the agent in a large-scale commercial beef feedlot clinical trial. Foodborne Pathog Dis 2008;5:589–98.

[30] McNeilly TN, Naylor SW, Mahajan A, Mitchell MC, McAteer S, Deane D, et al. *Escherichia coli* O157:H7 colonization in cattle following systemic and mucosal immunization with purified H7 flagellin. Infect Immun 2008;76:2594–602.

[31] McNeilly TN, Mitchell MC, Rosser T, McAteer S, Low JC, Smith DG, et al. Immunization of cattle with a combination of purified intimin-531, EspA and Tir significantly reduces shedding of Escherichia coli O157:H7 following oral challenge. Vaccine 2010;28:1422–8.

[32] Dziva F, Vlisidou I, Crepin VF, Wallis TS, Frankel G, Stevens MP. Vaccination of calves with EspA, a key colonisation factor of *Escherichia coli* O157:H7, induces antigen-specific humoral responses but does not confer protection against intestinal colonization. Vet Microbiol 2007;123:254–61.

[33] van Diemen PM, Dziva F, Abu-Median A, Wallis TS, van den Bosch H, Dougan G, et al. Subunit vaccines based on intimin and Efa-1 polypeptides induce humoral immunity in cattle but do not protect against intestinal colonisation by enterohaemorrhagic *Escherichia coli* O157:H7 or O26:H-. Vet Immunol Immunopathol 2007;116:47–58.

[34] Babiuk S, Asper DJ, Rogan D, Mutwiri GK, Potter AA. Subcutaneous and intranasal immunization with type III secreted proteins can prevent colonization and shedding of *Escherichia coli* O157:H7 in mice. Microb Pathog 2008;45:7–11.

[35] Judge NA, Mason HS, O'Brien AD. Plant cell-based Intimin vaccine given orally to mice primed with Intimin reduces time of *Escherichia coli* O157:H7 shedding in feces. Infect Immun 2004;72:168–75.

[36] Dean-Nystrom EA, Gansheroff LJ, Mills M, Moon HW, O'Brien AD. Vaccination of pregnant dams with Intimin (O157) protects suckling piglets from *Escherichia coli* O157:H7 infection. Infect Immun 2002;70:2414–8.

[37] Agin TS, Zhu C, Johnson LA, Thate TE, Yang Z, Boedeker EC. Protection against hemorrhagic colitis in an animal model by oral immunization with isogenic rabbit enteropathogenic *Escherichia coli* attenuated by truncating Intimin. Infect Immun 2005;73:6608–19.

[38] Smith MJ, Teel LD, Carvalho HM, Melton-Celsa AR, O'Brien AD. Development of a hybrid Shiga holotoxoid vaccine to elicit heterologous protection against Shiga toxins types 1 and 2. Vaccine 2006;24:4122–9.

[39] Wen SX, Teel LD, Judge NA, O'Brien AD. Genetic toxoids of Shiga toxin types 1 and 2 protect mice against homologous but not heterologous toxin challenge. Vaccine 2006;24:1142–8.

[40] Cataldi A, Yevsa T, Vilte DA, Schulze K, Castro-Parodi M, Larzábal, et al. Efficient immune responses against Intimin and EspB of EHEC after intranasal vaccination using the TLR2/6 agonist MALP-2 as adjuvant. Vaccine 2008;26:5662–7.

[41] Blanco JE, Blanco M, Alonso MP, Mora A, Dahbi G, Coira MA, et al. Serotypes, virulence genes, and intimin types of Shiga toxin (Verotoxin)-producing *Escherichia coli* isolates from human patients: prevalence in Lugo, Spain, from 1992 through 1999. J Clin Microbiol 2004;42:311–9.

[42] Olsvik Ø, Strockbine NA. PCR detection of heat stable, heat labile and Shiga-like toxin genes in *Escherichia coli*. In: Persing DH, Smith TF, Tenover FC, White TJ, editors. Diagnostic molecular microbiology. Principles and applications. Washington DC: American Society for Microbiology; 1993. p. 271–6.

[43] Paton AW, Paton JC. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx_1, stx_2, *eaeA*, enterohemorrhagic *E. coli hlyA*, rfb_{O111}, and rfb_{O157}. J Clin Microbiol 1998;36:598–602.

[44] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.

[45] Towbin H, Staehelin R, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350–4.

[46] Littell RC, Henry PR, Ammerman CB. Statistical analysis of repeated measures data using SAS procedures. J Anim Sci 1998;76:1216–31.

[47] Searle SR. Linear models. New York: J. Wiley and Sons; 1971.

[48] Bono A, Alvarez R, Buschiazzo D, Cantet RJC. Tillage effects on soil carbon balance in a semiarid agroecosystem. Soil Sci Soc Am J 2008;72:1140–9.

[49] Kenward MG, Roger JH. Small sample inference for fixed effects from restricted maximum likelihood. Biometrics 1997;53:983–97.

[50] Cray Jr WC, Moon HW. Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. Appl Environ Microbiol 1995;61:1586–90.

[51] Brown CA, Harmon BG, Zhao T, Doyle MP. Experimental *Escherichia coli* O157:H7 carriage in calves. Appl Environ Microbiol 1997;63:27–32.

[52] Snider TA, Fabich AJ, Washburn KE, Sims WP, Blair JL, Cohen PS, et al. Evaluation of a model for *Escherichia coli* O157:H7 colonization in streptomycin-treated adult cattle. Am J Vet Res 2006;67:1914–20.

[53] Cookson AL, Woodward MJ. The role of intimin in the adherence of enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 to HEp-2 tissue culture cells and to bovine gut explant tissues. Int J Med Microbiol 2003;292:547–53.

[54] Cornick NA, Booher SL, Moon HW. Intimin facilitates colonization by *Escherichia coli* O157:H7 in adult ruminants. Infect Immun 2002;70:2704–7.

[55] Dean-Nystrom EA, Bosworth BT, Moon HW, O'Brien AD. *Escherichia coli* O157:H7 requires intimin for enteropathogenicity in calves. Infect Immun 1998;66:4560–3.

[56] Luo W, Donnenberg MS. Analysis of the function of enteropathogenic *Escherichia coli* EspB by random mutagenesis. Infect Immun 2006;74:810–20.

[57] Tacket CO, Sztein MB, Losonsky G, Abe A, Finlay BB, McNamara BP, et al. Role of EspB in experimental human enteropathogenic *Escherichia coli* infection. Infect Immun 2000;68:3689–95.

[58] Gansheroff LJ, Wachtel MR, O'Brien AD. Decreased adherence of enterohemorrhagic *Escherichia coli* to HEp-2 cells in the presence of antibodies that recognize the C-terminal region of intimin. Infect Immun 1999;67:6409–17.

[59] Cook SR, Maiti PK, DeVinney R, Allen-Vercoe E, Bach SJ, McAllister TA. Avian- and mammalian-derived antibodies against adherence-associated proteins inhibit host cell colonization by *Escherichia coli* O157:H7. J Appl Microbiol 2007;103:1206–19.

[60] Van der Stede Y, Cox E, Van den Broeck W, Goddeeris BM. Enhanced induction of the IgA response in pigs by calcitriol after intramuscular immunization. Vaccine 2001;19:1870–8.

[61] van Etten E, Mathieu C. Immunoregulation by 1,25-dihydroxyvitamin D3: basic concepts. J Steroid Biochem Mol Biol 2005;97:93–101.

[62] Herremans TM, Reimerink JH, Buisman AM, Kimman TG, Koopmans MP. Induction of mucosal immunity by inactivated poliovirus vaccine is dependent on previous mucosal contact with live virus. J Immunol 1999;162:5011–8.

[63] Jeurissen SH, Claassen E, Van RN, Kraal G. Intra-intestinal priming leads to antigen-specific IgA memory cells in peripheral lymphoid organs. Immunology 1985;56:417–23.